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Polymer-stimulated ligation: enhanced ligation of oligo- and polynucleotides by T4 RNA ligase in polymer solutions

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ABSTRACT

The effects of macromolecular crowding were tested on several reactions catalyzed by T4 RNA ligase. The rate of cyclization of oligoriboadenylates was stimulated up to 10-fold by relatively high concentrations of several polymers (polyethylene glycol (PEG) 8000 or 20,000; bovine plasma albumin; Ficoll 70). In addition, higher concentrations of PEG 8000 or PEG 20,000 allowed the novel formation of large linear products from the oligoriboadenylates. Also stimulated by high concentrations of PEG 8000 were the rate at which T4 RNA ligase joined $p(dT)_{10}$ to oligoriboadenylates and the rate at which the enzyme activated $p(dT)_n$ by transfer of an adenylyl moiety from ATP to the oligonucleotides. These results with T4 RNA ligase are compared to earlier studies on the effects of crowding on DNA ligases.

INTRODUCTION

Previous studies of DNA ligases indicated striking changes in the enzymatic ligation of duplex segments of DNA in the presence of high concentrations of nonspecific polymers (1,2). We here extend those studies of macromolecular crowding to the RNA ligase produced in Escherichia coli upon infection with T4 bacteriophage (3). T4 RNA ligase uses a broad spectrum of polyribo- and polydeoxyribonucleotides of a wide range of sizes as substrates. The most rapid reactions demonstrated with this enzyme involve cyclization or oligomerization of relatively short single-stranded oligoribonucleotides. Rates of ligation of these materials are highly dependent upon their chain length; the most rapid reactions occur with substrates that are ca. 10 to 40 nucleotides in length (3,4). While typically such substrates yield circular products, linear aggregates can be formed if the oligoribonucleotides are either too short to cyclize or lack one of the termini needed for self-ligation, the missing terminus then being provided by a second species of oligomer. Oligodeoxyribonucleotides and DNA are also substrates for T4 RNA ligase. However, only exceedingly slow reactions have been observed for the joining between two oligodeoxyribonucleotides due to the limited rate at which the enzyme uses

these substrates as a source of 3'-hydroxyl termini. The reactions catalyzed by T4 RNA ligase are reviewed in references 5 and 6.

We have tested the effects of macromolecular crowding on certain of these reactions. We find that high concentrations of nonspecific polymers not only stimulate the rate of cyclization of small oligoriboadenylates, but in addition allow the novel formation of high molecular weight linear ligation products. Crowded conditions also stimulate the formation of hybrid molecules produced by the end-to-end ligation of $p(dT)_{10}$ to single-stranded oligoribonucleotides. In contrast, either in the presence or absence of concentrated polymer, T4 RNA ligase did not ligate significant amounts of single-stranded oligodeoxyribonucleotides to themselves under our conditions. Instead, polymer solutions increased the rate of accumulation of the adenylylated-oligodeoxynucleotide reaction intermediate (5,7).

MATERIALS AND METHODS

Materials

Micrococcal nuclease, poly(rA), poly(dT), dephospho-$(dA)_{10}$ and dephospho-$(dT)_{10}$ were from P-L Biochemicals. T4 RNA ligase (Cat. No. 8003, lots 21115, 31111 and 33112 with 1650, 3190 and 3800 circle-forming units/mg, respectively), Hae III endonuclease-digested ϕX174 RF DNA, T4 DNA ligase, and $p(dT)_{4-22}$ were from Bethesda Research Laboratories. *E. coli* alkaline phosphatase (BAPC), crystallized pancreatic DNase and calf thymus DNA were from Millipore. Polynucleotide phosphorylase (from *Micrococcus luteus*) and PEG 20,000 were from Sigma. PEG 200, PEG 1000 and PEG 8000 were from Baker. Crystallized bovine plasma albumin was from Miles and Ficoll 70 was from Pharmacia. Stock solutions of PEG, Ficoll or albumin were made in deionized water; the 50% albumin stock was adjusted to pH 7.6 with 0.08 volume of 1 N NaOH.

Methods

Preparation of ^{32}P-labeled oligomers. Partial digests of poly(rA) were prepared as Silber *et al*. (3) using several levels of micrococcal nuclease; partial digests of poly(dT) and of calf-thymus DNA were prepared with pancreatic DNase I. Digests were dephosphorylated with *E. coli* alkaline phosphatase at 65°, shaken with a mixture of phenol:chloroform:*i*-amyl alcohol::25:24:1, precipitated with 2 volumes of ethanol in the presence of 0.2 M sodium acetate, and the pellets washed with 95% ethanol. Polynucleotide kinase (P-L Biochemicals) and γ-^{32}P-ATP (2900 Ci/mmol, New England Nuclear) were used to phosphorylate the dephosphorylated DNase I partial digests or Hae III digest (8) as well as dephospho-$(dT)_{10}$ and dephospho-$(rA)_{10}$ (9). The $p(dT)_{4-22}$ was

labeled by the polynucleotide kinase exchange reaction under the conditions recommended by Bethesda Research Laboratories. Dephosphorylated micrococcal nuclease digests $(rA)_{9-60}$ and $(rA)_{40-500}$ were labeled with γ-^{32}P-ATP at 11 Ci/mmol (3).

Subfractions were isolated from ^{32}P-$(rA)_{9-60}$ on polyacrylamide gels (1.5 mm thickness vertical slab gels formulated as below). After electrophoresis (400 volts; Model SE apparatus, Hoefer Scientific) the gels were frozen and thawed twice and the appropriate segments cut from the gel. The gel pieces were crushed between two glass microscope slides and the pulverized residues were extracted for one day at room temperature in 0.5 ml of 0.3 M ammonium acetate, 0.1 mM EDTA with occasional mixing. After centrifugation, the supernatant fluids were dialyzed against deionized water and finally lyophilized before use. Over 70% of the radioactivity applied to the gel was reisolated by this procedure.

