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Preparation of a Circular Bihelical Deoxyribonucleic Acid Containing Repeating Dinucleotide Sequences*

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ABSTRACT: The preparation of a covalently closed, double-stranded DNA-like polymer containing alternating thymidylate and deoxyguanylate units in one strand and alternating deoxyadenylate and deoxycytidylate units in the complementary strands is described. The separated, single-stranded, complementary deoxyribopolynucleotides $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ were mixed under dilute conditions in the presence of the T4-polynucleotide ligase. The mixture was subsequently treated with DNA polymerase and the four deoxyribonucleoside triphosphates plus ligase. The resulting mixture of products was exhaustively digested with the exonuclease III of *Escherichia coli* and the resistant portion was separated by centrifugation to equilibrium in a cesium chloride density gradient. About 4% of the starting material was found to be in the double-stranded circular form, while another 8% was converted to the single-stranded circular polymers, poly (d(C-A)) and poly (d(T-G)). The double-stranded circular polymer has a buoyant density in alkaline cesium chloride about 30 mg/cm^3 greater than the average densities of its component strands, while its sedimentation velocity in alkaline sucrose is also significantly greater than that of the single-stranded circles. The double-stranded circular polymer molecules were visualized by electron microscopy and found to range from 150,000 to 600,000 in molecular weight.

The preparation of a variety of macromolecular, bihelical DNA-like polymers containing repeating nucleotide sequences has been reported from this laboratory (Byrd *et al.*, 1965; Wells *et al.*, 1965; Wells *et al.*, 1967a,b). All of the DNA-like polymers are transcribed by the DNA-dependent RNA polymerase of *Escherchia coli* under the usual conditions for the polymerase reaction; the transcription may be carried out, concurrently, of both strands by providing all of the four ribonucleoside 5′-triphosphates, or it may be restricted to either one of the two strands by controlling the supply of the triphosphates (Nishimura *et al.*, 1965; Kössel *et al.*, 1967; Morgan, 1970). The DNA-like polymers have been used (Morgan 1970) and continue to be used (P. Witonsky, A. R. Morgan, V. H. Paetkau, and R. Kleppe, unpublished experiments) to study the mechanism of transcription. It has been demonstrated that the rate and amount of transcription can be markedly influenced by the experimental conditions used, in particular, by the base composition of the strands in the DNA-like polymers.

It is clear that in biological transcription RNA polymerase must bind and subsequently initiate RNA synthesis at certain specific sites, presumably the promoter sites, only. According to the current views of the initiation of transcription, the specific binding of the RNA polymerase and the initiation of transcription are controlled by the σ or σ-like factors present in the "total" RNA polymerase (Burgess *et al.*, 1969; Bautz and Bautz, 1970; see *Cold Spring Harbor Symposium on Transcription*, 1970, in press, for comprehensive references). It may therefore be inferred that the transcription of most, if not all, of the synthetic DNA-like polymers with repeating nucleotide sequences is the consequence of one or more artificial circumstances, since these DNAs probably do not bear the authentic promoter regions. One strong possibility would be that the RNA polymerase binds and initiates transcription at the ends of the DNA-like polymers, and because of this facile artifactual binding, the need for the biologically meaningful binding at internal sites in these DNA-like polymers is obviated. Berg and coworkers (1965) have shown that the polymerase has a high affinity for the ends of DNA. This may

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be a consequence either of the "fraying" at the termini of double-stranded DNAs or of there being protruding single-stranded ends to which the polymerase may bind (*cf.* Richardson, 1966; Jones and Berg, 1966).

To study the mechanism of the initiation of transcription and, possibly, the nature of nucleotide sequences recognized by the RNA polymerase containing σ or analogous factors, we attempted to convert the double-stranded DNA polymers with repeating sequences to covalently closed, bihelical circles. Using the latter as substrates for the polymerase, one could hopefully begin to define *in vitro* conditions for transcription under which specificity may be brought out among the different defined, DNA-like polymers. Indeed, it would be a significant accomplishment to first define the experimental conditions (ionic strength, divalent ion concentration) under which the transcription of the circular form of some simple, repeating polymers is abolished. This line of thought is conceptually analogous to the cell-free synthesis of polypeptides as directed by synthetic ribopolynucleotides of repeating nucleotide sequences. At the artificially high Mg^{2+} ion concentration used in most of the early experiments, a requirement for the initiator codon and the corresponding tRNA was not evident, and a positive response was elicited from essentially all of the polymers tested for polypeptide synthesis. However, in subsequent experiments carried out at low Mg^{2+} ion concentration, the *in vitro* translational process could be restricted to only those messengers which contained the true initiator codons (Ghosh *et al.*, 1967; *Cold Spring Harbor Symposium on the Genetic Code*, 1966).

In addition to the main stimulus described above for preparation of the covalently closed double-stranded polymers, it is clear that the circular polymers could be of great use in physical studies of DNA. Covalently closed single-stranded and duplexed DNAs have now been shown to occur widely *in vivo* and, indeed, circularization seems to be an essential event at some stage in the replication cycle of DNA in the cell (see *Cold Spring Harbor Symposium*, 1968). The only circular DNA containing known nucleotide sequences described previously is that prepared from short-chain deoxyribopolynucleotides containing alternating A and T (Olivera *et al.*, 1968). These circular forms were topologically single-stranded and existed partly as bihelical entities because of self-complementary nucleotide sequence.

In the present paper, we report our initial work in this direction, which is concerned with the preparation of a covalently closed, double-stranded DNA-like polymer containing alternating T and G units in one strand and alternating A and C units in the complementary strand. The size of the circular polymer, $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$, is such that n is 120 or larger. Some physical properties of this polymer are also described.

Materials and Methods

Chemicals. Radioactively labeled nucleoside 5′-triphosphates were purchased from Schwarz BioResearch Inc.; unlabeled ones were also commercial products. The CsCl used was Sequanol Grade from Pierce Chemical Co. Agarose A-5M 100–200 mesh was a product of Bio-Rad Co.

Enzymes. DNA polymerase of *E. coli* was purified by the method of Jovin *et al.* (1969), the purification being carried through their last step (fraction 7), starting with fraction IV (Richardson *et al.*, 1964a) which was prepared in the laboratory of Dr. A. Kornberg some years ago.

Polynucleotide kinase from T4 phage-infected *E. coli* was prepared by Dr. S. Chang, essentially as described by Richardson (1965). Polynucleotide ligase was prepared from the same cells by Dr. N. K. Gupta by the method of Weiss *et al.* (1968a). In this work, a unit of ligase activity is defined as the conversion of 1 pmole of $[5'\text{-}^{32}P]dT_{10}$ (thymidine decanucleotide) to a form resistant to bacterial alkaline phosphatase per minute under the conditions described by Gupta *et al.* (1968).

Bacterial alkaline phosphatase was purchased from Worthington Biochemical Corp. and further treated by the method of Fiers and Khorana (1963) to inactivate any endonuclease. Exonuclease III was obtained as a by-product in the final purification step (G-100 chromatography) of DNA polymerase (Jovin *et al.*, 1969). One unit of activity is defined as the amount which would convert 1 nmole of DNA to an acid-soluble form in 30 min under the conditions of assay (Richardson *et al.*, 1964b).

Polynucleotides. High molecular weight $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was prepared by the extensive polymerization of deoxynucleoside triphosphates using a sample of the same DNA as template. The procedure used was a modification (A. R. Morgan and V. H. Paetkau, to be published) of the method of Wells *et al.* (1965). The molecular weight of the synthetic DNA-like polymer was estimated to be 310,000 by sedimentation velocity at neutral pH while the molecular weight of the single strands, corresponding to poly d(T-G) and poly d(C-A), was estimated by sedimentation in an alkaline medium to be 175,000. These and other properties of the double-stranded polymer used as the starting material in the present work are listed in Table I. The double-stranded polymer accepted essentially no ^{32}P label (1% of that introduced when the polymer was first treated with the bacterial alkaline phosphatase) when it was treated with polynucleotide kinase and $[\gamma\text{-}^{32}P]ATP$. Thus, the polymer did not bear a free -OH group at the 5′ end. Since it was a substrate for polynucleotide ligase, the polymer was assumed to carry 5′-phosphomonoester groups. This is to be expected because at least some 5′-exonucleolytic action of DNA polymerase would have occurred during the polymerization reaction to generate such ends (Deutscher and Kornberg, 1969). The molecular weight of the starting DNA-like polymer was also determined by the amount of ^{32}P incorporation on phosphorylation with polynucleotide kinase using $[\gamma\text{-}^{32}P]ATP$ of defined specific activity and the phosphatase-treated polymer as the substrate. The estimates of molecular weight of each strand thus obtained are included in Table I.

