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Enzymatic Joining of Polynucleotides

IX. A SIMPLE AND RAPID ASSAY OF POLYNUCLEOTIDE JOINING (LIGASE) ACTIVITY BY MEASUREMENT OF CIRCLE FORMATION FROM LINEAR DEOXYADENYLATE-DEOXYTHYMIDYLATE COPOLYMER*

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SUMMARY

A new method for the assay of polynucleotide joining activity is described; it measures the conversion of ^{3}H-labeled d(A-T) copolymer with 3′-hydroxyl and 5′-phosphoryl termini to a form resistant to exonuclease III. The method is rapid and precise and is suitable for assay of crude cell extracts. The product of the action of joining enzyme on the d(A-T) copolymer has the properties of a circular molecule. The optimal chain length of the d(A-T)$_n$ substrate is approximately 1000 nucleotides.

Polynucleotide joining enzymes (ligases), which catalyze the conversion of single strand interruptions in DNA to phosphodiester linkages accompanied by the hydrolysis of DPN or ATP, have been studied in several laboratories (1–6). These joining activities have been measured by a variety of assay methods: the change in sedimentation coefficient after covalent closure of hydrogen-bonded λ DNA circles (1, 2, 4); differential adsorption to hydroxylapatite after denaturation of hydrogen-bonded λ DNA dimers (3); conversion of an internally located 5′-^{32}P to a form resistant to alkaline phosphatase (2, 5); linkage of a polynucleotide strand to a second one attached to cellulose (6); and restoration of transforming activity to DNA previously treated with pancreatic DNase (7). Several assays which measure the first step in the ligase-catalyzed reaction have also been described; the formation of the enzyme-adenylate intermediate from DPN has been used for the *Escherichia coli* enzyme (8), and an ATP-PP$_i$ exchange reaction has been used to assay the T4-induced ligase (9).

Although these methods have clearly been adequate for the detection and quantitative assay of DNA joining activities, they do have several significant limitations. The joining assays either utilize substrates which are difficult to prepare or the assays themselves are laborious. Moreover, the methods which rely on the partial reaction do not measure the true catalytic capacity of the enzyme. Finally, for those assays which depend on a ^{32}P-labeled substrate the polynucleotide must be prepared at frequent intervals to maintain the specific radioactivity required for adequate sensitivity.

This paper describes a method for the rapid and sensitive assay of joining activity that works well in crude extracts. It utilizes a long lived substrate that is easy to prepare in large quantities. The method is based on the previous finding that the joining enzyme can catalyze the formation of single stranded circular molecules from d(A-T)[1] oligomers with 3′-hydroxyl and 5′-phosphoryl termini (10) and measures the conversion of ^{3}H-labeled d(A-T)$_n$ to a form which is resistant to exonuclease III.

EXPERIMENTAL PROCEDURE

Materials

Nucleotides and Enzymes—dATP and dTTP were purchased from Calbiochem, DPN from Sigma, and ^{3}H-dTTP (4 to 10 Ci per mmole) from Schwarz BioResearch; γ-^{32}P-ATP was prepared according to Glynn and Chappell (12). *E. coli* joining enzyme was Fraction VI of Anraku, Anraku, and Lehman.[2] *E. coli* DNA polymerase (Fraction VII) and exonuclease III were isolated according to Jovin, Englund, and Bertsch (13) and Richardson and Kornberg (14), respectively. Polynucleotide kinase was prepared according to Richardson (15). *E. coli* alkaline phosphatase was isolated by the procedure of Malamy and Horecker (16) and assayed by the method of Garen and Levinthal (17). Pancreatic DNase obtained from Worthington (Code D, 2650 units per mg) was dissolved at a concentration of 1.0 mg per ml in cold 0.02 N HCl and frozen in small batches which were thawed as needed, used once, and discarded. The enzymes were diluted as required into the following buffers—DNase: 0.01 M potassium phosphate (pH 7.4), 5 mM $MgCl_2$, and bovine plasma albumin (1 mg per ml); purified joining enzyme: 0.05 M Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 1 mM EDTA, and bovine plasma albumin (0.5 mg per ml) (crude extracts of *E. coli*,

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[1] The revised tentative rules of IUPAC-IUB for abbreviations have been followed (11). d(A-T) refers to a double stranded copolymer composed of alternate deoxyadenylate and deoxythymidylate residues. The term oligomer (or oligo) is used for chains composed of fewer than 500 nucleotide residues; the term polymer (or poly) is reserved for chains containing more than 500 residues.

[2] Y. Anraku, N. Anraku, and I. R. Lehman, manuscript in preparation.