RNA ligase assays. Unless otherwise specified, T4 RNA ligase was assayed in polypropylene tubes (Eppendorf) in mixtures (final volume of 10 µl) containing 50 mM Tris·HCl buffer (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5 mM ATP, T4 RNA ligase dilution (1 µl), 5'-^{32}P-oligonucleotides and other additions as indicated. The T4 RNA ligase was diluted in 10 mM Tris·HCl buffer (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 25% glycerol, and 0.2 mg/ml bovine serum albumin. After incubation, 1 µl of 0.2 M EDTA was added to each tube. Where indicated, incubation mixtures were further treated with an excess of E. coli alkaline phosphatase (10 µl containing 2 µg of phosphatase; 35 min incubation at 65°) to remove unreacted 5'-^{32}P-phosphate groups. Samples were then diluted with an equal mixture of 10 M urea, 0.05% xylene cyanol, heated 5 min at 100°, cooled in ice water, and aliquots taken for electrophoresis.

Gels (10) contained 11.5% acrylamide, 0.4% bis-acrylamide, 7 M urea and were in a buffer of 0.05 M Tris, 0.05 M boric acid, 1.4 mM EDTA. Vertical slab gels of 0.4 mm thickness (BRL apparatus) were run at 500 V. Gels were evaluated by autoradiography at -70° with XAR-5 film (Eastman Kodak) and "Lightning-plus" screens (DuPont).

RESULTS

We will consider in turn the effects of concentrated polymer solutions on the ability of T4 RNA ligase to ligate oligoribonucleotides, oligodeoxyribonucleotides, and then mixtures of these two classes of materials.

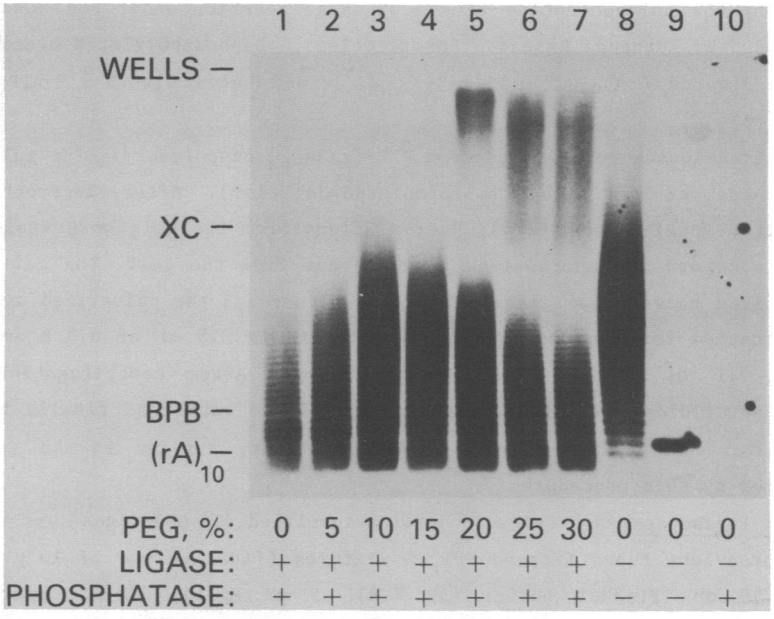

Figure 1. Effects of PEG 8000 concentration on ligation of 5'-^{32}P-(rA)$_{9-60}$ by T4 RNA ligase. Ligase assay mixtures (see Methods) contained 0.5 µg of 5'-^{32}P-(rA)$_{9-60}$ and the indicated (w/v) concentrations of PEG 8000 in lanes 1-8 and 10. Where indicated, T4 RNA ligase (30 ng) was added, and the mixtures incubated 30 min at 20°. Non-reacted ^{32}P was removed by phosphatase treatment where indicated (see Methods). Lane 9 contained 5'-^{32}P-(rA)$_{10}$. Samples were prepared for the polyacrylamide gel as in Methods. The positions of migration of xylene cyanol (XC) and bromphenol blue (BPB) are indicated.

Ligation of Oligoriboadenylates

The routine substrates used to assay the effects of macromolecular crowding on T4 RNA ligase were two partial enzymatic digests of poly(rA) with overlapping distributions of chain lengths. The heterogeneity in chain lengths in each of these digests facilitated a survey over a wide range of substrate sizes. The 5'-termini of the chains within each digest were labeled with ^{32}P. In the following, these digests will be referred to as (rA)$_{9-60}$ and (rA)$_{40-500}$ to indicate the approximate ranges of chain lengths which contribute significant amounts of radioactivity. For characterization of the products of the reactions, subfractions of these labeled digests were isolated on polyacrylamide gels and used as substrates for the ligase. Product formation from any of these substrates was assayed both by formation of labeled species with altered electrophoretic mobilities and by appearance of labeled material that was resistant to dephosphorylation by *E. coli* alkaline phosphatase.