Further, as expected, poly($d(T\text{-}G) \cdot d(C\text{-}A)$) separated into two bands in an alkaline CsCl preparative gradient (Figure 1). The densities at the centers of the two bands, measured pycnometrically after fractionation of the material in the gradient, were 1.84–1.85 for $d(T\text{-}G)_n$ and 1.70–1.71 $d(C\text{-}A)_n$. These values were slightly higher than the ones given in Table I, which were obtained in analytical runs, using $d(A\text{-}T)_n$ as a marker (assumed density 1.722, Wells and Blair, 1967). The polymer used in the alkaline density gradient as well as in many of the experiments described below on circularization contained distinctive radioactive labels in the two strands. Thus the d(C-A) strand was labeled with ^{14}C in the cytosine ring while the d(T-G) strand contained ^{3}H in thymine. In the separation shown in Figure 1, there was no overlap of the two labels, showing clean separation of the two strands. The results are in agreement with those of Wells and Blair (1967).

"Open channel" scintillation counting was used to measure the total amounts of polymer present in various assays or preparative steps. The specific activities of ^{3}H- and ^{14}C-labeled strands were the same in this channel, about 70,000 cpm per OD_{260} unit of DNA. Thus open channel counts reflect

TABLE I: Properties of the Starting Polymer $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$.

Molecular Weight[a]	
Sedimentation velocity at neutral pH	310,000
Sedimentation velocity at alkaline pH	175,000
Daltons of DNA per 5′-^{32}P Added by Polynucleotide Kinase	
Without alkaline phosphatase pretreatment	11,000,000
With phosphatase pretreatment at 37°	139,000
With phosphatase pretreatment at 65°	144,000
Density in Alkaline CsCl[b]	
$d(T\text{-}G)_n$	1.823
$d(C\text{-}A)_n$	1.692

[a] Method of Studier (1965). [b] Determined by analytical ultracentrifugation, using $d(A\text{-}T)_n$ as a reference (see Methods) and also by the isoconcentration method (Vinograd, 1963). The values given are the average obtained by the two methods.

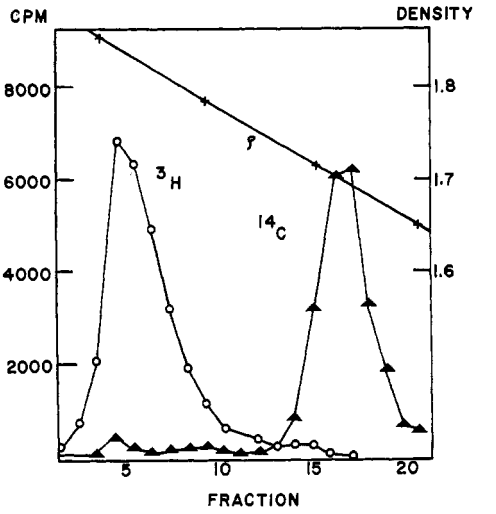

FIGURE 1: Separation of $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ in an alkaline CsCl gradient. Three OD_{260} units of this DNA, containing ^{3}H-labeled thymine (1100 cpm/nmole of thymine in ^{3}H-restricted channel) and ^{14}C-labeled cytosine (1100 cpm/nmole of cytosine in ^{14}C-restricted channel), were centrifuged in a solution of CsCl of initial density 1.797 g/ml and initial pH *ca.* 12.5. After 72 hr of centrifugation at 40,000 rpm in an SW 65 Ti rotor, at 23°, the gradient contents were divided into 0.20-ml fractions. The radioactive material and densities were determined as indicated in Materials and Methods. The cpm values are per 0.050 ml of each fraction: (○) ^{3}H-restricted channel counts; (▲) ^{14}C-restricted channel counts; (+) density.

the total amount of nucleotide, without regard for the individual strands. All molar concentrations of polynucleotides refer to nucleotide phosphate groups.

The 5′-phosphate group of the DNA-like polymer was removed by treatment with bacterial alkaline phosphatase, essentially by the method of Weiss *et al.* (1968b). The phosphatase was removed by extraction with phenol and chloroform–isoamyl alcohol (24:1) (Sevag *et al.*, 1938), and the DNA was passed through a G-75 Sephadex column. The labeling of the 5′-OH groups of this DNA with ^{32}P from [γ^{32}P]ATP was carried out by the method of Weiss *et al.* (1968b). The 5′-^{32}P-labeled DNA-like polymer was isolated as described above.

In those cases where 5′-^{32}P-labeled DNA-like polymer was subjected to bacterial alkaline phosphatase to determine the extent of ^{32}P made phosphatase resistant by ligase, the measurement was carried out as follows. After the phosphatase treatment, calf thymus DNA was added as a carrier, the DNA was precipitated with ice-cold 5% trichloroacetic acid, and the precipitate was sedimented by centrifugation. The acid-soluble radioactivity in the supernatant was measured, and in some assays, the DNA in the pellet was redissolved with alkali, and its radioactivity was also measured after neutralization of the solution.

Ligase Reaction. The incubation mixture for the joining reaction was a slight modification of the one described by Weiss *et al.* (1968a). It contained 0.035 M Tris (Cl), pH 7.6, 0.1 mM ATP, 0.01 M dithiothreitol, and 0.01 M $MgCl_2$. The temperatures and polymer concentrations, as well as other details of individual reactions, are described in the legends to the figures.

Degradation with Exonuclease III. Degradation with exonuclease III was carried out in a solution containing 0.05 M Tris (Cl^-), pH 8.0, 3 mM $MgCl_2$, and 1 mM dithiothreitol. The units of the enzyme used in different experiments are given in legends to the appropriate figures. Aliquots were taken at timed intervals and pipetted onto filter paper disks. The disks were thoroughly washed with cold 5% trichloroacetic acid, ethanol–ether (1:1), and ether and counted in a scintillation counter. In some large-scale reactions, the hyperchromicity of the DNA solution was monitored with a Gilford spectrophotometer during the exonuclease III reaction. All reactions were done at 37°. The per cent of material reported as being exonuclease III resistant was taken from the plateau of each reaction with respect to time.

Assays for Determination of Circularization of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. (a) The first assay used was to measure the conversion of the ^{32}P of 5′-^{32}P-labeled polymer to a phosphatase-insensitive form (see above). This assay however did not distinguish between "linear" end-to-end oligomerization of the polymer and the intramolecular cyclization reaction (see Results).

More reliable assays involved the determination of the extent of degradation of the ^{3}H- and ^{14}C-labeled polymer by exonuclease III or by DNA polymerase in the absence of deoxynucleoside triphosphates. The portion of the polymer insensitive to these nucleolytic degradations would be expected to be in the circular form (single-stranded circles consisting of $d(T\text{-}G)_n$ or $d(C\text{-}A)_n$, or the double-stranded circles). It was experimentally determined that the synthetic polymer, $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was completely degraded by exonuclease III (see also below under Results) if the prior treatment with polynucleotide ligase was omitted. Conditions for the degradation by exonuclease III have been given above. For completion of the exonucleolytic degradation following the ligase reaction, it was found essential to completely deproteinize the reaction mixtures. The ligase appeared to protect the polymer against exonuclease III action. The deproteinization procedure is described below.

