Enzymic Joining of Polynucleotides

IV. Formation of a Circular Deoxyadenylate–Deoxythymidylate Copolymer

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The polynucleotide-joining enzyme from Escherichia coli catalyzes an intramolecular joining reaction with linear deoxyadenylate–deoxythymidylate oligomers leading to the formation of single-stranded circular molecules. The rate of circle formation from oligomers in the range of 34 to 60 nucleotide residues increases with increasing chain length and temperature. The smallest circle thus far synthesized is composed of 34 to 36 nucleotides.

1. Introduction

A striking feature of recent studies on the structure of DNA has been the ubiquity of circular forms. A wide variety of naturally occurring single- and double-stranded circular DNA's has been discovered, including an intracellular form of DNA from phage λ (Young & Sinsheimer, 1964; Bode & Kaiser, 1965) and DNA's from phage ϕX174 (Sinsheimer, 1959; Burton & Sinsheimer, 1963), polyoma virus (Dulbecco & Vogt, 1963; Weil & Vinograd, 1963), HeLa cells (Radloff, Bauer & Vinograd, 1967), trypanosomes (Riou & Paoletti, 1967) and animal cell mitochondria (Borst & Ruttenberg, 1965; Kroon, Borst, Van Bruggen & Ruttenberg, 1966). In the case of λ DNA, covalently closed circular molecules may be derived from the corresponding hydrogen-bonded forms by the action of a DNA-joining enzyme (Gellert, 1967; Olivera & Lehman, 1967; Gefter, Becker & Hurwitz, 1967), and a similar situation may exist with respect to other circular DNA's.

In this communication, we describe the synthesis of a single-stranded circular form of the alternating copolymer, dAT† by means of the polynucleotide-joining enzyme from Escherichia coli. These circles are formed from linear molecules by an intramolecular joining reaction, which can occur as a consequence of the self-complementary repeating nucleotide sequence of poly dAT. Thus, when the 3'-hydroxyl group of a linear dAT chain assumes a position immediately adjacent to the 5'-phosphoryl terminus of the same chain, a phosphodiester bond can be formed between the two termini and a circle is generated (Fig. 1).

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‡ Abbreviations used: the revised tentative rules of IUPAC-IUB are followed. Reference to this and some additional comments are given in the preceding paper. The following additional abbreviations are used: d(pTpA)ₙ and d(pTpA)₂₁, dAT oligomers bearing 5'-phosphoryl termini containing 19 and 21 adenine–thymine base pairs, respectively; d-TpA(pTpA)₂₁ and d-TpA (pTpA)₂₀, dAT oligomers without 5'-phosphoryl termini containing 19 and 21 adenine–thymine base pairs, respectively.

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FIG. 1. Conformation of dAT oligomers in solution.

Species I represents the completely random coil observed at elevated temperatures; species II represents the symmetrical hairpin which is probably the most stable (and predominant) form below the helix-coil transition; species III (a, b and c) represent partially melted forms which may have a transient existence; species IV is presumably the conformation which can be enzymatically converted to the closed circular form (species V). The distribution of the various species is probably not static (Scheffler et al., 1968) and the rate of circle formation is probably determined by the concentration of conformation IV.

The dAT circle is unique in that it is single-stranded and at the same time double-helical. Although this form is unlike any circular DNA thus far described, it is of interest because of the simplicity of its structure and the fact that a single molecular species of defined chain length can be readily obtained. Aside from their intrinsic interest, these chemically defined polynucleotides lacking termini may be useful as models of circular DNA's for enzymological and physical chemical studies.