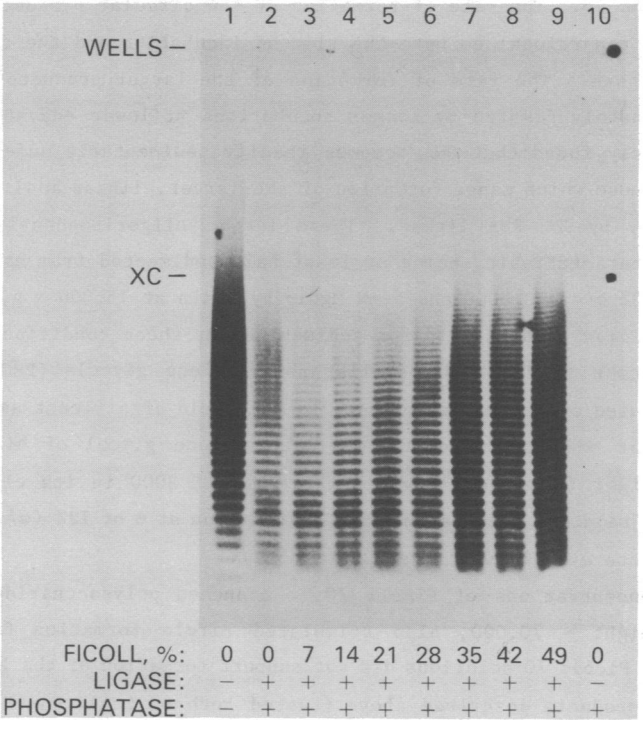

Figure 2. Effects of Ficoll 70 concentration on ligation of 5'-^{32}P-(rA)$_{9-60}$ by T4 RNA ligase. Ligase assay mixtures (see Methods) contained 0.5 µg of 5'-^{32}P-(rA)$_{9-60}$ and the indicated (w/v) concentrations of Ficoll 70. Where indicated, T4 RNA ligase (30 ng) was added, and the mixtures incubated 30 min at 20°. Non-reacted ^{32}P was removed by phosphatase treatment where indicated (see Methods). Samples were prepared for the polyacrylamide gel as in Methods. XC, xylene cyanol.

Survey of the effects of polymers. The influence of PEG 8000 on the ligation of (rA)$_{9-60}$ by T4 RNA ligase is shown in Figure 1. The behavior of (rA)$_{40-500}$ was similar. The substrate before ligation is in lane 8. The phosphatase-resistant products formed in the presence of increasing concentrations of PEG 8000 are in lanes 1-7. At lower PEG 8000 levels (5-10% w/v) there is a stimulation in the rate of formation of monomeric circles of relatively short chain length (<80 bases) relative to the rate in the absence of the PEG. Those circles ≤ 42 bases migrate more rapidly than do the corresponding substrate chains, as described in the next section. At higher PEG 8000 concentrations (15-30% w/v), circle formation is diminished relative to that at 5-10% (w/v) PEG. In 20-30% (w/v) PEG a second class of larger, more slowly migrating products appears that is characterized below as relatively large, linear

addition products. The rate of formation of the circular products in 20% w/v PEG 8000 is proportional to both the time of incubation and the concentration of T4 RNA ligase. The rate of formation of the larger products is somewhat disproportionately favored by longer incubations at lower enzyme levels. We have previously found that DNA becomes readily sedimentable under conditions similar to those which cause formation of the larger, linear addition products from $(rA)_{9-60}$ by T4 RNA ligase. These larger oligoriboadenylate products share this characteristic, being at least half sedimented from assay mixtures containing 20% w/v PEG 8000 and 5 mM $MgCl_2$ by 5 min at 15,000 x g, whereas the smaller, circular products did not sediment under these conditions.

In contrast to PEG 8000, smaller polyethylene glycols (PEG 200 or PEG 1000) stimulated circle formation but did not yield significant amounts of the larger, linear addition products. A polyethylene glycol of higher average molecular weight (PEG 20,000) was similar to PEG 8000 in its effects on the ligation of $(rA)_{9-60}$, stimulating circle formation at 6 or 12% (w/v) and giving larger products at 19% (w/v) (data not shown).

High concentrations of Ficoll 70, a branched polysaccharide of average molecular weight = 70,000, also stimulated circle formation from $(rA)_{9-60}$ (Figure 2). Ficoll 70 solutions did not support formation of the higher molecular weight products described above (tested both on $(rA)_{9-60}$ (Figure 2) and $(rA)_{40-500}$; data not shown). Sucrose, the monomer of Ficoll, neither stimulated nor inhibited circle formation (tested at 10, 30, 50 and 70% w/v sucrose with $(rA)_{9-60}$; data not shown).

Concentrated solutions (8 to 38% w/v) of bovine plasma albumin stimulated the rate of circle formation from $(rA)_{9-60}$ by 2- to 4-fold (data not shown).

Identification of the products. The substrate for these experiments, $^{32}p\text{-}(rA)_{9-60}$, was prepared according to Silber et al. (3). T4 RNA ligase was demonstrated by those authors to yield single-stranded circular products in ligation reactions with such a substrate in dilute, i.e. non-crowded, solutions. Our control reactions in the absence of background polymers are consistent with their observations in that we also find such products to be resistant to alkaline phosphatase. These circles have distinctive electrophoretic mobilities, those shorter than ca. 42 bases moving more rapidly than linear molecules of the same length and those longer then ca. 43 bases moving slower than the comparable linear molecules (Figure 3). When PEG 8000 is added, more of the same circular products are produced as judged by the exact correspondence in rates of electrophoretic migration. In addition, larger products are formed. Both types of products were characterized by their