(b) Electron microscopy was used to confirm the formation of, and further characterize, the circular DNAs. Electron micrographs were prepared by Dr. R. B. Inman using a modification (Inman *et al.*, 1965; Inman, 1967) of the method of Kleinschmidt *et al.* (1962) and the spreading solution contained HCHO (see method B in Materials and Methods, Mitra *et al.*, 1967). We are grateful to Dr. Inman, Laboratory of Biophysics, University of Wisconsin, for all the results obtained by this technique and described in this paper.

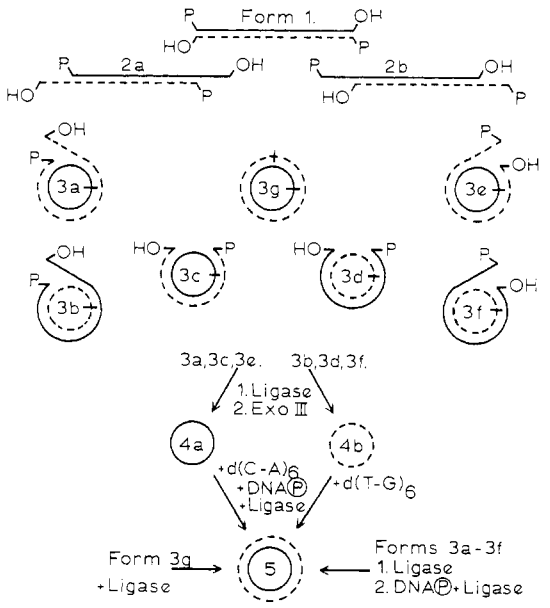

FIGURE 2: Theoretical considerations for circularization of the linear $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ polymer. Form 1 represents the bihelical, linear DNA used as the starting material. Forms 2a and 2b consist of partially bihelical molecules with either the 5′ ends (indicated by -P throughout) or the 3′ ends (indicated by -OH throughout) protruding. The various possible circular configurations resulting from intramolecular hydrogen bonding between complementary ends are shown as forms 3a to 3g. In each of 3a to 3f, one strand has formed a complete circle, as explained under Results, and the second strand contains either a gap between the 5′ and 3′ ends, or a single-stranded overlap (either the 5′ or 3′ end of either strand) giving four possibilities. Figure 3g represents the presumably rare case of a molecule in which both strands are of identical length, and thus capable of forming a completely bihelical circle, form 5. Following ligase joining of hydrogen-bonded, complete circles, the incomplete, or protruding strand can either be "repaired" with DNA polymerase and ligase (to product form 5), or degraded with an exonuclease to produce form 4: (——) $d(T\text{-}G)_n$; (--------) $d(C\text{-}A)_n$.

Removal of Proteins and Small Molecules from the DNA-Like Polymers after Enzymatic Reactions. The chloroform–isoamyl alcohol and the phenol treatments described above were effective in that all the noncircular, polynucleotidic material was subsequently sensitive to exonucleolytic degradation. These methods were, however, not convenient for handling many samples. A faster method for removing proteins and small molecules, which was used in experiments of either analytical or preparative nature, was as follows. The sample was evaporated, if necessary, to 0.3 ml. Then EDTA (equal to twice the Mg^{2+} content of the sample) and sodium dodecyl sulfate (final concentration 0.1%) were added and the sample was incubated at 37° for 10 min. The high molecular weight polymer was then recovered as the excluded peak from a column (0.6 × 30 cm) of Agarose A-5M, 50–100 mesh, equilibrated, and developed with 0.005 M Tris (Cl^-), pH 8.0, plus 0.1 mM EDTA, at room temperature. In this way, the polymer was recovered in about 30 min, without need for either extraction with organic solvents, or time-consuming dialyses. Proteins and dodecyl sulfate were well separated from the excluded peak. Numerous columns could be developed simultaneously by one person, and the same columns were used repeatedly over a period of many months at room temperature. When not in use, they were protected with a 0.02% azide solution. They were occasionally washed with dilute alkali.

Determination of the Radioactive and Nonradioactive Polymers. Fractions containing radioactive polymer obtained from CsCl or sucrose gradients were analyzed by pipetting aliquots onto filter paper disks (Bollum, 1959; Nishimura *et al.*, 1964). These were washed with 5% trichloroacetic acid, ethanol–ether (1:1), and ether and placed in a toluene-based liquid scintillation vial. Nonradioactive fractions were analyzed in a Gilford spectrophotometer after being diluted to 1 ml. Densities of undiluted fractions from CsCl gradients were measured with a calibrated 100-μl pipet immediately after fractionation of the gradient.

Results

(1) *Theoretical Considerations.* The starting material, $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$, obtained from DNA polymerase reactions is probably in the form 1 (Figure 2). There are few, if any, internal nicks and the polymer does not appear to be in an end-to-end aggregated form as shown by the molecular weight determinations of the double-stranded polymer and of the single strands obtained from it (Table I). In order to bring about intramolecular circularization, lateral displacement of one strand relative to the other would be required to generate single-stranded complementary regions at opposite ends of each molecule (forms 2a and 2b, Figure 2). The latter could (a) revert to form 1 by "creeping," *i.e.*, by lateral displacement of the strands relative to each other (*cf.* Olivera and Lehman, 1968), (b) undergo end-to-end aggregation, or (c) circularize intramolecularly to give any one of the seven structures represented by forms 3a–3g in Figure 2.

In order to produce the presumed intermediates, forms 2a and 2b, $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was either denatured by heating and quickly cooled, or else physically separated into its component strands and these were allowed to recombine. We presumed that the strands of $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ would in general not be perfectly aligned when they recombined, but rather, that only a portion of each strand would be part of a bihelical state, thus leading to at least the temporary existence of either form 2a (in which 3′ ends protrude as single-stranded regions) or 2b (in which 5′ ends protrude). Under dilute conditions, some of 2a and 2b was expected to circularize, to one of the forms 3. In the unlikely case that the protruding "sticky" ends in forms 2 were of identical length in a given molecule, it could form a perfectly bihelical circle (3g), analogous to the hydrogen-bonded, circular form of λ phage DNA (Hershey, 1970). Form 3g would be converted into a covalently closed, double-stranded circular form (5) by ligase.

More generally, one strand of forms 2a and 2b would be longer than the other. Two types of circles could therefore result. Either (i) the shorter strand could form a complete circle, using the longer strand as a template in the region of joined ends, or (ii) the longer strand could form a complete circle, using the shorter strand as a template in this region. In case i the longer strand would be left projecting with either a 3′ (forms 3a and 3b) or a 5′ (forms 3e and 3f) end. In case ii, the shorter strand would be unable to complete the circle and would be left with a gap (forms 3c and 3d). In each case, the ability of the two strands to slip laterally with respect to each other could bring the complete circular strand to the configuration in which the 5′-phosphate and the 3′-OH groups were juxtaposed and susceptible to the action of the ligase. This reaction would be analogous to the formation of circular, covalently closed λ phage DNA (Gellert, 1967). Incubation with ligase would convert all of forms 3a–3f into forms consisting of one covalently closed circle and one strand which was still susceptible to exonucleolytic degradation. Thus, the steps

TABLE II[a]

Experiment	Procedure	Exonuclease III Resistant DNA (%)
1	Heat and cool, 6 units of ligase/nmole of DNA, 60-min incubation	12
2	(Heat and cool, 6 units of ligase/nmole of DNA, 60-min incubation), three times	25
3	Heat and cool, 0.5 unit of ligase/nmole of DNA, 3-hr incubation; then heat and cool, 0.5 unit of ligase/nmole of DNA, 2-hr incubation; then heat and cool, 1 unit of ligase/nmole of DNA, 5-hr incubation	7
4	(Heat and cool, no ligase, 60-min incubation) three times	1

[a] The ligase reaction mixture is described in Materials and Methods. All incubations were at 37°. The DNA concentration in experiments 1, 2, and 4 was 0.067 OD_{260} unit/ml (10 nmoles/ml), and the reaction volume was 0.30 ml. Experiment 3 was done using 0.13 OD_{260} unit/ml of DNA, on a 9-ml scale. Heat-and-cool cycles consisted of keeping the sample in a boiling water bath for 5 min, followed by immersion in a 37° bath. The exonuclease III degradation was done following isolation of the DNA by the sodium dodecyl sulfate–Agarose method. Four units of exonuclease III per nmole of DNA were added, and incubation was at 37° under the conditions given in Materials and Methods. At 45 min, aliquots were taken to measure acid-precipitable counts. Open channel scintillation counting was used to measure total polynucleotidic material. The samples used for exonuclease-resistance tests contained between 600 and 1100 cpm each in open channel counting. Where multiple heating-and-cooling steps are indicated, the exonuclease resistance refers to the material isolated after the last incubation.