2. Materials and Methods

(a) Polynucleotides

The dAT copolymer was prepared in a primed synthesis using E. coli DNA polymerase (Schachman, Adler, Radding, Lehman & Kornberg, 1960). It was degraded to a mixture of shorter units (chain length approximately 150) by treatment with micrococcal nuclease followed by E. coli alkaline phosphatase, as described previously for the dA and dT homopolymers (Olivera & Lehman, 1967). Homogeneous fractions of dAT oligomers \(d(pT)pA\) and \(d(pT)pA\) were prepared from pancreatic DNase digests of dAT copolymers by preparative disc gel electrophoresis (Elson & Jovin, 1968; Scheffler, Elson & Baldwin, 1968). The \(d(pT)pA\) was contaminated to a small extent (10% or less) with \(d(pT)pA\) and \(d(pT)pA\). After treatment with alkaline phosphatase, the unfractonated mixture, as well as the \(dT(pT)pA\) and \(dT(pT)pA\), were labeled at their 5'-termini with \(^{32}P\) by treatment with \(\gamma^{32}P\)-labeled ATP and polynucleotide kinase (Richardson, 1965).

(b) Enzymes

Crystalline pancreatic DNase was obtained from Worthington Biochemical Corp. E. coli exonuclease III (Richardson & Kornberg, 1964); E. coli exonuclease I (Lehman & Nussbaum, 1964); E. coli endonuclease I (De Waard & Lehman, 1966); E. coli DNA polymerase (and exonuclease II) (Jovin, Englund & Bertsch, manuscript in preparation) were prepared and assayed as described previously. Fraction VI of E. coli polynucleotide-joining enzyme
ENZYMATIC JOINING OF DNA. IV

Olivera & Lehman, 1967 was used for most of the experiments; in a few instances, where indicated, a more purified preparation obtained after DEAE-Sephadex chromatography of fraction V by Dr Y. Anraku was used. All other enzymes and reagents are described in the preceding paper (Olivera & Lehman, 1968).

(c) Enzyme assays

Polynucleotide-joining enzyme assays were performed using a reaction mixture containing 10 mM-Tris-HCl, pH 8.0, 2 mM-MgCl₂, 1 mM-EDTA, 0.01 mM-NAD, 5',32P-labeled dAT oligomer and bovine plasma albumin, 50 µg/ml. The concentrations of enzyme and polynucleotide were varied as indicated in the legends to the Tables and Figures. Conversion of the 5',32P-group to a form in which it was insusceptible to phosphatase was measured as described previously (Olivera & Lehman, 1967). Conversion of the 5',32P-group to an acid-soluble form was determined in the following way: 0.1 ml. of carrier salmon sperm DNA (2.5 mg/ml.), 0.1 ml. of 0.1 M-PP₁, and 0.5 ml. of cold 3.5% perchloric acid-0.35% uranyl acetate were added per 0.1 ml. of reaction mixture. After 10 min at 0°C the resulting precipitate was collected on a Whatman glass filter (GF/C 2.4 cm) and washed 3 times (10-ml. portions) with cold 1 N-HCl, 3 times with 1 N-HCl-0.1 M-PP₁, and once with absolute ethanol. The filter was dried and its radioactivity determined.

(d) Physical methods

Analytical polyacrylamide gel electrophoresis was performed by the method of Jovin, Chrambach & Naughton (1964), modified (Elson & Jovin, 1968) to allow acrylamide concentrations up to 20%. Molecular weight measurements were carried out using a Spinco model E ultracentrifuge equipped with a photoelectric scanner (Scheffler et al., 1968). Melting curves were measured according to the procedure described by Inman & Baldwin (1962). Radioactivity was determined using a Nuclear-Chicago gas-flow counter equipped with a Micromil window. Optical density measurements were made with a Zeiss PMQ spectrophotometer.

3. Results

(a) Formation of circles from a mixture of dAT oligomers

Treatment of 5',32P-labeled dAT oligomers (average chain length, 150) with polynucleotide-joining enzyme under standard assay conditions led to a substantial conversion of the 5',32P-group to a form in which it was insusceptible to E. coli alkaline phosphatase, indicating that it had been incorporated into a phosphodiester bond (Fig. 2(a)). This result might have been produced in at least two ways: (1) the two ends of a single oligomer could have folded back upon each other so as to bring the 5'-phosphoryl and 3'-hydroxyl ends of the same strand in correct juxtaposition to each other, generating a circular molecule; or (2) the dAT chains could have formed staggered hydrogen-bonded aggregates which were joined to form long linear structures.