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Salmonella typhimurium, and T4-infected *E. coli* to be assayed for joining enzyme were diluted into the same buffer, except that 0.01 M β-mercaptoethanol was added for the T4-infected cell extracts); DNA polymerase and exonuclease III: 0.05 M Tris-HCl (pH 7.4), 0.10 M ammonium sulfate, 0.01 M β-mercaptoethanol, and bovine plasma albumin (1 mg per ml).

Bacterial and Phage Strains—The *E. coli* strains used were 1100 (an endonuclease I-deficient strain from Professor H. Hoffman-Berling (18)), B, and CR63. Phages T4r$^+$, T4*am*N82 (gene 44), and T4*am*E605 (gene 30) were provided by Dr. R. S. Edgar and Dr. John Drake; *E. coli* CR63 served as the permissive and *E. coli* B as the nonpermissive host for the amber mutants. *S. typhimurium* LT2 strains EL2, SL1561, and EL10 were provided by Dr. Esther Lederberg.

Methods

Preparation of Crude Extracts—Bacteria were grown in yeast extract-phosphate-glucose medium (19) at 37°, *E. coli* was grown with aeration, and *S. typhimurium* in standing culture. When the absorbance at 595 mμ reached 0.8 to 1.0 (about 5 × 10^8 cells per ml) the cells were harvested by centrifugation in the cold. T4-infected *E. coli* B were prepared by infecting *E. coli* B growing in H broth (20) with a multiplicity of 5 at a cell density of 5 × 10^8 per ml, incubating at 37° for 15 min (except for T4*am*N82 infection for which incubation was continued for 60 min), pouring the cultures over crushed ice, and harvesting the cells by centrifugation. The uninfected and infected cell pellets were suspended in a buffer composed of 0.05 M glycylglycine (pH 7.0), 1 mM EDTA, and 1 mM glutathione to 0.1 to 0.2 g, wet weight, per ml and were disrupted with five 30-sec bursts with a Mullard sonic drill. The extracts were then centrifuged at 15,000 × *g* for 20 min and the pellets were discarded. This procedure yielded extracts with a protein concentration of 10 to 20 mg per ml.

End Group Labeling of Synthetic DNA—End group labeling and number average length analysis with polynucleotide kinase and γ-^{32}P-ATP were performed as described by Weiss, Live, and Richardson (21).

Preparation of d(A-T)$_n$ Substrate—The d(A-T) copolymer was prepared by a modification of the method of Schachman *et al.* (22). It was found convenient to prepare unlabeled d(A-T)$_n$ and labeled d(A-T)$_n$ of high specific radioactivity separately in order to permit dilution to attain varying specific radioactivities without changing the concentration of nucleotide (concentration of polynucleotides are expressed as equivalents of nucleotide phosphorus); no significant difference between labeled and unlabeled preparations was noted. Unlabeled d(A-T)$_n$ was prepared in an incubation mixture (200 ml) containing 0.06 M potassium phosphate (pH 7.4), 6 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.5 mM dATP, 0.5 mM dTTP, and 3.5 μM d(A-T) copolymer treated with pancreatic DNase (see below). The reaction was started by the addition of 4.0 ml of DNA polymerase (500 units per ml) and incubated at 37°. ^{3}H-Labeled d(A-T)$_n$ was prepared in the same manner except that a 20-ml reaction volume was used and ^{3}H-dTTP was present at a final specific radioactivity of 0.1 mCi per μmole in the reaction mixture. The reaction was followed by recording the absorbance at 260 mμ in a 0.2-cm cuvette against a blank with an A_{260} of about 1. When the A_{260} achieved a minimum (after 4 to 5 hours), the reaction was stopped by adding solid NaCl to a final concentration of 1.0 M and heating at 70° for 25 min. The product was dialyzed against 1.0 M NaCl, 1 mM EDTA until the A_{260} of the dialysis buffer was less than 0.002. It was concentrated 3-fold by dialysis against solid polyethylene glycol (Carbowax 6000), and dialyzed against 40 volumes of 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA (2 changes). Applying a molar extinction coefficient of 6700 (23), this procedure yielded d(A-T)$_n$ preparations that were about 1 mM in nucleotide with an over-all yield of 30 to 40%. The number average length of the polymers was about 5000 nucleotides.