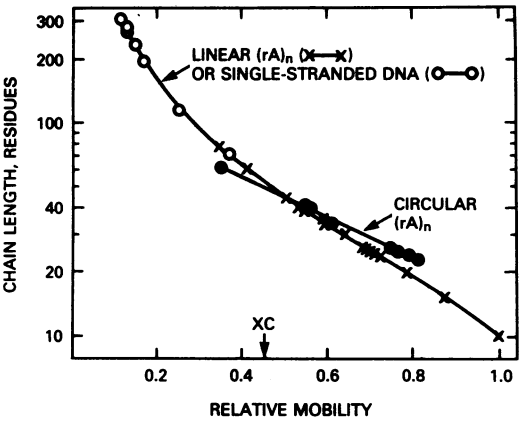

Figure 3. Mobility of circular and linear oligoriboadenylates and of single-stranded DNA on 12% polyacrylamide-7M urea gels. The single-stranded DNA points are from a 5'-^{32}P-labeled heat-denatured (5 min, 100°; quenched) Hae III restriction nuclease digest of φX174 RF DNA. The linear $(rA)_n$ points are taken from the series of bands given by 5'-^{32}P-$(rA)_{9-60}$; the absolute sizes of the individual bands was determined by the position of migration of 5'-^{32}P-$(rA)_{10}$ in an adjacent lane. The circular $(rA)_n$ points are ligase products from isolated subfractions of 5'-^{32}P-$(rA)_{9-60}$ whose sizes were determined by counting bands in adjacent lanes of 5'-^{32}P-$(rA)_{9-60}$ and 5'-^{32}P-$(rA)_{10}$. The circular nature of these products is described in the text. All mobilities are expressed relative to that of the 5'-^{32}P-$(rA)_{10}$. The similar mobilities of single-stranded DNA and of $(rA)_n$ of the same chain length are consistent with the observations of Maniatis et al. (10) for this size range and the present type of gel.

responses to E. coli alkaline phosphatase and polynucleotide phosphorylase (Figure 4). Amounts of alkaline phophatase which totally removed the radioactivity from the substrate had little effect on either class of product, as expected for ligated material (Figure 4B). Polynucleotide phosphorylase degraded both the substrate and the larger class of product to small oligomers (Figure 4A), consistent with a linear structure of the substrate and the large products (see below). In contrast, the small products were resistant to polynucleotide phosphorylase as expected for circles. The substrate $(rA)_{9-60}$ has the bulk of its radioactivity in ca. 50 resolvable species of different chain lengths. The circular products formed with this substrate in polymer solutions also contain their radioactivity in about 50 resolvable bands, indicating circle formation in polymer solutions is occurring for essentially all of the chain lengths present in the substrate preparation.

More evidence for the linear nature of the slowly migrating products formed in 15-30% PEG 8000 was obtained from reactions employing substrates

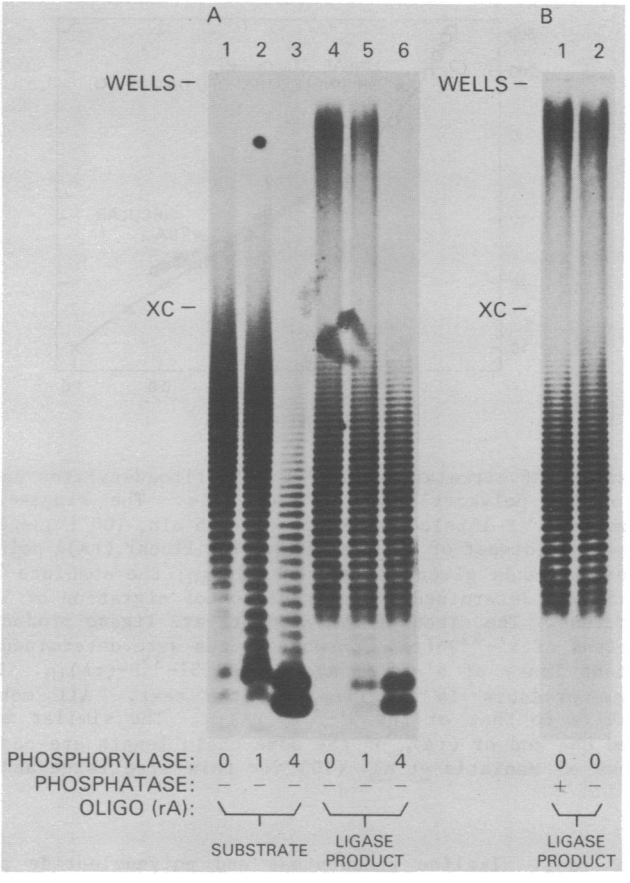

Figure 4. Sensitivity of the products of T4 RNA ligase on 5'-^{32}P-(rA)$_{9-60}$ to (A) polynucleotide phosphorylase or to (B) E. coli alkaline phosphatase. In preliminary incubations as in Methods but 15-fold increased in scale, T4 RNA ligase (2.3 ng) was incubated with 5'-^{32}P-(rA)$_{9-60}$ (7.5 µg) for 30 min. As shown in (B) essentially all of the substrate had been ligated as judged by the phosphatase-resistance of the ^{32}P (see Methods). Aliquots of a similar preparation but without ligase treatment and of the ligase product are compared in (A) for sensitivity to polynucleotide phosphorylase. The polynucleotide phosphorylase reaction mixtures contained 10 µl of the preliminary incubation mixtures in a final volume of 30 µl containing 83 mM Tris•HCl buffer, pH 8.2, 5 mM $MgCl_2$, 10 mM K_2HPO_4, 0.2 mg/ml bovine serum albumin and amounts of polynucleotide phosphorylase indicated (0, 0.01 or 0.04 phosphorylase units in lanes 1, 2 or 3 and in lanes 4, 5 or 6, respectively). Samples were prepared for the polyacrylamide gel as in Methods. XC, xylene cyanol.

whose chain length varied by < 2 bases. With such substrates, the distribution of products between the circles discussed above and various larger species could be clearly seen to be functions of both the concentration of PEG 8000 and the chain length of the oligomer. The effects of PEG 8000