That circular molecules were, in fact, produced was demonstrated by treating the product with E. coli exonucleases I or III, each of which requires a free 3'-terminus to initiate hydrolysis and is capable of degrading poly dAT completely to acid-soluble fragments. As shown in Figure 2(b), there was excellent agreement between the percentage of total 32P which became insusceptible to alkaline phosphatase and that which was insusceptible to exonuclease. Therefore, circular oligo-dAT molecules were the exclusive product of the joining reaction.

(b) Formation of circles from dAT oligomers of defined chain length

When d(pTpA)₅₇ labeled in its 5'-phosphate with 32P was treated with polynucleotide-joining enzyme (fraction V), approximately 25% of the molecules were
Fig. 2. Formation of dAT circles from a mixture of linear dAT oligomers.

A preparation of dAT oligomers (1·2 × 10^{-5} M in termini, average chain length 135) in a volume of 1·0 ml. was incubated with 3 units of polynucleotide-joining enzyme in standard reaction medium; except that 0·05 ml. of a boiled crude extract of E. coli (Olivera & Lehman, 1967) served as a source of NAD; an identical control reaction mixture was prepared which lacked joining enzyme. At the times indicated, 0·05-ml. portions of each reaction mixture were removed, heated for 2 min at 100°C, then treated with alkaline phosphatase to assay for phosphodiester bond formation. After 60 min, the remainder of both reaction mixtures was heated for 2 min at 100°C and subjected to exonucleases I or III. For treatment with exonuclease III, 0·1 ml. of the reaction mixture was mixed with 0·2 ml. of a solution composed of 0·1 mM-Tris-HCl, pH 8·0, 1 mM-MgCl₂ and 10 mM-β-mercaptoethanol; the indicated amounts of exonuclease III were then added. For treatment with exonuclease I, 0·1 ml. of reaction mixture was mixed with 0·2 ml. of a solution composed of 0·1 mM-glycine, pH 9·5, 10 mM-MgCl₂ and 2·5 mM-β-mercaptoethanol; 30 units of exonuclease I were added. Since 1 unit of exonuclease I is equivalent to 10 units of exonuclease III, this point is plotted as 300 units on the abscissa. After reaction with exonuclease, the fraction of 32P remaining acid-soluble was determined as described in Materials and Methods.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Perchloric acid-uranyl acetate-insoluble 32P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>22</td>
</tr>
<tr>
<td>Exonuclease I</td>
<td>24</td>
</tr>
<tr>
<td>Endonuclease I</td>
<td>17</td>
</tr>
<tr>
<td>Endonuclease I, followed by exonuclease I</td>
<td>2</td>
</tr>
<tr>
<td>Pancreatic DNase</td>
<td>17</td>
</tr>
<tr>
<td>Pancreatic DNase followed by exonuclease I</td>
<td>&lt;0·5</td>
</tr>
<tr>
<td>Exonuclease II</td>
<td>20</td>
</tr>
</tbody>
</table>

A preparation of 8·4 × 10^{-8} M (in termini) 32P-labeled d(pTpA)₁₉ in a volume of 0·3 ml. was treated for 30 min with 0·4 unit of polynucleotide-joining enzyme under standard reaction conditions as described in Materials and Methods. At the end of the reaction, the mixture was heated at 100°C for 2 min. Portions (0·01 ml.) of the reaction mixture were diluted with standard assay buffer to a final volume of 0·1 ml. They were then treated with the enzymes indicated for 20 min at 37°C. Reaction mixtures treated with exonucleases I or II contained 0·05 ml. of 0·05 mM-glycine buffer (pH 9·5) which was added after incubation with the joining enzyme. The levels of enzymes added (in units) were: alkaline phosphatase, 4; exonuclease I, 28; endonuclease I, 0·4; and exonuclease II, 25; 10 µg of pancreatic DNase were used. After incubation, acid-soluble 32P was determined as described in Materials and Methods. The acid-soluble 32P found after endonuclease treatment probably represents low-molecular weight degradation products of the endonucleases which were still insoluble in perchloric acid-uranyl acetate.
converted to circles as judged by the extent of conversion of the $^{32}$P to a phosphatase- and exonuclease I- and II-insensitive form. The relatively low extent of conversion is most probably the result of contamination of this particular preparation of fraction V with exonucleases, either exonuclease I (Lehman, 1960) or oligonucleotide diesterase (Jorgensen & Koerner, 1966). Each of these enzymes would be expected to degrade rapidly the dAT oligomer substrate but not the circular product. It was, in fact, found that approximately 75% of the $^{32}$P added initially was in the form of 5'-mononucleotides at the conclusion of the reaction with the joining enzyme. A more purified preparation of the polynucleotide-joining enzyme (DEAE-Sephadex fraction) is essentially free of exonuclease activity and with such preparations conversions in excess of 80% have been observed (see below).