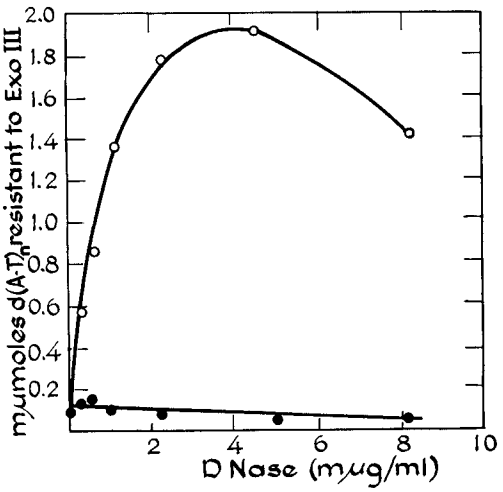

FIG. 1. Calibration curve for DNase digestion of ^{3}H-d(A-T) copolymer to form joining enzyme substrate. Reaction mixtures (0.11 ml) containing 0.09 M Tris-HCl (pH 8.06), 0.01 M $MgCl_2$, 1 mM EDTA, and 0.83 mM ^{3}H-d(A-T)$_n$ (1670 cpm per nmole) were incubated at 37° for 35 min in the presence of pancreatic DNase at the concentrations indicated. The DNase was inactivated by heating to 75° for 30 min and the product was tested as a substrate in the joining enzyme assay as described under "Methods." ●, blank values obtained from the assay in the absence of joining enzyme; ○, exonuclease III-resistant d(A-T)$_n$ corrected for the appropriate blank when 0.015 unit of purified *E. coli* joining enzyme was included in the assay.

Because such polymer preparations were almost inert in the joining reaction and were relatively poor primers for the DNA polymerase, they were treated with pancreatic DNase in order to find a length or a concentration of 3′-hydroxyl and 5′-phosphoryl termini (or both) that would maximize the formation of exonuclease III-resistant d(A-T)$_n$ under conditions chosen to perform the joining assay. Fig. 1 presents a typical calibration curve for the DNase digestion.

The incubation mixture used for large scale preparation of substrate contained (in 10 ml) 0.09 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, 1 mM EDTA, 0.83 mM ^{3}H-d(A-T) copolymer (1670 cpm per nmole), and pancreatic DNase, 4.5 mμg per ml. The reaction mixture was incubated at 37° for 35 min, heated to 75° for 30 min to inactivate the DNase, and then rapidly chilled in ice to favor intramolecular helix formation. The substrate prepared in this manner had a number average chain length of 700 to 1000 nucleotides residues as determined by end group labeling with polynucleotide kinase.

Polynucleotide Joining Enzyme Assays—The (dA)$_n$·(dT)$_n$ assay of Olivera and Lehman was performed as previously described (2) except that DPN was present at 26 μM. The (dA)$_n$·(dT)$_n$ was prepared by annealing 3′-hydroxyl and 5′-phosphoryl-oligo-dT (number average chain length of 180, labeled at the 5′-termini with ^{32}P at a specific radioactivity of

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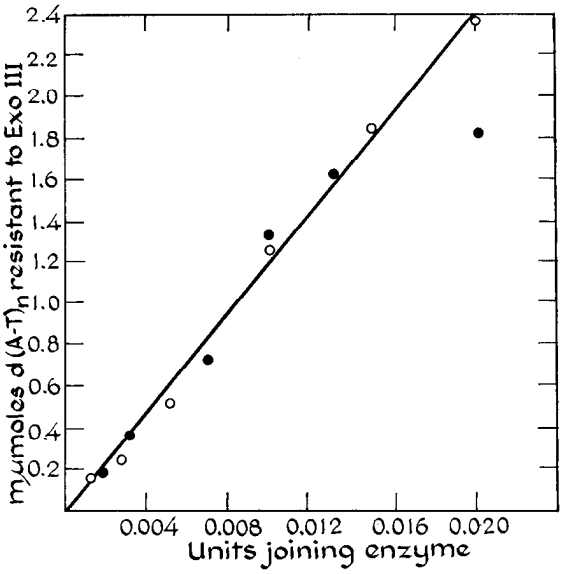

FIG. 2. Linearity of the $d(A\text{-}T)_n$ assay with purified *E. coli* joining enzyme. The assays were performed as described under "Methods" with ^3H-$d(A\text{-}T)_n$ with a specific radioactivity of 1670 cpm per nmole and contained the indicated amounts of enzyme. The different *symbols* represent assays performed on different days. The units shown were determined with the $(dA)_n \cdot (dT)_n$ method of Olivera and Lehman (2) to show that the $d(A\text{-}T)_n$ method is as sensitive as the $(dA)_n \cdot (dT)_n$ assay which is linear over the same range of enzyme.

4000 cpm per μμmole) to poly dA (number average chain length >2000).