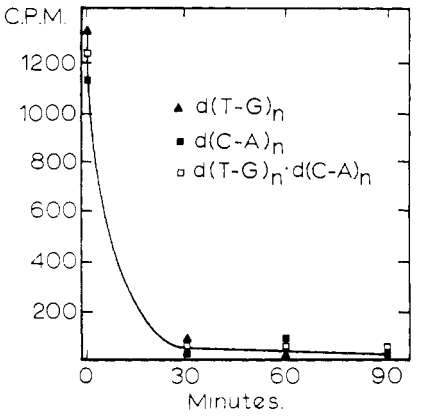

FIGURE 3: Degradation of linear double- and single-stranded DNAs by exonuclease III. Conditions for degradation are given in Materials and Methods. The exonuclease III to DNA ratio was 3.5 units/nmole for $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and twice this for the single-stranded polymers.

indicated in Figure 2 would give rise to single-stranded circles, form 4. On the other hand, DNA polymerase, in the presence of the four deoxynucleoside triphosphates, would be expected to (i) remove protruding 3′ or 5′ ends (Lehman and Richardson, 1964; Deutscher and Kornberg, 1969) and/or (ii) fill gaps by repair onto 3′-OH ends (Richardson *et al.*, 1964c). Ligase would then complete the circularization of the second strand, leading from forms 3a–3f to form 5. Forms 4a and 4b, in the presence of their complementary oligonucleotides as primers, could also be repaired by DNA polymerase and, in the presence of ligase, also go to form 5. Various routes of conversion into forms 4 and 5 were studied, and are described below.

(2) *Circularization by Heating and Cooling of Poly(d(T-G)·d(C-A))*. In initial experiments, when ^{32}P-labeled $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was treated with polynucleotide ligase, 30–60% (depending upon the conditions) of the ^{32}P label was converted to a phosphatase-resistant form. The extent of phosphatase resistance increased with increasing concentration of the polymer, suggesting that end-to-end aggregation rather than circularization was responsible for the phosphatase insensitivity. The product of the ligase reaction in these experiments was almost completely degraded by the exonucleolytic activity of DNA polymerase (degradation carried out in the absence of added deoxynucleoside triphosphates). Electron microscopy of the ligase-treated products indicated only a very small amount of circular material; the majority of the DNA seemed to consist of "welded," or end-to-end joined, and often branched, material. This would explain the high percentage of ^{32}P in an alkaline phosphatase insensitive form and also the observed slower rate of degradation, compared to untreated polymer, by the exonucleolytic action of DNA polymerase.

As already mentioned in Materials and Methods, poly (d(T-G)·d(C-A)) was found to be completely degraded by exonuclease III. Results of a typical experiment are shown in Figure 3. Not only was the double-stranded polymer degraded completely but the separated single strands corresponding to $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ were also degraded (Figure 3). (The naturally occurring DNAs are degraded only to the extent of about 40% by this exonuclease; Richardson *et al.*, 1964b). Because the circular DNAs would be resistant to exonuclease III, further assays for circularization were performed by exhaustive degradation with exonuclease III of the ligase-treated reaction mixtures after complete deproteinization (see Methods).

In the first method for circularizing the DNA, the starting polymer was diluted to a concentration of 10 nmoles/ml, heated in a boiling water bath for 5 min, cooled to 37° quickly, and then subjected to ligase. A single round of heating and cooling, followed by the addition of 6 units of ligase per nmole of DNA, converted 12% of the polymer to a form insensitive to exonuclease III (Table II). When the heating and cooling were done twice more, each cooling step being followed by restoring the same level of ligase, the exonuclease III resistance increased to 25%. A control sample, carried through three cycles of heating and cooling, but without any ligase, was 1% exonuclease III resistant.

This procedure required a relatively high level of ligase to produce a reasonable yield of exonuclease-resistant DNA. Adding 0.5 unit of ligase per nmole of DNA converted only 4% into an exonuclease III resistant form. (As seen below, when the formation of circles was achieved by adding $d(C\text{-}A)_n$ to a solution of $d(T\text{-}G)_n$, the half-saturation level occurred at 0.35 unit of ligase per nmole of DNA).

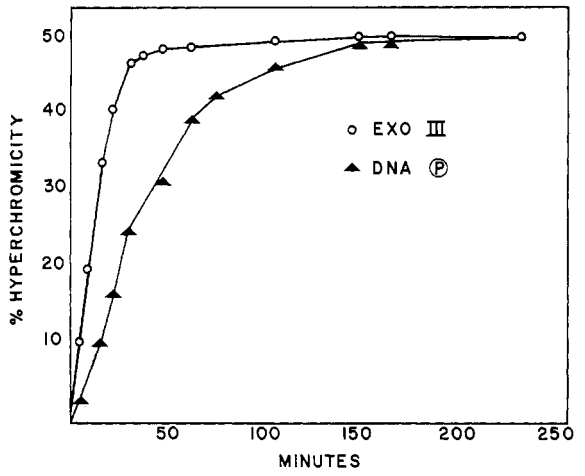

FIGURE 4: Degradation of ligase-treated $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ with exonucleases. The polymer was partially converted into circles by heating and cooling, followed by ligase (see Results, 2). It was then subjected either to exonuclease III or DNA polymerase action in parallel cuvets at 37°. The reaction conditions for both samples were as described for exonuclease III degradation in Materials and Methods. Each cuvet, of volume 1.0 ml, contained 0.22 OD_{260} unit of ligase-treated polymer. The ratio of exonuclease III to DNA was 1.4 units/nmole. The ratio of DNA polymerase to DNA was 3 units/nmole synthesis (Richardson *et al.*, 1964a). The optical density at 260 nm was monitored in a Gilford recording spectrophotometer.

Experiment 3 in Table II refers to a large-scale reaction mixture, on 1.2 OD_{260} units of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ in 9 ml. In this case, only 0.5, 0.5, and 1.0 unit of ligase/nmole of DNA were added after each of three heat-and-cool steps. The excluded peak from the Agarose 5M column was 7% resistant to exonuclease III. On examination in the electron microscope, it appeared to consist of long, branched molecules, many of them up to ten times as long as the starting DNA before heating and cooling. About 10% of the DNA appeared to be in circular forms. (The DNA used in the preparation of circles contained no detectable circles when examined by electron microscopy and was relatively uniform in length).

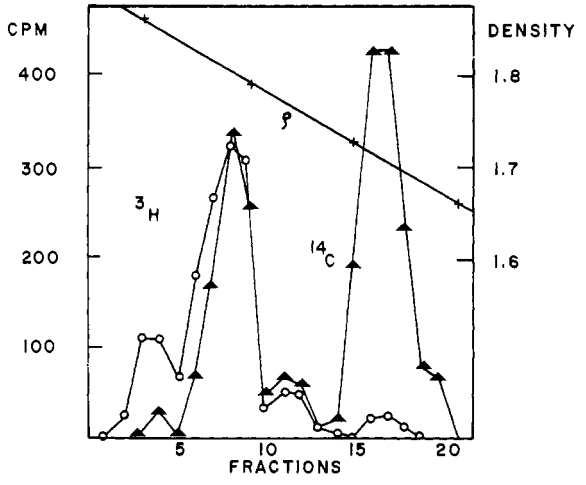

FIGURE 5: Alkaline CsCl gradient analysis of circular $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ prepared by heating and cooling followed by ligase. The starting polymer and conditions for analysis were the same as in Figure 1: (○) ^{3}H-restricted channel counts (poly(T-G)); (▲) ^{14}C-restricted channel counts (poly(C-A)); (+) density.