The effect of endonuclease treatment provided further proof for the circularity of the dAT oligomers. A product was prepared from d(pTpA)$_{21}$ which was insusceptible to both phosphatase and exonuclease. Upon treatment with *E. coli* endonuclease I or pancreatic DNase, essentially all (>98%) of the $^{32}$P was converted to an exonuclease-sensitive form (Table 1).

\[(c)\] Physical properties of linear and circular d(pTpA)$_{21}$

Circles were generated from d(pTpA)$_{21}$, then isolated by extraction of the reaction mixture with phenol followed by dialysis. The circular product was subjected to polyacrylamide gel electrophoresis and found to have a mobility very close to the linear d(pTpA)$_{21}$. Its sedimentation properties were examined in the ultracentrifuge.

### Table 2

**Physical properties of linear and circular d(pTpA)$_{21}$**

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Linear</th>
<th>Circular</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\frac{2 , RT}{\alpha^2} \left( \frac{d \ln A}{d \alpha^2} \right)$</td>
<td>$5520 \pm 50$</td>
<td>$5600 \pm 50$</td>
</tr>
<tr>
<td>$T_m$ (°C)</td>
<td>$38.0 \pm 0.2$</td>
<td>$37.2 \pm 0.2$</td>
</tr>
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</table>

The reaction mixture (3.8 ml.) for the preparation of circular d(pTpA)$_{21}$ was composed of 3.5 x 10$^{-6}$ M (in termini) 5',32P-labeled d(pTpA)$_{21}$, 4.0 µM-NAD, 10 mM-Tris-HCl (pH 8.0), 2 mM-MgCl$_2$, 1 mM-EDTA, bovine plasma albumin, 40 µg/ml. and 12 units of polynucleotide-joining enzyme. After incubation for 60 min at 37°C the NAD concentration was raised to 6 µM, an additional 6 units of enzyme were added and the mixture was incubated for 30 min more. A value of 30% conversion (as judged by measurement of phosphatase-insensitive $^{32}$P) was reached at 60 min and did not increase upon further incubation. The reaction mixture was extracted twice with 1-ml. portions of redistilled, water-saturated phenol and the phenol removed by extraction of the aqueous phase with ether. The sample was dialyzed against 30% Carbowax until its volume was reduced to 1.0 ml., then dialyzed against 0.05 M-sodium perchlorate-0.01 M-sodium cacodylate buffer (pH 7.0). To remove contaminating linear oligomers, the sample was redialyzed against a solution composed of 0.1 M-glycine buffer (pH 9.5), 0.01 M-MgCl$_2$ and 2.5 mM-$\beta$-mercaptoethanol, then treated with exonuclease I (50 units) for 30 min at 37°C. The phenol and ether extractions were repeated and the sample redialyzed against 5 mM-sodium perchlorate, 1 mM-sodium cacodylate, 1 mM-EDTA (pH 7.0). This buffer was used for the measurement of melting curves. To prevent aggregation of the linear molecules, molecular weight determinations were performed in a solvent composed of 0.5 M-sodium perchlorate and 0.02 M-sodium hydroxide.