The assay utilizing ^3H-$d(A\text{-}T)_n$ as substrate contained (in 0.1 ml) 0.03 M Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1.2 mM EDTA, 26 μM DPN, bovine plasma albumin (50 μg per ml), 0.16 mM ^3H-$d(A\text{-}T)_n$ (1670 cpm per nmole), and 0.002 to 0.02 unit of joining enzyme (as determined by the $(dA)_n \cdot (dT)_n$ assay). For assay of T4-ligase DPN was replaced by 0.2 mM ATP and the reaction mixture was supplemented with dithiothreitol to a final concentration of 10 mM. After incubation at 30° for 30 min the reaction was terminated by boiling for 2 min. Ten microliters of 0.1 M β-mercaptoethanol and 150 units of exonuclease III were added and the mixture was incubated at 37° for 30 min. The reaction mixture was chilled in ice, and 0.2 ml of 0.1 M Tris-HCl (pH 8), 50 μl of 0.25 mM calf thymus DNA, and 0.4 ml of cold 7% perchloric acid were added. After 10 min at 0° the mixture was filtered on a Whatman GF/C 2.4-cm glass filter previously soaked in 0.1 M sodium pyrophosphate. The filter was washed five times with 10 ml of cold 1 N HCl and three times with 10 ml of cold 95% ethanol and then dried and the radioactivity was determined in a scintillation counter. In the absence of joining enzyme less than 0.4% of the added ^3H remained acid-precipitable. One unit of joining activity is defined as the amount converting 100 nmoles of $d(A\text{-}T)_n$ to an exonuclease III-resistant form in 30 min under conditions of the assay; 1 $d(A\text{-}T)_n$ unit defined in this manner is approximately equal to 1 $(dA)_n \cdot (dT)_n$ unit as defined by Olivera and Lehman (2).

Large Scale Preparation of $d(A\text{-}T)_n$ Circles—$d(A\text{-}T)_n$ circles were made in a reaction mixture identical with that used for the joining enzyme assay except that an excess of purified enzyme was used and the reaction was terminated when a limit was reached as measured by exonuclease III resistance (under these conditions up to 80% of the nucleotides could be converted to an exonuclease III-resistant form). The joining enzyme was then inactivated by heating at 100° for 2 min and the $d(A\text{-}T)_n$ product was digested with exonuclease III until no further decrease in acid-precipitable radioactivity was observed. The exonuclease III was inactivated by heating at 100° for 2 min and the denatured protein was removed by centrifugation. The supernatant solution was then dialyzed against 500 volumes of 1 M NaCl, 1 mM EDTA, 0.02 M Tris-HCl (pH 8.1) (two changes), then against solid polyethylene glycol to concentrate, and finally against 500 volumes of 0.02 M Tris-HCl (pH 8.1), 1 mM EDTA (two changes).

TABLE I

Joining enzyme activities of crude extracts measured by $d(A\text{-}T)_n$ assay

Extracts were prepared and joining enzyme assays were performed as described under "Methods." All values represent the average of at least three determinations on the linear portion of the assay curve. The values for the *Escherichia coli* and T4-infected cell extracts may not be directly comparable with the *Salmonella typhimurium* values because two different substrate preparations were used to assay these two sets of extracts. The joining activity in the *E. coli* and *S. typhimurium* extracts were found to be dependent on DPN; ATP-dependent activity was detected only in extracts of T4-infected cells.

Extract	Cofactor	Specific activity
		units/mg
E. coli 1100	DPN	1.7
E. coli CR63	DPN	1.7
E. coli B	DPN	1.7
	ATP	<0.006
T4+-infected *E. coli* B	DPN	1.1
	ATP	2.1
T4 *am*N82-infected *E. coli* B	DPN	0.88
	ATP	12
T4 *am*E605-infected *E. coli* B	ATP	<0.03
S. typhimurium EL2	DPN	1.2
S. typhimurium SL1561	DPN	1.2
S. typhimurium EL10	DPN	<0.005

Other Methods—A Zeiss PMQII spectrophotometer was used for all optical measurements. ^3H was counted in a toluene-based scintillation fluid in the Nuclear-Chicago Unilux spectrometer. ^{32}P was counted in a Nuclear-Chicago model 186 gas flow counter equipped with a micromil window. Protein was determined by the method of Lowry *et al.* (24). Sucrose density gradient centrifugation was performed according to the procedure of Martin and Ames (25).

RESULTS

Linearity of Assay with Purified Joining Enzyme—Fig. 2 shows that the assay is linear over a 10-fold range in enzyme concentration and that the method is reproducible. It should be noted, however, that the assay is reproducible only as long as the same substrate preparation (DNase digest) is used; variations of up to 40% have been observed from one substrate preparation to another. When necessary, different substrate preparations were cross-calibrated by assaying the same enzyme preparation with the different DNase digests.