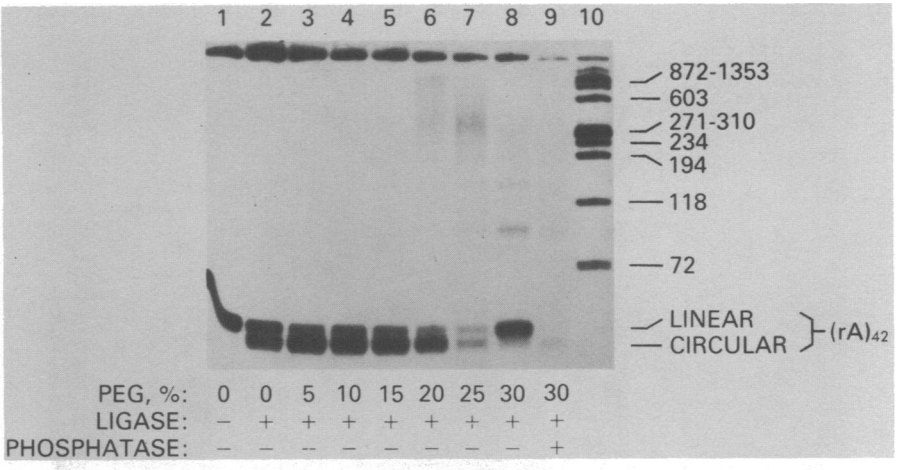

Figure 5. Effects of PEG 8000 concentration on ligation of 5'-^{32}P-$(rA)_{42}$ by T4 RNA ligase. Ligase assay mixtures (see Methods) contained 5'-^{32}P-$(rA)_{42}$ (3 ng, 300 cpm) and the indicated (w/v) concentrations of PEG 8000 in lanes 1-9. Where indicated, T4 RNA ligase (0.25 µg) was added, and the mixtures were incubated for 60 min at 20°. Non-reacted ^{32}P was removed by phosphatase treatment in lane 9 (see Methods). Lane 10 contained 5'-^{32}P-labeled Hae III nuclease-digested φX174 RF as size standard. Samples were prepared for the polyacrylamide gel as in Methods. Intensity in the wells of this gel is an artifact and was not present on other gels with the same material.

concentration on the products formed from $(rA)_{42}$ is shown in Figure 5. From 0 to 15% w/v PEG 8000, only the circular monomer was formed. When the PEG 8000 concentration was raised further, the fraction of circular monomers progressively decreased and more slowly migrating material appeared. This latter material contained bands which migrated as expected for the linear dimer, trimer and tetramer to within experimental error (± 3 residues) on gels standardized as in Figure 3. Similar experiments have resolved linear products through the hexamer (data not shown). The unresolved zone of more slowly moving products presumably contains still larger oligomers. The effect of substrate chain length on the product distribution for ligation in 20% w/v PEG 8000 is shown in Figure 6. Isolated fractions containing molecules of 23 to 41 bases in length formed monomer circles. The $(rA)_{61}$ also formed monomer circles but in addition formed a series of linear oligomer bands as just mentioned and the continuum of more slowly migrating materials. Oligomers of 80 bases in length formed primarily linear products.

Certain combinations of chain lengths of oligoadenylates with concentrations of PEG 8000 generate products which migrate as much larger species on

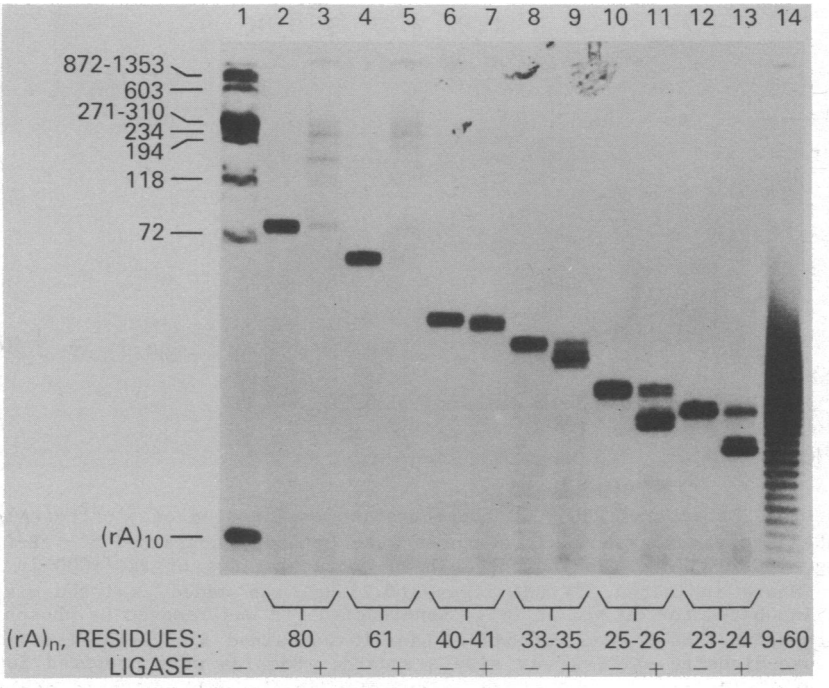

Figure 6. Products of T4 RNA ligase in 20% PEG 8000 solution on 5'-^{32}P-oligoriboadenylates of different chain lenths. Ligase assay mixtures (lanes 2-13) contained 5'-^{32}P-oligoriboadenylate fractions (3 to 10 ng, 350 cpm) of the indicated chain lengths, or (lane 14) 5'-^{32}P-$(rA)_{9-60}$. All lanes contained 20% (w/v) PEG 8000 in ligase assay mixtures as in Methods. Where indicated, T4 RNA ligase (0.25 μg) was added, and the mixtures incubated for 60 min at 20°. Lane 1 contained 5'-^{32}P-labeled Hae III nuclease-digested φX174 RF and 5'-^{32}P-$(rA)_{10}$ as size standards. Samples were prepared for the polyacrylamide gel as in Methods.