Methods for the determination of relative molecular weights and measurement of melting curves are given in Materials and Methods.
(Scheffler et al., 1968). From the trace of the absorbance versus distance, the quantity \(2RT/\omega^2 \cdot (\ln A/dr^2)\) was obtained which is proportional to the weight-average molecular weight at \(r\) (\(R\) = gas constant, \(T\) = absolute temperature, \(\omega\) = angular velocity, \(A\) = absorbance and \(r\) = distance from the center of rotation). In separate runs, at the same temperature and at the same speed, the molecular weights of the linear \(d(pTpA)_{21}\) and the circular form derived from it were identical within an experimental error of less than 5% (Table 2). The identity of molecular weights of the linear and circular molecules together with the close correspondence in

![Graph](image)

**Fig. 3.** Sucrose density-gradient sedimentation of dAT oligomer mixture after treatment with polynucleotide-joining enzyme.

5\(^{\circ}\)P-labeled dAT oligomers were prepared from a pancreatic DNase digest of dAT copolymer by treatment first with phosphatase, then polynucleotide kinase and \([\gamma\text{-}32P]ATP\). The unfraccionated mixture of oligomers (\(2 \times 10^{-6} \text{ M in } \text{termini}\) was incubated with 0.22 unit of joining enzyme in 0.20 ml. of standard reaction medium for 75 min at 37\(^{\circ}\)C. An additional 0.22 unit was added at 45 min. The particular preparation of fraction V of joining enzyme used in this experiment appeared to be relatively free of exonuclease activity. Alkaline sucrose-gradient sedimentation was then performed on equal portions of the reaction mixture treated with phosphatase (---O---) and untreated with phosphatase (---O---), as well as on the original [5\(^{\circ}\)P]dAT oligomer mixture (---O---) (untreated with joining enzyme or phosphatase). The sedimentation medium was a linear gradient from 5 to 20% sucrose, containing 0.7 m-NaCl-0.3 m-NaOH. Sedimentation of the samples was performed on a Spinco model L centrifuge, using an SW29 rotor at 38,000 rev./min at 20\(^{\circ}\)C for 57 hr (Martin & Ames, 1961). Nine fractions were collected from each tube and counted. The Norit-adsorbable radioactivity (---O---) was determined in fractions obtained from the reaction mixture treated with phosphatase. The normalized radioactivity distributions are plotted on the upper panel and the fraction of radioactivity remaining Norit-adsorbable after phosphatase treatment (i.e. percentage conversions to circles) is plotted on the lower panel.
electrophoretic mobility in polyacrylamide gels excludes the possibility that circular dimers or higher oligomeric circles were formed to a significant extent†.

The temperature melting curves of the linear and circular forms of d(pTpA) were measured at pH 7.0 in medium of low ionic strength (5 mM-sodium perchlorate, 1 mM-sodium cacodylate and 1 mM-EDTA). The midpoint of the transition for the linear oligomer was approximately 1°C below that of the high-molecular weight dAT polymer, whereas the $T_m$ for the circular form of d(pTpA) was nearly 20°C above that of the polymer (Table 2).

![Graph showing comparison of rate of dAT circle formation with rate of joining of dT oligomers.](image)

(d) Comparison of rate of dAT circle formation with rate of joining of dT oligomers

To compare the rate of dAT circle formation with the rate of joining of dT oligomers, it was necessary to prepare a mixture of dAT oligomers capable of reacting to the same extent as the (dA)-(dT) homopolymer pair. The following procedure was devised. High-molecular weight dAT copolymer was degraded with pancreatic DNase. This treatment yields a mixture of oligomers bearing thymine at their 5'-phosphoryl and adenine at their 3'-hydroxyl termini (Scheffler et al., 1968); hence, all the chains may assume a conformation in which the two termini of a hairpin structure are directly adjacent to each other (Fig. 1). The mixture of dAT oligomers was treated sequentially with phosphatase and polynucleotide kinase in the presence of [γ-32P]ATP to label the 5'-termini with 32P. Three reaction mixtures containing labeled dAT oligomers were prepared; one was incubated with joining enzyme, then treated with phosphatase, the second treated only with joining enzyme and the third incubated in the absence of either enzyme. The samples were then sedimented in a sucrose density-gradient (Fig. 3). It is apparent that only the more rapidly sedimenting oligomers in the mixture were efficiently (>70%) converted to circles. Although

† The observation that a relatively homogeneous population of circular d(pTpA) was formed despite the extensive (presumably exonucleolytic) degradation of the linear oligomers suggests that the contaminating nuclease acted by an "all or none" mechanism, forming a non-dissociable enzyme–substrate complex which released mononucleotides as it progressed along the dAT chain.
Fig. 5. Effect of chain length and of temperature on the rate of dAT circle formation.