Assay of Crude Extracts—The $d(A\text{-}T)_n$ method has been used to analyze a number of crude extracts for polynucleotide joining activity. A summary of the results is presented in Table I.

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The three *E. coli* strains tested were found to have identical DPN-dependent joining enzyme activities. Essentially none of the substrate was made acid-soluble (<10%) by incubation with amounts of *E. coli* extract near the upper limit of linearity for the assay (9 μg of protein).

The d(A-T)$_n$ assay was also capable of detecting the T4-induced polynucleotide ligase described by Weiss and Richardson (5), Becker *et al.* (26), and Cozzarelli *et al.* (6). This activity was dependent on the presence of ATP and was absent in extracts prepared from *E. coli* B or *E. coli* B infected with T4*am*E605, a mutant of T4 defective in the structural gene for the T4 ligase (27). It was present at a 6-fold higher level in extracts prepared from *E. coli* B infected with T4*am*N82, a mutant which fails to terminate the synthesis of "early" phage-induced enzymes (28). The DPN-dependent activity present in the extracts of infected cells is presumably due to the host enzyme. About 25% of the substrate was converted to acid-soluble products when incubated under assay conditions (with ATP as cofactor) with 7.0 or 3.5 μg of protein from the T4$^+$- or T4*am*N82-infected cell extracts, respectively.

Since the product of the action of joining enzymes on d(A-T)$_n$ is a circular molecule (Reference 10; see also below), it should be highly sensitive to endonucleolytic attack. It might therefore be argued that the d(A-T)$_n$ assay of joining activity in crude extracts does not measure the absolute joining activity, but rather reflects the ratio of joining to endonucleolytic activities. To test this possibility, purified ^{3}H-d(A-T)$_n$ circles were incubated with crude extracts (5 μg of protein) from *E. coli* strains B or 1100 or T4$^+$-infected *E. coli* B under standard assay conditions (except that DPN or ATP was omitted and the d(A-T)$_n$ circles were present at a concentration of 0.074 mM). In each of these cases less than 15% of the label became sensitive to exonuclease III. This figure is probably within the accuracy of the measurement and suggests that under these conditions significant endonucleolytic degradation of the d(A-T)$_n$ does not occur. Provided that there are no DPN- or ATP-dependent endonucleases present (29, 30), this indicates that the assay is a reliable measure of joining activity in these extracts.

The (dA)$_n$·(dT)$_n$ assay previously reported (2) was also used to assay the three *E. coli* and the T4-infected cell extracts. With this method it was possible to show that *E. coli* 1100 and CR63 have similar levels of joining enzyme (1.4 units per mg); however, it was difficult to detect activity in *E. coli* B and in T4-infected cell extracts, presumably because of the rapid degradation of the substrate (2).

Several strains of *S. typhimurium* were also analyzed for their content of joining enzyme. As shown in Table I extracts of EL2 and SL1561 have the same level of DPN-dependent joining activity. Less than 10% of the substrate was made acid-soluble upon incubation with 10 μg of protein from each of these extracts. On the other hand, there was no activity detectable in EL10. The absence of measurable activity in EL10 is due to extensive degradation of the d(A-T)$_n$ by this extract; 80% of the substrate became acid-soluble when incubated with 10 μg of protein under assay conditions.

Circular Nature of Product—The d(A-T)$_n$ substrate was incubated with either purified joining enzyme or *E. coli* B extract, and the kinetics of degradation was followed with an even more extensive exonuclease treatment than used in the assay (5-fold). As shown in Fig. 3, after an initial rapid degradation by exonuclease III a form of d(A-T)$_n$ remained which was completely