0.8% agarose gels (data not shown) or on 12% acrylamide-7M urea gels. The sizes of these products ranged up to at least 500 nucleotides in length, corresponding to at least a 10-fold increase in length relative to the substrate for linear products. To determine if indeed these very long chains are still linear molecules or whether they have ultimately cyclized to give large rings, we have isolated the large products formed by ligation of the substrate 5'-^{32}P-$(rA)_{13-50}$ (obtained from Sephadex G-75 chromatography of 5'-^{32}P-$(rA)_{9-60}$) in 20% w/v PEG 8000. The median sizes of the substrate and isolated large product were estimated by densitometry of autoradiograms to be 32 and 242 bases, respectively. This corresponds to an 8-fold increase in size due to ligation. Treatment of the isolated large linear product with an excess of *E. coli* alkaline phosphatase released 11% of the ^{32}P into an acid-soluble form. If

the assumption is made that all of the large product is, in fact, linear, this extent of hydrolysis corresponds to a 9-fold increase in size compared to the substrate. The agreement between these two estimates of the degree of polymerization indicates that the assumption is correct and that a substantial fraction of the large product is linear.

Influence of temperature or reaction components on the ligation of oligoriboadenylates. The amounts and proportions of products made in 20% w/v PEG 8000 from $(rA)_{9-60}$ were not significantly different at 20° vs. 37°. At 0°, however, the yield of products was reduced by ca. 10-fold.

Formation of products in 20% w/v PEG 8000 was essentially unchanged by increasing the concentration of Mg^{++} from 5 mM to 10 mM. Decreasing the level to 2.5 mM Mg^{++}, however, prevented formation of large products (see Discussion). Mn^{++} at 5 or 10 mM in place of Mg^{++} yielded no detectable products.

The rate of circle formation in the ligase reaction was approximately proportional to the concentration of $(rA)_{9-60}$ when tested at 0.2, 1 and 5 times that used in the standard assay system. The rate of formation of larger products from $(rA)_{9-60}$ was increased by at least an order of magnitude in going from 0.2 to 1 times the standard level and was little further changed at a still five-fold higher $(rA)_{9-60}$ concentration.

Effects of several monovalent cations were tested. The standard assay procedure mixture contains 50 mM Tris·HCl buffer, pH 7.8, as well as 5 mM KCl from the enzyme diluent. Decreasing the concentration of the Tris buffer from 50 mM to 10 or 20 mM made little difference in the amounts or types of products made from $(rA)_{9-60}$ in 20% w/v PEG 8000. In contrast, the presence of relatively low levels of Na^{+} (20, 50 or 100 mM) completely stopped production of large products, although formation of small circles was slightly increased. At 5 mM NaCl or KCl, the products were similar to those in the absence of these salts.

Thymidylate Oligomers or Denatured DNA as Substrates for T4 RNA Ligase

Either in the absence or in the presence of concentrated PEG 8000 solutions, the major product we observe of T4 RNA ligase action on oligothymidylates or on denatured DNA is the activated intermediate formed by transfer of an adenylyl moiety from ATP to the 5'-phosphate group of the oligonucleotide (5). While the rate of formation of this intermediate can be stimulated 3- to 10-fold by addition of PEG 8000, this was not accompanied by any obvious accumulation of ligated products. Slow joining reactions between oligodeoxyribonucleotides have been demonstrated by several groups (7,11).

Formation of the activated intermediate was assayed by the conversion of

Table I: PEG 8000 Stimulates Formation of Adenylylated-oligo(dT) by T4 RNA Ligase.

Substrate	Addition	Phosphatase-resistant Radioactivity, %	
		- Acid Hydrolysis	+ Acid Hydrolysis
5'-^{32}P-$(dT)_{4-22}$	None	2	2
	Ligase	20	3
	Ligase + PEG	54	2
5'-^{32}P-$(dT)_{n>600}$	None	1	7
	Ligase	7	4
	Ligase + PEG	90	6

Reaction mixtures as in Methods, but scaled to 50 µl volume, contained either 5'-^{32}P-$(dT)_{4-22}$ (1300 cpm) or 5'-^{32}P-$(dT)_{n>600}$ (700 cpm), and 20% w/v PEG 8000 where indicated. T4 RNA ligase (0.8 µg) was added where indicated, and the mixtures were incubated for 2 hrs. at 20°. Aliquots of these samples were assayed for acid-lability as in legend to Figure 7, treated with phosphatase (as Methods), and the Norit-adsorbable ^{32}P was measured (24).

the radioactivity of 5'-^{32}P-labeled oligodeoxyribothymidylates to a phosphatase-resistant form. This phosphatase-resistant labeled material was distinguished from that generated by ligation in two ways: First, the anhydride linkage of the intermediate has a much greater acid-lability than does the phosphodiester linkage of the oligothymidylate substrates or products (7) (Table I). Second, the relatively minor shift in electrophoretic mobility of the substrate due to treatment with the ligase was that expected for addition of a single nucleotidyl residue to the oligonucleotide. This mobility shift was reversed and the substrate band regenerated upon acid hydrolysis (Figure 7).