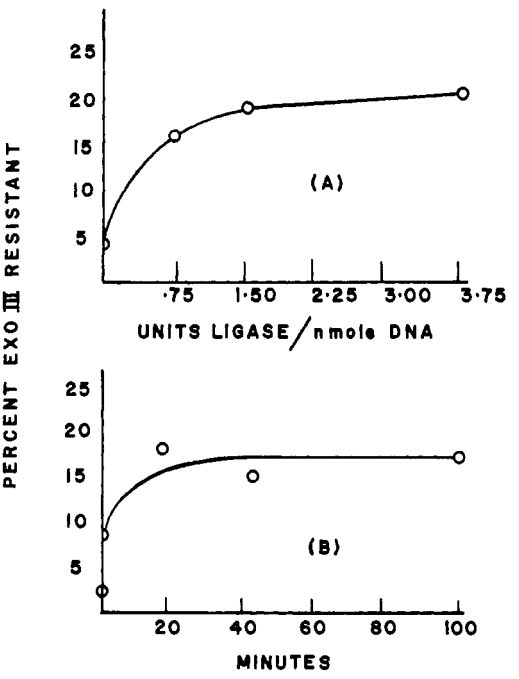

FIGURE 6: Yield of exonuclease III resistant DNA as a function of time and ligase level. Each point on the graphs involved a single (6B) or duplicate (6A) experiments of 0.90-ml volume, containing 0.025 OD_{260} unit per ml of $d(T\text{-}G)_n$ to which was added an equal amount of $d(C\text{-}A)_n$ at zero time. Either the indicated amount (6A) or 1 unit/nmole of DNA (6B) of ligase was also present initially. Incubation was at 23° for either 2 hr (6A) or the indicated time (6B). Other conditions of the ligase reaction are given in Materials and Methods. Each sample was isolated by the sodium dodecyl sulfate–Agarose method and subjected to exonuclease III degradation. Three units of exonuclease III were added per nmole of DNA and were then incubated for 40 min at 37°, and acid-precipitable material was measured in the usual way, using open channel scintillation counting to measure the total amount of circular DNA.

To examine this material further, it was degraded with exonucleases, one portion with exonuclease III, the other with DNA polymerase. The kinetics of degradation are shown in Figure 4. Of the two portions 5 and 6%, respectively, proved to be resistant and were recovered in the excluded peak of an Agarose-5M column. This material was pooled and centrifuged to equilibrium in an alkaline CsCl solution.

Four peaks can be seen in the distribution at density equilibrium (Figure 5). Beginning at the highest density end, there is a small peak at ρ 1.85, containing only ^{3}H, and corresponding to $d(T\text{-}G)_n$. (In this polymer, thymine was labeled with ^{3}H, cytosine with ^{14}C.) The second peak, of density 1.80, contained equal amounts of ^{3}H and ^{14}C. The starting polymer showed no ^{14}C at this density (Figure 1). The third very small peak, of density *ca.* 1.77, probably corresponds to a contamination with the hybrid-density polymer, containing both $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$, which has been described earlier (Paetkau, 1969). Finally, there is the fourth, large, peak of ^{14}C-containing material, with a density of 1.71, corresponding to $d(C\text{-}A)_n$.

The polymers in the second and fourth peaks of Figure 5 were pooled separately and dialyzed to remove alkali and CsCl. The samples were then treated with exonuclease III (18 units of exonuclease per nmole of DNA, incubated at 37° for 30 min) and isolated on Agarose-5M columns. The polymers appearing in the excluded peak of the Agarose columns, that is, material undegraded by exonuclease III, accounted for 70 and 80% of the total material in the second and fourth

TABLE III[a]

Step	Experiment 1[b,d]		Experiment 2[c,e]	
	cpm	% of Original	cpm	% of Original
Ligase plus DNA polymerase	224,000	(100)	224,000	(100)
Concentration	140,000	63	210,000	94
Exonuclease III degradation	24,000	11	28,700	13
Alkaline sucrose sedimentation I			7,800	3.5
II			11,500	5.1
Alkaline CsCl gradient $d(T\text{-}G)_n$	4,800	2.2	2,800 (I)	1.3
			4,000 (II)	1.8
$d(T\text{-}G)_n \cdot d(C\text{-}A)_n$	5,000	2.2	4,600 (I)	2.1
			2,000 (II)	0.9
$d(C\text{-}A)_n$	7,600	3.4	1,300 (I)	0.6
			6,700 (II)	3.0
Dialysis of pooled fractions				
$d(T\text{-}G)_n \cdot d(C\text{-}A)_n$	1,500	0.7	3,200	1.4
$d(T\text{-}G)_n + d(C\text{-}A)_n$	5,100	2.3	9,100	4.1

[a] The conditions of the ligase and DNA polymerase reactions were the same in both experiments. The volume in the ligase reaction (first step) was 40 ml and contained 2.0 OD_{260} units each of $d(T\text{-}G)_n$ and $d(G\text{-}A)_n$, after mixing. Ligase was present at a ratio of 0.75 unit per nmole of DNA. Other conditions were as given in Materials and Methods. After 1 hr at 24°, the pH was lowered to 6.95 with potassium phosphate (final concentration 0.07 M) and the four deoxynucleoside 5′-triphosphates were added to a final concentration of 0.1 mM, thereby increasing the volume to 48 ml. The temperature was decreased to 10°, 3 units/ml of DNA polymerase and 9 units/ml of ligase were added, and the mixture was incubated for 3 hr. All recoveries are based on open channel counting, which showed equal specific activities for (^{3}H-labeled) $d(T\text{-}G)_n$ and (^{14}C-labeled) $d(C\text{-}A)_n$. The amounts of the double-stranded circular form in the alkaline CsCl gradients were calculated by summing up the ^{14}C (restricted channel) counts appearing in the $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ circles region, and converting this number to the corresponding open channel counts which would be contained by a double-stranded polymer. [b] In experiment 1, the mixture was then concentrated with a Diaflo pressure cell (UM 1 membrane), and the sodium dodecyl sulfate–Agarose method used to remove proteins and small molecules. [c] In experiment 2 the mixture was concentrated instead by dialysis against polyethylene glycol 6000–7500, followed by the sodium dodecyl sulfate–Agarose column step. [d] In experiment 1, the high molecular weight material excluded from the Agarose column following exonuclease III degradation was centrifuged to equilibrium in an alkaline CsCl gradient (see Figure 7). [e] In experiment 2, the exonuclease III resistant material was first sedimented in an alkaline sucrose solution, and the faster sedimenting (I) and slower sedimenting (II) portions separately banded in alkaline CsCl (see Figure 9). The double-stranded circular DNA from I and II was pooled before dialysis.

peak, respectively. Based on its exonuclease insensitivity, bouyant density (see Discussion), and composition, material in the second peak of Figure 5 is concluded to be the double-stranded, covalently-closed, circular form of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. The material in the fourth peak is assumed to be circular $d(C\text{-}A)_n$.

The recovery of the double-stranded, covalently circular form of $d(TG)_n \cdot d(CA)_n$ at this stage was about 0.6% of the starting material (700 cpm in open channel counting out of 120,000 cpm).

(3) *Circularization by Mixing Separated Strands Followed by Ligase.* Single-stranded, linear $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ were prepared in alkaline CsCl gradients (*cf.* Figure 1). When $d(C\text{-}A)_n$ was added back to a solution of $d(T\text{-}G)_n$ plus ligase, at 24°, up to 20% of the DNA was converted into an exonuclease III resistant form. Clearly, in order to promote intramolecular circularization over bimolecular end-to-end aggregation, it was important to carry out the mixing and joining experiments under dilute conditions. In the present experiment, the yield of exonuclease III resistant material was the same at 0.004 and 0.04 OD_{260}/ml of polymer (final concentration). This system was saturated by a much lower level of ligase (Figure 6A) than the one described above which involved heating and cooling; 50% of the maximum joining occurred at 0.35 unit of ligase per nmole of DNA. The time course of the reaction indicated that the maximum amount of joining had occurred by 20 min (Figure 6B). The rather high background of exonuclease III resistant material seen in the absence of ligase (3–5%) was reduced to about 1% if EDTA was added prior to the addition of $d(C\text{-}A)_n$. The addition of $d(C\text{-}A)_n$ in eight equal portions, rather than all at once, had no appreciable effect on the yield of exonuclease III resistant material.