Three reaction mixtures (0.25 ml. each) containing $2 \times 10^{-6}$ M (in termini) [5'-32P]dAT oligomers (average chain length of approximately 40) prepared from a pancreatic DNase digest of dAT copolymer as described in Materials and Methods were treated with 1.6 units of polynucleotide-joining enzyme (DEAE–Sephadex fraction) at the temperatures and for the time-periods indicated. A fourth reaction mixture lacking joining enzyme served as a control. After incubation, the reactions were stopped by heating for 2 min at 100°C. A portion (0.05 ml.) from each reaction mixture was treated with 4 units of phosphatase for 30 min at 42°C. Then 0.22 μmole (nucleotide) of a mixture of unlabeled dAT oligomers (from the pancreatic DNase digest which served as a source of the 5'-32P-labeled oligomers) was added together with 0.01 ml. of a saturated solution of sucrose and 0.01 ml. of 0.01% bromphenol blue. The mixture was then subjected to polyacrylamide gel electrophoresis (Elson & Jovin, 1968). A second 0.05-ml. portion not incubated with phosphatase was also run under these conditions. After electrophoresis, the gels were stained with toluidine blue to reveal the bands of oligomers (Plate I). Each band was then cut out and dried and the 32P determined. The radioactivity in the bands of the gel of the phosphatase-treated sample was compared with that in the corresponding bands of the sample not treated with phosphatase to determine the percentage conversion of the 32P to a phosphatase-insensitive form. (This was possible only up to the 19th band, after which resolution was inadequate to permit individual bands to be cut out.) Comparison of 32P in bands from samples not treated with phosphatase with comparable bands from the reaction mixture lacking joining enzyme (dashed line), indicated that nucleolytic degradation of the dAT oligomers in the course of reaction with joining enzyme was negligible. The assignment of chain lengths to the individual bands was accomplished (1) by running a parallel gel of dAT oligomers to which was added known 5'-32P-labeled d(pTpA)$_{13}$ and (2) by adding approximately 0.20 A$_{260}$ unit of unlabeled d(pTpA)$_{13}$ to the mixture of dAT oligomers and determining that the stain intensity of band number 3 increased after electrophoresis and staining with toluidine blue.

The chain lengths of these oligomers is not known precisely, they are probably in the range of 45 to 60 nucleotides (see Fig. 5).

As shown in Figure 4, at 30°C the rate of formation of dAT circles from the fractionated oligomers (fractions 3 and 4) was approximately fourfold less than the rate of joining of dT oligomers hydrogen-bonded to dA chains when examined at equal oligomer concentrations. At 20°C the rates were nearly equivalent.

(e) Dependence of circle formation on oligomer chain length

To evaluate the effect of chain length of dAT oligomers on the rate of circle formation, the following experiment was performed. Mixtures of 5'-32P-labeled dAT oligomers (average chain length approximately 40) were incubated with the DEAE–Sephadex fraction of the polynucleotide-joining enzyme at 20, 30 and 40°C.
A separate portion of the oligomer mixture not treated with enzyme served to evaluate the distribution of chain lengths in the digest. After incubation, two equal portions were withdrawn from each reaction mixture, one of which was treated with alkaline phosphatase. A mixture of unlabeled linear dAT oligomers was added to each sample; they were then subjected to acrylamide gel electrophoresis and stained (Plate I). Each of the stained bands corresponds to a single species of d(pTpA)_n (Elson & Jovin, 1968). The values of n were determined either by comparison with a homogeneous ^32P-labeled oligomer of known chain length or, alternatively, by adding a relatively large amount of unlabeled oligomer of known chain length to a sample before electrophoresis, then determining which of the resulting bands acquired a more intense stain. The bands were cut out of the gels and the percentage of conversion to circles at each chain length was determined by a comparison of the ^32P in the samples treated with phosphatase and those left untreated (Fig. 5). Since the level of ^32P in the bands derived from the control reaction (lacking both joining enzyme and phosphatase) was very similar to that in the samples treated with joining enzyme but not phosphatase, we may infer that degradation of the dAT oligomers (by contaminating nucleases) in the course of the reaction did not occur to a significant extent.