T4 RNA ligase also apparently adenylylated single-strand DNA of heterogeneous sequence. Substrates were either a denatured 5'-^{32}P-Hae III endonuclease digest of ϕX174 RF DNA or a series of denatured 5'-^{32}P-labeled pancreatic DNase I digests of calf thymus DNA of differing average molecular weights. With either type of substrate ligase converted radioactivity to a phosphatase-resistant form which migrated very similarly to the original substrates on gel electrophoresis. The rate of formation of these products on denatured DNA was stimulated 3- to 5-fold by addition of 20% w/v PEG 8000 (data not shown).

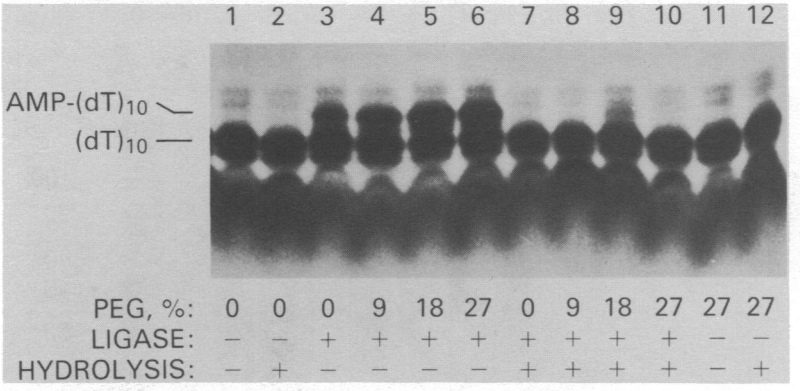

Figure 7. Effects of PEG 8000 concentration on product formation from 5'-^{32}P-$(dT)_{10}$ by T4 RNA ligase and the acid-lability of the product. Ligase assay mixtures (24 µl volume as in Methods) contained 5'-^{32}P-$(dT)_{10}$ (5 µg) and the indicated (w/v) concentrations of PEG 8000. Tubes were incubated for 2 hr at 20° with T4 ligase (67 ng) as indicated. In the subsequent hydrolysis, an equal volume of 2N H_2SO_4 was added to the indicated tubes, which were then heated for 15 min at 100°, cooled and neutralized with NaOH. Non-hydrolyzed samples received the equivalent amount of Na_2SO_4 and were not heated. Samples were prepared for the polyacrylamide gel as in Methods. The 5'-^{32}P-$(dT)_{10}$ bands migrated ca. 16 cm. AMP-$(dT)_{10}$ is adenylylated-5'-^{32}P-$(dT)_{10}$ and $(dT)_{10}$ is 5'-^{32}P-$(dT)_{10}$.

Ligation of 5'-^{32}P-$(dT)_{10}$ to Oligoriboadenylates

The major product formed by T4 RNA ligase action on a mixture of 5'-^{32}P-$(dT)_{10}$ and 3',5'-hydroxyl $(rA)_{9-60}$, whether in the presence or absence of PEG 8000, was the activated intermediate, adenylylated-^{32}P-$(dT)_{10}$, as might be expected from the preceding section. There was, in addition, a small amount of a second type of product whose formation was strongly stimulated by high concentrations of PEG 8000 (Figure 9). This product migrated as a population of chains of ca. 25-50 bases in length, a result consistent with joining of single $(dT)_{10}$ molecules onto members of the population of $(rA)_{9-60}$ molecules. End-to-end joining of the $(dT)_{10}$ to itself was not detected; it would have yielded discrete bands of n = 20, 30,... bases in length such as those shown in lane 9 of Figure 8 which were generated by T4 DNA ligase (12). Such a reaction for T4 RNA ligase would be unlikely under these conditions in any case based on the preceding section.

DISCUSSION

We have previously described effects of macromolecular crowding upon the joining of DNA strands by DNA ligases. Two DNA ligases, namely those from rat

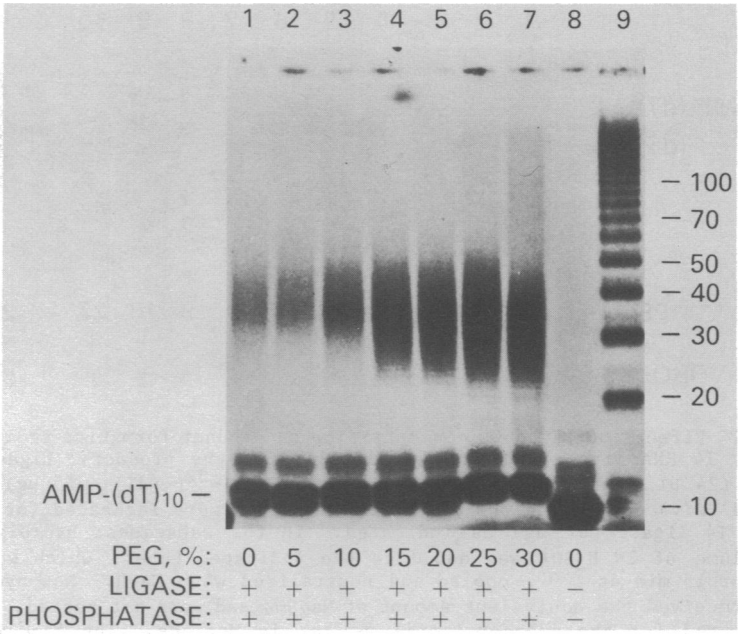

Figure 8. Effects of PEG 8000 concentration on ligation of 5'-^{32}P-$(dT)_{10}$ to 3',5'-hydroxyl$(rA)_{6-90}$ by T4 RNA ligase. Ligase assay mixtures (see Methods) contained 5'-^{32}P-$(dT)_{10}$ (0.04 µg), 3',5'-hydroxyl$(rA)_{9-60}$ (0.1 µg) and the indicated concentrations (w/v) of PEG 8000. T4 RNA ligase (0.15 µg) was added for lanes 1-7 and the mixtures were incubated 2 hrs at 20°. Non-reacted ^{32}P was removed by phosphatase treatment (see Methods) where indicated. Samples were prepared for the polyacrylamide gel as in Methods. For lane 9, T4 DNA ligase (2×10^{-4} unit) was added and the sample was phosphatase treated before electrophoresis. The chain lengths of the phosphatase-resistant ^{32}P-labeled oligomers formed in the T4 DNA ligase products of lane 9 are indicated in the margin. AMP-$(dT)_{10}$ is adenylylated-5'-^{32}P-$(dT)_{10}$.