A reaction (40-ml volume) was carried out by adding an equivalent amount of $d(C\text{-}A)_n$ to a solution of 3 nmoles/ml of $d(T\text{-}G)_n$ containing 7.2 units/ml of ligase. After 40 min at 24°, the reaction was stopped with EDTA and the solution was concentrated on a Buchler Evapo-Mix apparatus at room temperature. The DNA was isolated by the usual sodium dodecyl sulfate–Agarose-5M method and the excluded peak was subjected to the exonucleolytic action of DNA polymerase. The change in OD_{260} was monitored, the reaction was terminated after coming to a plateau (6 hr), and the polymers were again isolated on an Agarose column. The material in the excluded peak, which was 14% of the total, was centrifuged to equilibrium in alkaline CsCl. Less than 2% of the material in the CsCl gradient banded at the double-stranded circle density; the rest of the sample constituted single-

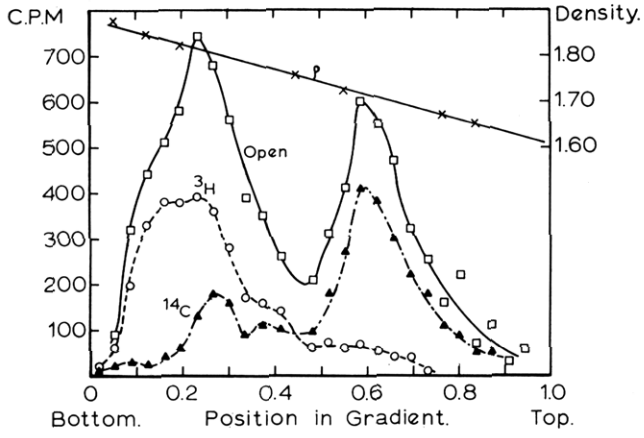

FIGURE 7: Alkaline CsCl gradient analysis of circular DNA prepared by ligase and DNA polymerase action on recombined d(T-G)$_n$ and d(C-A)$_n$. The conditions of centrifugation and analysis, and the specific activities, were the same as in Figure 1, except that the cpm values are for 0.10 ml of each fraction. The reaction conditions are given in Table III, experiment 1: (○) ^{3}H (poly(T-G)); (▲) ^{14}C (poly(C-A)); (□) open channel counts (poly(T-G) plus poly(C-A)); (×) density. The specific activities of both strands were about 1.4 times as high in the open channel mode as in their respective restricted channels. Fractions from 0.18 to 0.35 in the gradient were pooled, dialyzed, and used for electron microscopy (see Figure 8).

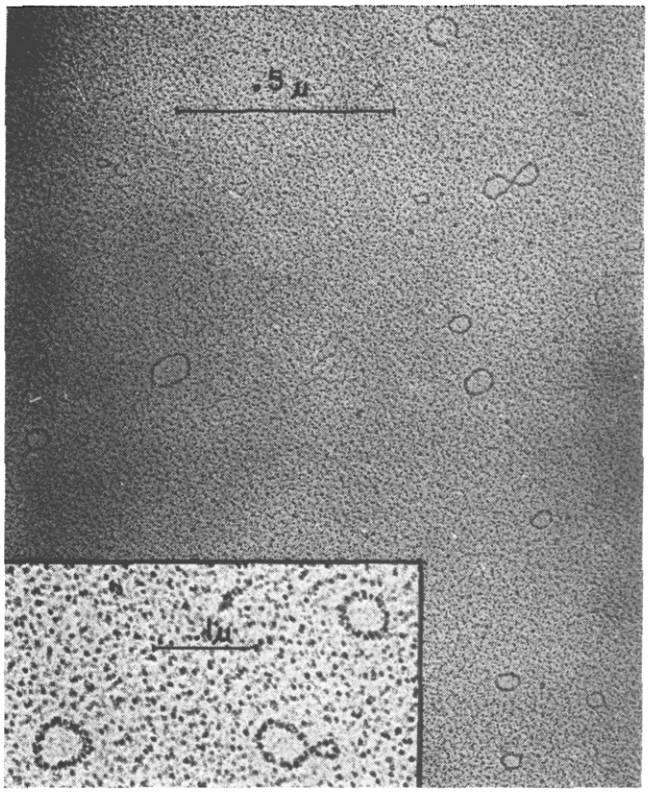

FIGURE 8: Electron micrographs of circular d(T-G)$_n$·d(C-A)$_n$. The material indicated in the legend to Figure 7 was used to prepare electron micrographs (see Materials and Methods). The inset shows an enlarged view of three typical circular molecules.

stranded circular d(T-G)$_n$ and circular d(C-A)$_n$. The preceding steps correspond to the formation of forms 4a and 4b in Figure 2.

In order to prepare double-stranded cricular material from the isolated single-stranded circles (corresponding to the conversion of forms 4a or 4b into form 5 in Figure 2), 1.5 nmoles of the circular d(C-A)$_n$ obtained above was treated with 65 units of DNA polymerase per nmole of DNA at 22° (Richardson *et al.*, 1964c). The system contained [^{3}H]TTP and d-GTP (each 0.1 mM) plus the chemically snythesized primer d(T-G)$_6$ (Ohtsuka *et al.*, 1965). The ratio of primer to circular DNA was 1:2, measured as nucleotide phosphate. Fifteen units of ligase per nmole of DNA were present, and the reaction volume was 1.25 ml. Most of the ^{3}H-labeled product from this reaction, when examined in an alkaline CsCl gradient, was linear, exonuclease III sensitive, d(T-G)$_n$. About one-third of the ^{3}H label banded at the position of double-stranded circles, but this was only 40% exonuclease III resistant. The remaining 60% of this band was probably an overlap from the linear d(T-G)$_n$ band.

This method, therefore, produced reasonably good yields of the single-stranded circles corresponding to d(T-G)$_n$ and d(C-A)$_n$ (*ca.* 14%) but, as carried out in this experiment, was unsatisfactory for the preparation of double-stranded circular material.

(4) *Circularization by Mixing Separated Strands Followed by Ligase plus DNA Polymerase.* Of the methods tried, the most efficient for preparation of double-stranded closed circles as well as single-stranded circles was the one described in Table III. An equivalent amount of d(C-A)$_n$ was added to a ligase reaction mixture containing 6 nmoles of d(T-G)$_n$ and 9 units of ligase per ml at 24°. After an hour, the pH was lowered to 6.95, the temperature was decreased to 10°, and the four deoxynucleoside 5′-triphosphates were added to 0.1 mM concentration. DNA polymerase (3 units/ml) and more ligase (9 units/ml) were then added, and the mixture was incubated for 3 hr to promote the repair and joining of incomplete strands in circular molecules (forms 3a–3f being converted into form 5 in Figure 2). The reaction mixture was then concentrated either by using the Diaflo pressure cell or by dialysis against polyethylene glycol 6000–7500. To remove noncircular DNA, the samples were subjected to degradation by exonuclease III; 11 and 13%, respectively, of the two samples described in Table III were subsequently recoverable as high molecular weight DNA by Agarose chromatography.

In the first experiment, the DNA was then directly subjected to an alkaline CsCl equilibrium sedimentation step. The results are shown in Figure 7. The relative amounts of d(T-G)$_n$ circles, d(T-G)$_n$·d(C-A)$_n$ circles, and d(C-A)$_n$ circles were calculated from the distribution of ^{3}H and ^{14}C counts, corresponding to thymine and cytosine, respectively. Of the circular DNA, 28% was double-stranded, representing a conversion and recovery of 2.2% of the starting material to double-stranded, covalently circular d(T-G)$_n$·d(C-A)$_n$. There was a subsequent loss in the dialysis step, probably because of the very small amount of material present.