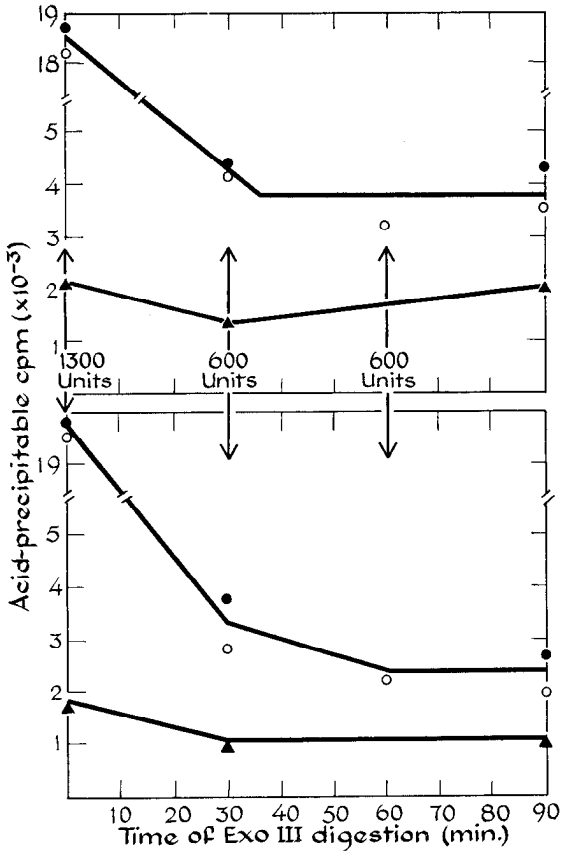

FIG. 3. Resistance of the d(A-T)$_n$ product to exonuclease III prior to and following treatment with pancreatic DNase. Standard reaction mixtures for the d(A-T)$_n$ assay (except that the final volume was 1.0 ml) containing either 0.2 unit of purified *E. coli* joining enzyme (*upper plot*) or 93 μg of protein from an *E. coli* B extract (*lower plot*) were incubated at 30° for 30 min and heated at 100° for 2 min. β-Mercaptoethanol (0.1 ml, 0.1 M) was added followed by 1300 units of exonuclease III, and at zero time the tubes were placed at 37°. An additional 600 units of exonuclease III were added at 30 and 60 min. At the times indicated, samples were removed as follows. (*a*) Aliquots, 0.10 ml, were precipitated immediately with perchloric acid as described under "Methods" (○——○). (*b*) Aliquots, 0.2 ml, were heated at 100° for 2 min to inactivate the exonuclease III and pancreatic DNase was added to a final concentration of 0.61 mμg per ml. After incubation for 30 min at 37° the DNase was inactivated by heating at 100° for 2 min; 0.10 ml of this preparation was then precipitated with perchloric acid as above (●——●). To the remainder of the DNase-treated sample was added 120 units of exonuclease III; after incubation at 37° for 30 min, it was precipitated with perchloric acid (▲——▲). All values are corrected for dilution due to the addition of the various enzyme solutions by normalizing to values for 0.10 ml of reaction mixture at zero time. In the absence of joining enzyme or extract 90 cpm/0.10 ml of reaction mixture were perchloric acid-precipitable after 30 min of digestion with exonuclease III.

resistant to further exonuclease digestion; furthermore, mild treatment of this product with pancreatic DNase, in a manner which did not affect its acid precipitability, significantly increased its sensitivity to further treatment with exonuclease. This result, coupled with the fact that the conditions used to prepare the substrate strongly favor intramolecular hydrogen bonding,[3] suggests that the product molecules are formally

[3] R. L. Baldwin, personal communication.

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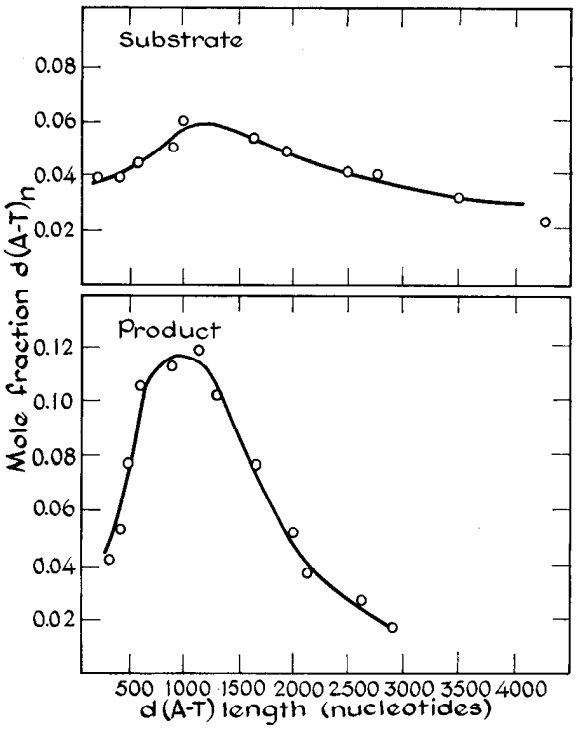