liver nuclei and Escherichia coli, catalyze blunt-end ligation of DNA under crowded conditions, a type of reaction which is undetectable with these enzymes under conventional assay conditions (1). A third DNA ligase, that from T4-infected E. coli, can catalyze this reaction in the absence of background molecules, but its rate is increased by over a thousand-fold in the presence of high concentrations of polymers and concomitantly the distribution of products is shifted to favor linear rather than circular molecules. These dramatic results prompted us to test the effects of crowding on the reaction catalyzed by T4 RNA ligase. Smaller but still significant effects were observed.

There are interesting parallels between the effects with the DNA ligases and the present results with T4 RNA ligase. The similarities are all the more

striking in view of the differences in substrates between the relatively rigid segments of double-stranded DNA joined by the DNA ligases and the highly flexible molecules of single-stranded RNA joined by T4 RNA ligase. With both DNA and RNA ligases, the responses can be divided into two ranges depending on the concentrations of background molecules. Relatively low concentrations of a variety of polymers increase the rates of joining of several types of substrates by either T4 DNA or T4 RNA ligase by 3- to 10-fold. The products tend to be circular when the characteristics of the substrates involved do not preclude cyclization. The products made by T4 RNA ligase under these conditions from $(rA)_{9-60}$ are entirely circular. Circular products are also made by T4 DNA ligase from long DNA segments (4400 base pairs) at such relatively low polymer concentrations. When T4 DNA ligase joins small blunt-ended DNA segments (e.g., 10 base pairs long) at similar polymer concentrations, a series of products of increasing chain length are formed. At least the bulk of these products is linear, presumably because the ability of relatively short double-stranded DNA molecules to circularize is severely limited by restrictions on twisting and bending of the duplex molecules (13).

At higher polymer concentrations, both T4 DNA ligase and T4 RNA ligase tend to make large linear products. These have been most strikingly demonstrated in polyethylene glycol solutions with either ligase and to a lesser extent with a variety of other polymers for T4 DNA ligase. The large products of either ligase in concentrated polyethylene glycol solutions sediment readily and are apparently highly aggregated while in the presence of the polyethylene glycol. This behavior of the products is reminiscent of earlier descriptions of "psi DNA" (14,15) and indeed the conditions eliciting the sedimentable behavior are minor extrapolations in Mg^{++} and polyethylene glycol concentrations from those yielding psi DNA (16). The sedimentability of the large products made by the RNA ligase from oligoriboadenylates may well be a manifestation of the phase-separation behavior of poly(rA) studied by Eisenberg and Felsenfeld (17).

The ligase stimulations in both low and high concentrations of polymers presumably stem from excluded volume effects (18). The nonspecific effect of the polymers and the requirement for relatively concentrated solutions of polymers is consistent with this idea. Formation of the large linear products can be readily understood in terms of the high effective concentration of substrate termini due to macromolecular crowding (1,19). The stimulation of formation of circular products by crowding is probably a result of changes in tertiary structure which increase the compactness of the polynucleotide

substrates. Further studies on the effects of crowding on model substrates which can undergo both intermolecular and intramolecular joining should help in understanding these effects.

In concentrated PEG 8000 solutions, formation of hybrid molecules by ligation of 5'-phosphate terminated $(dT)_{10}$ to nonphosphorylated $(rA)_{9-60}$ (20, 21) is significantly stimulated. In contrast, oligodeoxynucleotides are not good substrates for self-ligation by T4 RNA ligase, either under conventional conditions or in the presence of high concentrations of PEG 8000. These substrates tend to be converted to their adenylyl derivatives which are then slow to be ligated (7,11,20). High polymer concentrations do, however, increase the rate of the activation reaction up to 10-fold.

Polymer-stimulated ligation is likely to be effective for many other substrates and combinations of substrates, not only among the large number of materials known to be ligated by T4 RNA ligase but also including other materials which are inactive as substrates under conventional assay conditions. The materials to be tested can be readily screened by ligation at a variety of PEG 8000 concentrations (0, 5,..., 30% w/v) in the presence of 5 mM Mg^{++}. When the Mg^{++} concentration is significantly lower, either intentionally or due to chelation by other reaction components such as nucleotides or EDTA, the most striking effects on ligation have not been obtained (1,2)--presumably due to an absence of the "psi" aggregation phenomenon mentioned above.

Finally, we note that RNA ligase of the type used in this study has only been found in bacteriophage-infected Escherichia coli (5). In view of the ability of macromolecular crowding to elicit enzymatic activities which are undetectable under conventional assay conditions (1), it would be of interest to test other prokaryotic and eukaryotic sources under crowded conditions for this type of RNA ligase. In addition, distinctly different RNA ligases which join substrates containing 2'-phosphate or 2',3'-cyclic phosphate groups, have been isolated from a variety of sources (22,23). A comparison of the response to macromolecular crowding of such RNA ligases to that of the T4 RNA ligase might be informative.

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ABBREVIATION:

PEG, polyethylene glycol.

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