The material indicated in the legend to Figure 7 was pooled and dialyzed. From the amount of ^{3}H and ^{14}C in this sample, it was expected to have only half as much d(C-A)$_n$ as d(T-G)$_n$, yet electron micrographs showed about 80% as double-stranded, circular material (Figure 8). If the excess d(T-G)$_n$ were missing because of a characteristic of the sample preparation for electron microscopy, one would expect the material from the d(T-G)$_n$ region (ρ 1.85) to behave similarly; yet, in electron micrographs of this material, the expected, single-stranded, circles were readily apparent.

In experiment 2 of Table III, the exonuclease III resistant polymer was sedimented through an alkaline sucrose gradient before banding in alkaline CsCl (Figure 9). The faster sedi-

menting material (I) was enriched to the extent of 52% in double-stranded circles, and contained 70% of the total double-stranded, circular DNA. The overall per cent of double-stranded, covalent circles in I plus II was 31.

(5) *Some Properties of the Circular DNA-Like Polymers.* Several electron micrographs of the circular DNA represented in Figure 8 were examined, and 53 circles, selected at random, were measured. The weight-average molecular weight was 360,000, and the number-average molecular weight was 320,000. The sizes observed varied, corresponding to molecular weights of from 150,000 to 600,000. A double-stranded circular DNA of molecular weight 150,000 consists of 240 base pairs. Because of the topological constraint, there would be an average angle of 1.5° between the planes of neighboring base pairs.

The high-magnification inset in Figure 8 shows no evidence of "looping out," nor were such regions visible in other circles examined at this magnification. In addition, there are no sharp bends in the circular form. Both of these characteristics argue against there being any differences in the lengths of the two strands of the circles. As a control, electron micrographs of single-stranded, circular d(T-G)$_n$ showed an irregular contour (unpublished observations).

The bouyant density of d(T-G)$_n$·d(C-A)$_n$ circles was 30 mg/cm^3 greater in alkaline CsCl than the average densities of d(T-G)$_n$ and d(C-A)$_n$ (*cf.* Figures 5, 7, and 9, top frame).

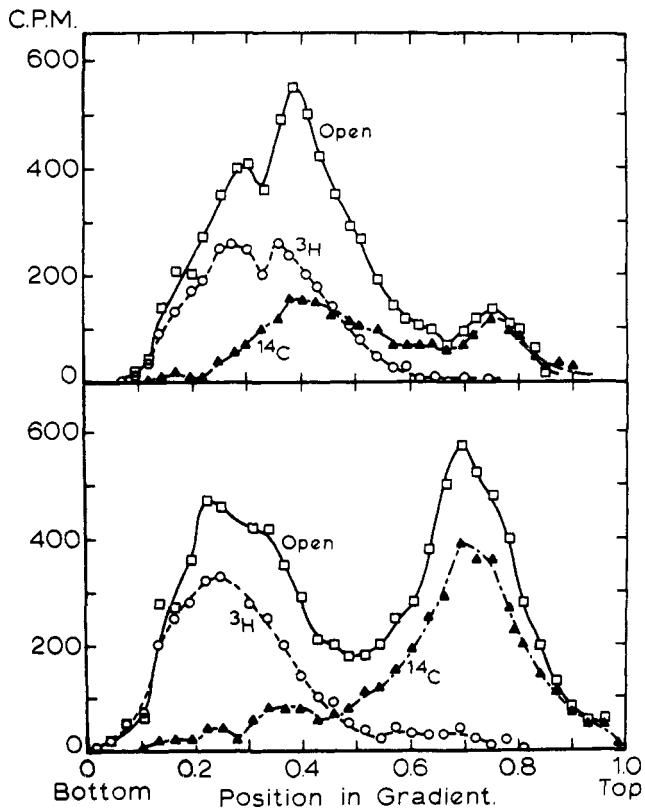

FIGURE 9: Alkaline CsCl gradient analysis of circular DNA. The starting polymer and conditions of centrifugation and analysis are the same as in Figure 1, except that the cpm values are for 0.10 ml of each fraction. The experimental conditions of preparation are given in Table III, experiment 2. The upper frame represents the equilibrium banding of material (41% of the total) corresponding to the faster sedimenting material in an alkaline sucrose gradient (see Results). The ^{14}C-containing polymer at position 0.4 in the gradient is double-stranded, circular, d(T-G)$_n$·d(C-A)$_n$. The lower frame represents the slower sedimenting material from the alkaline CsCl: (○) ^{3}H-restricted channel counts (poly(T-G); (▲) ^{14}C-restricted channel counts (poly(C-A)); (□) open channel counts (poly(T-G) plus poly(C-A)).

Discussion

The present paper has described the successful preparation, in very limited amounts, of circular single-stranded and double-stranded DNA-like polymers with defined repeating nucleotide sequences. It was expected that forms 3a to 3f (Figure 2) in which the two strands of the circularized molecules are of unequal length, and thus unable to form perfect duplexed circles, would predominate over form 3g, in which ligase could seal up both strands. This was in fact observed, the predominant circular structure formed in the presence of ligase alone being covalently closed in only one strand. The combined use of ligase and DNA polymerase in the presence of the four deoxyribonucleoside 5′-triphosphates led to the formation in significant amounts of the double-stranded circular polymer.

The most efficient way to generate double-stranded, circular d(T-G)$_n$·d(C-A)$_n$ studied to date appears to be (i) separation of the linear strands in an alkaline CsCl gradient, (ii) recombination under dilute conditions in the presence of polynucleotide ligase (thereby producing forms 2a and 2b, then various of the forms 3, and covalently joined species similar to form 3), and (iii) repair and/or excision of mismatched ends by DNA polymerase, then sealing of the second strand by ligase. For purification, degradation by exonuclease III to remove noncircular DNA, crude separation of faster and slower sedimenting material in an alkaline sucrose gradient, and finally, separation in an alkaline CsCl bouyant density gradient are most suitable. If the 30% of circles which appear to be double-stranded in alkaline CsCl (Table III) are representative of the ligase- and DNA polymerase-treated material, about 3.6% of the total DNA would be double-stranded circles after the joining reaction is complete. Another 8% would be single-stranded closed circles.

Another method of separating double-stranded from nicked circles would be the use of an ethidium bromide–CsCl gradient (*cf.* Pikó *et al.*, 1968). This technique was tried on one sample of circular DNA, but large losses invalidated the experiment (unpublished observations). Certainly this elegant technique is suggested, especially for the preparation of defined, repeating circular DNAs in which the component strands do not separate as readily as do d(T-G)$_n$ and d(C-A)$_n$ in alkaline CsCl.

The present study needs to be extended for preparation of the circular polymers in practically useful amounts. It is expected that the rather large losses attending purification observed in this work will diminish when the reactions are carried out on a larger scale. The principles for further work seem to be clear. One line which needs further investigation is the preparation of the double-stranded circles using the single-stranded circles as templates (going from forms 4a and 4b to form 5, Figure 2), because the single-stranded circles are produced in larger proportion and, presumably, their yield could be improved further.

The double-stranded, circular polymer was found to be heterogeneous in size. This was to be expected. First, the starting materials were not monodisperse with respect to molecular weight. Second, the mixing of the separated single-stranded d(C-A)$_n$ and d(T-G)$_n$ would lead to linear duplexes with a wide variation in the relative lateral displacements of the strands; linear elongation by end-to-end joining could also occur prior to, or simultaneously with, circularization of the

duplexes and the result would be the formation of circular molecules of variable size.

When the mixture of single- and double-stranded circles of experiment two, Table III, was centrifuged through an alkaline sucrose gradient, there was no clear separation between single- and double-stranded circles (unpublished observations). This was a result of the variability in molecular weights. However, the faster sedimenting 41% of this material contained 70% of the total double-stranded, circular DNA. It is to be expected that the double-stranded circular DNA would sediment faster than single-stranded circles; Pikó *et al.* (1968) showed that the double-stranded, cyclic form of mitochondrial DNA from sea urchin has a sedimentation coefficient 3.8 times as high as does the single-stranded, cyclic form in an alkaline medium.