FIG. 4. Length distribution analysis of substrate and product. The 5′-phosphate was removed from the ^{3}H-d(A-T)$_n$ substrate (8420 cpm per nmole) with *E. coli* alkaline phosphatase, and the resulting 5′-OH-d(A-T)$_n$ was labeled with ^{32}P (1860 cpm per $\mu\mu$mole) according to Weiss *et al.* (21). Alkaline phosphatase present in the reaction mixture was inhibited by adding inorganic phosphate at 2.3 mM (21). The polynucleotide kinase was inactivated by heating at 100° for 2 min and denatured protein was removed by centrifugation. A portion of the supernatant fluid was removed and dialyzed extensively against 1 M NaCl, 1 mM EDTA, 0.1 M potassium phosphate (pH 7.0) to remove the γ-^{32}P-ATP (substrate). The remainder was incubated in a volume of 0.40 ml with 0.08 unit of purified joining enzyme under standard conditions for the d(A-T)$_n$ assay (except inorganic phosphate was present at 4 mM). After digestion of the unreacted material with exonuclease III, the material was dialyzed as described above (product). The ^{3}H-^{32}P-labeled substrate and product were then subjected to zone centrifugation in density gradients of 5 to 20% sucrose, 1 M NaCl, 0.1 M Tris-HCl (pH 8.1), 0.05 M potassium phosphate (pH 8.0), 0.01 M EDTA at 50,000 rpm for 9.5 hours at 5° in a Spinco model L2-65B ultracentrifuge with an SW56 rotor. Fractions of about 0.15 ml were collected on Whatman GF/C filters; these were dried and washed twice with 10 ml of cold 0.1 M sodium pyrophosphate, 1 N HCl, twice with 10 ml of cold 1 N HCl, and three times with 10 ml of cold ethanol to remove sucrose and any γ-^{32}P-ATP that remained. The filters were dried and ^{3}H and ^{32}P were determined. The substrate and product migrated at approximately the same rate as determined by the positions of the peaks of radioactivity; however, the substrate was more disperse than the product, as shown by peak widths. The specific radioactivities of ^{3}H and ^{32}P were used to calculate the number average length for each fraction. The fraction of total ^{32}P contained in a given fraction is denoted as the mole fraction of molecules. The results for the ^{3}H-^{32}P substrate are presented in the *upper plot*, those for the circular molecules in the *lower*. For the d(A-T)$_n$ substrate the mole fraction of molecules with a number average length less than 120 nucleotides was 0.49; with a length greater than 4300 it was 0.06. For the circles the mole fraction with a length less than 260 nucleotides was 0.15; with a length greater than 2900 it was 0.03. Since no ^{32}P was lost from the d(A-T)$_n$, the inorganic phosphate effectively inhibited the alkaline phosphatase present throughout the experiment.

circular and single stranded. This result confirms the previous report (10) that the action of joining enzyme on d(A-T) oligomers generates single stranded circles.

Length Distribution Analysis of Substrate and Product—The substrate used in the assay is an unfractionated DNase digest composed of d(A-T)$_n$ molecules of various chain lengths. To characterize further the action of *E. coli* joining enzyme on d(A-T)$_n$, a length distribution analysis was performed on the substrate and product molecules to determine whether some limited range of substrate length is preferentially converted to circles.

The distribution analysis was performed as follows. The 5′-termini of the ^{3}H-d(A-T)$_n$ substrate were labeled with ^{32}P with polynucleotide kinase and γ-^{32}P-ATP. Part of the ^{3}H,^{32}P-labeled d(A-T)$_n$ substrate was converted to circles with limiting amounts of joining enzyme so that the amount of circle formation would be determined by the rate of catalysis. The substrate and product were then centrifuged in sucrose density gradients to fractionate the molecules on the basis of size and the fractions were collected and analyzed for their ratio of ^{3}H to ^{32}P. In any fraction the amount of ^{3}H should be directly proportional to the nucleotide content while the ^{32}P would be proportional to the number of d(A-T) copolymer molecules; therefore, the ^{3}H:^{32}P ratio should be directly proportional to the number average length of the polymer molecules. Fig. 4 presents the results of such an experiment.

The mean lengths of substrate and product calculated from the data in Fig. 4 are identical (1060 and 1030 nucleotides, respectively, weighted on the basis of 5′-termini); however, the size distribution of substrate is much broader than that of the product. Since the ^{3}H-^{32}P product was prepared under conditions in which the amount formed would be proportional to the rate of enzyme action, the presence of a preferentially joinable length

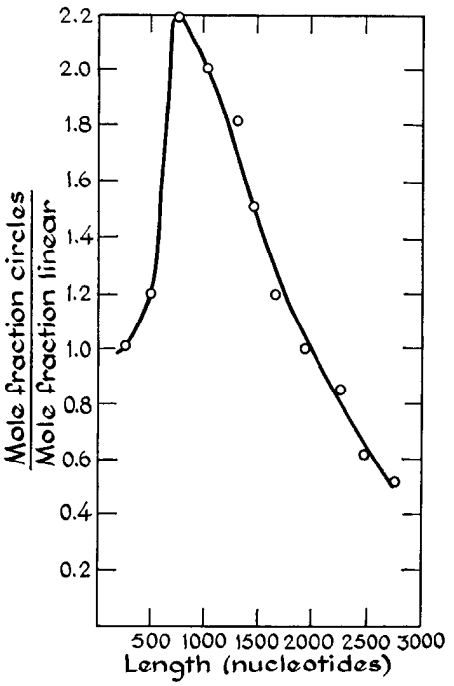

FIG. 5. Relative joinability of d(A-T)$_n$ as a function of length. Values for the mole fraction of molecules of a given length were read off the curves of Fig. 4 and are plotted as a ratio of mole fraction of circles to mole fraction of linear molecules against length.