Three conclusions may reasonably be drawn from this experiment, at least with regard to the conditions examined. (1) At a given temperature the rate of circle formation shows a strong dependence on the chain length of the oligomer, reaching a broad maximum at a value of n of approximately 25; (2) the rate of reaction for an oligomer of given chain length is strongly dependent upon temperature; and (3) there is a lower limit in size (n of 17) below which circle formation is extremely slow or does not occur at all; this limit appears to be independent of temperature.

Circular molecules can also be generated from high-molecular weight dAT copolymer. Incubation of dAT copolymer (0.5 μmole of termini) of chain length greater than 10,000 with 0.3 unit of joining enzyme for 75 minutes at 37°C led to the conversion of more than 60% of the linear molecules to a circular form. Hence, there is no obvious upper limit to the chain length at which circle formation can occur.

4. Discussion

It is clear that single-stranded circular forms of the dAT copolymer and smaller oligomers can be generated in reactions catalyzed by the polynucleotide-joining enzyme. Although the product is very similar to the linear molecule from which it is derived in both molecular weight and electrophoretic mobility, it does not contain a free 3'-terminus as judged by its insusceptibility to exonucleases I, II and III, and, in the case of d(pTpA)_21, it has a T_m nearly 20°C higher than the linear molecule. These results also eliminate the possibility that diester bonds have been formed between aggregated chains.

The temperature dependence of the rate of circle formation is obviously complex. The increased rate at higher temperatures is probably related not only to the overall activation energy of the joining reaction itself (i.e. formation of the diester bond) but also to the temperature dependence of chain slippage; i.e. the rate of

† Circles generated from d(pTpA)_21 migrated with a mobility equal to or slightly greater than that of the corresponding linear oligomer. Since the discrepancy would be expected to decrease in the case of the higher oligomers, the difference in mobility was neglected in the calculations of the relative conversions.
Plate I. Photograph of stained polyacrylamide gel after electrophoresis of dAT oligomers (for details see legend to Fig. 5).

The heavy band (band 1) contains unresolved oligomers up to and including d(pTpA)$_{11}$; band 2 is d(pTpA)$_{12}$ and each successive band is one d(pTpA) unit longer. The direction of migration is from the top to the bottom of the gel.
interconversion of the various conformations of the oligomeric helices with loops at different locations relative to the ends of the strands (Fig. 1 and Scheffler et al., 1968). Thus, the longer oligomers would be expected to have a greater tendency to assume stable conformations with multiple loops in which the 5'-phosphoryl and 3'-hydroxyl termini are properly aligned. On the other hand, shorter oligomeric species with two loops may be relatively unstable, so that the lower limit in size may correspond to that length below which the lifetime of the joinable species is too short to permit phosphodiester bond synthesis. An obvious difficulty with this hypothesis is the observed temperature-independence of the lower limit of chain length at which joining occurs. However, a chain length specificity of the enzyme may be superimposed on a temperature-dependent slippage process so that steric requirements of the enzyme itself may prohibit closure of smaller oligomers regardless of the temperature of the reaction.

The finding that at 30°C the rate of dAT circle formation was fourfold lower than the rate of joining of dT oligomers in the (dA)·(dT) homopolymer pair, whereas at 20°C the two rates were nearly equivalent, would suggest that there may be differences in the mechanism of chain slippage in these two types of polymers. However, further work is clearly needed to establish this point.

The dAT circles of known and homogeneous chain length are easily prepared and constitute an interesting and potentially useful series of model compounds for enzymological and physical studies. For example, the absence of termini permits a direct quantitative estimate of "end effects" in thermal and pH melting. Similarly, such defined circular molecules may be excellent templates for studies of the initiation of synthesis by DNA and RNA polymerases. They may also be useful as highly sensitive substrates for the detection and assay of endonucleases.

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