The buoyant density of the double-stranded circular polymer in an alkaline cesium chloride gradient was about 30 mg/cm^3 higher than the average density of the two strands in the same medium. This result is similar to that reported previously for covalently circular polyoma DNA, which showed an increase of 19 mg/cm^3 in buoyant density relative to that of its linear component strands (Vinograd *et al.*, 1968). The enhanced density in alkali is a consequence of the peculiarly twisted, "tightened" structure which must be generated upon denaturation of covalently circular, double-stranded DNA by the high pH.

One hypothesis relating DNA structure to the control of polymerase activity is that polypyrimidine clusters in one strand of a bihelical DNA act like promoter regions, in the sense that polymerase binds to such regions with high affinity, and initiates transcription (Szybalski *et al.*, 1966). Relevant to this model, the repeating dinucleotide DNA $d(T\text{-}C)_n \cdot d(G\text{-}A)_n$, which contains only pyrimidines in one strand and only purines in the complementary strand, is transcribed asymmetrically by RNA polymerase; the polypyrimidine strand is transcribed at an initial rate up to 20 times that of the polypurine strand (Morgan, 1970). The other repeating dinucleotide DNA $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ is transcribed with only a slight, and variable, asymmetry. It contains alternating purines and pyrimidines in both strands, and the promoter hypothesis would predict that its transcription would not favor either strand, since neither contains the requisite polypyrimidine structure. The fact that it is transcribed at all, even though it has no polypyrimidine (*i.e.* promoter-like) regions, may well be related to its relatively low molecular weight. The consequent high frequency of free ends would provide sites for RNA polymerase to initiate transcription (Berg *et al.*, 1965). The synthesis of circular forms of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and $d(T\text{-}C)_n \cdot d(G\text{-}A)_n$ on a larger scale will permit us to test whether a DNA lacking ends and polypyrimidine regions can be transcribed at all.

Acknowledgments

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Synthesis of 2′(3′)-*O*-Glycyl Derivatives of Cytidylyl-(3′–5′)-inosine and 2′-Deoxycytidylyl-(3′–5′)-adenosine*

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ABSTRACT: The synthesis of the title compounds, potential substrates for ribosomal peptidyl transferase, is described. Orthoester **1**, prepared from inosine and ethyl *N*-benzyloxycarbonylaminoorthoacetate using methanesulfonic acid as catalyst, was condensed with *N*-acetyl-2′,5′-di-*O*-acetylcytidine 3′-phosphate (**2**) in the presence of dicyclohexylcarbodiimide in pyridine to give intermediate **3**. The latter was hydrolyzed in 80% formic acid at 0° to afford the 2′(3′)-*O*-(*N*-benzyloxycarbonyl)glycyl derivative **4a**. Hydrogenolysis of compound **4a** in 80% acetic acid at 0° using palladium catalyst gave the title derivative **4b**. Similarly, the *N*-dimethyl aminomethylene orthoester of adenosine 5′-phosphate (**5**) was condensed with *N*-dimethylaminomethylene-5′-*O*-(4-methoxy)trityl-2′-deoxycytidine (**6**) in the presence of dicyclohexylcarbodiimide in pyridine to afford (after removal of *N*-dimethylaminomethylene groups) the protected derivative **7**. Compound **7** was detritylated and hydrolyzed in 80% acetic acid at room temperature to the 2′(3′)-*O*-(*N*-benzyloxycarbonyl)glycyl derivative **8a** which was hydrogenolyzed (Pd) in 80% acetic acid at 0° to the title product **8b**.

Previous communications in this series (Rychlik *et al.*, 1967, 1969, 1970a) dealt with the problem of the substrate specificity of ribosomal peptidyl transferase, an enzyme which is believed to be responsible for the biosynthesis of all proteins in living organisms. We have found, using, as simple models, aminoacyl nucleosides and nucleotides instead of aminoacyl tRNA, that the substrate specificity of the enzyme depends on three major factors: (a) character of the nucleoside (base and sugar) moiety of a given aminoacyl derivative; (b) nature of the aminoacyl residue comprising a given aminoacyl nucleoside or nucleotide; (c) nature and chain length of oligonucleotide attached to the 5′ position of a given aminoacyl nucleoside.

Whereas 2′(3′)-*O*-glycyladenosine (A-Gly[1]) itself is inactive in the peptide chain transfer reaction catalyzed by peptidyl ribosomal transferase, it is a fairly good acceptor of the peptide chain when either a uridylyl-3′ or, still better, cytidylyl-3′ residue[2] is attached to the 5′-hydroxyl group of the parent compound. On the other hand, UpU-Gly, which does not contain any nucleoside units typical of the terminal trinucleotide of aminoacyl tRNA, exhibits no activity in peptide chain transfer. In order to pursue in greater detail the problem of acceptor activity of aminoacyl oligonucleotides in peptide chain transfer reaction catalyzed by peptidyl ribosomal transferase, we undertook the synthesis of two additional derivatives, CpI-Gly and dCpA-Gly. The former represents a relatively minor structural alteration in the molecule of CpA-Gly which was found to be a good substrate for peptidyl ribosomal transferase (Rychlik *et al.*, 1967). It is interesting to note that a similar structural change in the terminal unit of tRNA, *i.e.*, enzymic deamination of CpCpA to CpCpI, resulted in retention of amino acid acceptor activity of modified tRNA (Li and Su, 1967). Moreover, as shown previously (Rychlik *et al.*, 1969) I-Phe retains about 30% of transfer activity of A-Phe as determined in a ribosomal system containing poly(lysyl)- or *N*-acetylphenylalanyl-tRNA's as donors of peptide (or *N*-acylaminoacyl) residue.

In the case of dCpA-Gly the structure of the penultimate nucleoside unit of CpA-Gly is modified by replacing the 2′-hydroxyl group with hydrogen atom. As noted earlier (Rychlik *et al.*, 1969) a similar structural change in the terminal residue (A-Phe) led to almost complete loss of transfer activity in dA-Phe. The comparison of the effect of

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[1] Abbreviations used are: A-Gly, 2′(3′)-*O*-glycyladenosine; CpI-Gly, cytidylyl-(3′–5′)-(2′)3′-*O*-glycylinosine; dCpA-Gly, 2′-deoxycytidylyl-(3′–5′)-2′(3′)-*O*-glycyladenosine; CpCpA, cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-adenosine; CpCpI, cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-inosine; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; dA-Phe, 2′-deoxy-3′-*O*-L-phenylalanyladenosine; CpA, cytidylyl-(3′–5′)-adenosine; UpU-Gly, uridylyl-(3′–5′)-2′(3′)-*O*-glycyluridine; UpA-Gly, uridylyl-(3′–5′)-2′(3′)-*O*-glycyladenosine; CpA-Gly, cytidylyl-(3′–5′)-2′(3′)-*O*-glycyladenosine; CpI, cytidylyl-(3′–5′)-inosine; dCpA, 2′-deoxycytidylyl-(3′–5′)-adenosine; dC, 2′-deoxycytidine; A, adenosine; Cp, cytidine 3′-phosphate; Gly, glycine; I, inosine; pA(OEt)(CH_2NHZ), 2′,3′-*O*-(*N*-benzyloxycarbonyl)aminomethylethoxymethyleneadenosine 5′-phosphate; I(OEt)(CH_2NHZ), 2′,3′-*O*-(*N*-benzyloxycarbonyl)aminomethylethoxymethyleneinosine; MeOTrdCpA(OEt)(CH_2NHZ), 5′-*O*-(4-methoxy)trityl-2′-deoxycytidylyl-(3′–5′)-2′,3′-*O*-(*N*-benzyloxycarbonyl)-aminomethylethoxymethyleneadenosine; CpI-(ZGly), cytidylyl-(3′–5′)-2′(3′)-*O*-(*N*-benzyloxycarbonyl)glycylinosine; dCpA(ZGly), 2′-deoxycytidylyl-(3′–5′)-2′(3′)-*O*-(*N*-benzyloxycarbonyl)adenosine.

[2] The latter resembles most closely the terminal unit of aminoacyl tRNA.