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would result in a greater mole fraction of molecules of this size in the product than in the substrate. Such preferential joining does appear to occur; 65% of the product molecules were between 500 and 2000 nucleotides in length while only 26% of the substrate molecules were in this range. Thus the optimal length of 3′-OH, 5′-P-d(A-T)$_n$ for joining is approximately 1000 nucleotides; furthermore, it seems that the DNase calibration curve used for substrate preparation (Fig. 1) has selected for molecules of this size.

DISCUSSION

The d(A-T)$_n$ joining enzyme assay reported here has several advantages over previously described methods. The substrate is easily prepared in large quantities and can be labeled with a long lived isotope. The d(A-T)$_n$ assay can be made much more sensitive than those methods which measure the incorporation of a ^{32}P-labeled phosphomonoester group into a phosphodiester linkage since on the average 1000 nucleotides are made resistant to exonuclease III by the formation of a single phosphodiester bond. Fig. 2 shows that with d(A-T)$_n$ with a relatively low specific radioactivity (1670 cpm per nmole of nucleotide) the d(A-T)$_n$ assay is as sensitive as the (dA)$_n$·(dT)$_n$ method which uses a ^{32}P-labeled substrate (4000 cpm per $\mu\mu$mole of phosphate). Moreover, while the specific radioactivity of the d(A-T)$_n$ can easily be increased 100-fold over that used here, a similar increase in the specific radioactivity of the 5′-^{32}P would be technically impossible at the present time.

The d(A-T)$_n$ assay method is not limited to purified enzyme preparations and yields reliable values for joining activity in a number of crude extracts. The absence of significant nucleolytic degradation by most *E. coli* extracts makes d(A-T)$_n$ an ideal substrate for screening various strains of this organism for joining enzyme mutants. In only one case did the method fail to detect any joining activity in a crude extract (*S. typhimurium* EL10); this was presumably due to extensive degradation of the substrate by nuclease in the extract. For those cases in which the d(A-T)$_n$ method indicates abnormally low levels of joining activity, a few simple controls are immediately available: d(A-T)$_n$ circles can be used to test the extract for competing endonuclease activity while the linear substrate can be used to measure both endo- and exonucleolytic activities.

In the initial studies of polynucleotide joining enzyme-catalyzed formation of d(A-T)$_n$ circles from the corresponding linear oligomers (in the range of about 40 to 100 nucleotides), a strong dependence of rate and extent of joining on chain length was observed (10); this report extends that observation to polymeric molecules (chain length >500). If the data from the two graphs in Fig. 4 are replotted as molecular length against the ratio of mole fraction of circles of a given length to the mole fraction of linear substrate molecules of that length, a unimodal curve is obtained (Fig. 5). It rapidly increases from a value of 1.0 for a length of 250 nucleotides, peaks at a value of 2.2 for a length between 750 and 1000 nucleotides, and then rapidly decreases to values less than 1.0 for lengths greater than 2000 nucleotides. A possible explanation for the first portion of the curve is that, as substrate length increases to about 1000 nucleotides, the equilibrium concentration of linear d(A-T)$_n$ molecules with their termini properly aligned for joining (Form IV of Reference 10) increases so as to become a significant fraction of molecules of that length; this is in contrast to short d(A-T) oligomers (on the order of 10 nucleotides long) in which the stable form is a hairpin configuration (31). The rapid decrease in the rate of sealing which occurs as substrate length increases beyond 1000 nucleotides can be explained in several ways. If one postulates that for very long molecules (>1000 nucleotides) the stable configuration at equilibrium is one in which the 3′-hydroxyl and 5′-phosphoryl termini are apposed, and thus properly aligned for joining, then the decreased rate of sealing of these molecules might be due to a failure to achieve the stable configuration under the conditions used in these experiments. This is not unreasonable since the rate of chain slippage, which would determine the rate of equilibration, decreases with increasing chain length (31). An alternative explanation is that in very long d(A-T)$_n$ chains (>1000 nucleotides) cloverleaf branching of the molecules can stabilize a structure in which a single stranded region (a gap) is present between the 3′-hydroxyl and 5′-phosphoryl termini so that joining cannot occur.

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