scriptional diversity. However, parallel experiments with human brain nucleic acids indicate that highly diverse populations of RNA molecules are not restricted to a particular part of the brain. On the contrary, several different regions of the human brain show transcriptional activities even more diversified than those presently reported for mouse brain (L. Grouse and B. J. McCarthy, in preparation, 1971).

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Enzymatic and Physical Studies on $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ and $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$*

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ABSTRACT: Studies on the synthesis and characterization of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ are reported. The DNA is rereplicated much more efficiently at high pH values (9.3) than at lower pH values (7.4); in contrast, the sequence isomeric DNA, $(dI)_n\cdot(dC)_n$, is rereplicated more rapidly at the lower pH value. $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ possesses unusual X-ray diffraction and circular dichroism properties; hence, additional physical and enzymatic studies with this polymer are reported. The DNA is an efficient template or substrate for a variety of DNA-metabolizing enzymes including polymerases, nucleases, kinase, and ligase. DNA ligase from T4-infected *E. coli* readily forms circular $(dI\text{-}dC)_n$ in high yield; this reaction may be used as a sensitive assay for DNA ligase. Viscometric studies on linear $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ show that it readily undergoes a salt-facilitated strand rearrangement prior to the helix to coil transition, as shown for $(dA\text{-}dT)_n\cdot(dA\text{-}dT)_n$. Pancreatic DNase specifically cleaves $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ at the IpC linkage to give oligomers which are terminated at the 5′ end with pC residues. The effect of chain length on the melting and circular dichroism properties is reported. Studies on the synthesis of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ are reported. The synthetic reaction demonstrates linear kinetics with either $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ or $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ as templates and with either the *Micrococcus luteus* or the *Escherichia coli* DNA polymerase. The rate of the synthetic reaction is a function of the template nucleotide concentration and not the number of free 3′-OH ends. $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ is the most thermostable DNA found to date. Titration studies indicate that, at neutral pH, the base pairs are the normal Watson–Crick type. This DNA is insusceptible to degradation by exonuclease I.

A systematic study on the properties of DNA polymers is being performed in an effort to understand the effect of primary nucleotide sequence on the properties and structure of DNA (for a summary of these studies, see Wells *et al.*, 1970). We postulated that certain types of nucleotide sequences may serve as recognition sites for the regulation of genetic information. Fourteen different double-stranded DNAs contain-

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ing defined repeating nucleotide sequences have been synthesized. For these investigations to provide the maximum information, all possible members of the complete set of the simplest DNA polymers must be studied in a comparative fashion. At the outset of our work, neither $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ nor $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ had been synthesized.

We recently reported the synthesis and characterization of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (Grant *et al.*, 1968). Once this DNA was fully characterized it was used as a template for the synthesis of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$.

These two polymers are particularly interesting since (a) they both possess self-complementary structures, (b) $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ apparently has an unusual structure (Mitsui *et al.*, 1970), and (c) $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ is the most thermostable DNA found to date.

Materials and Methods

Nucleotides. Nucleoside triphosphates were purchased from Schwarz BioResearch, Orangeburg, N. J., and were at least 95% pure. ITP and dITP were prepared by nitrous acid deamination of ATP and dATP, respectively (Inman and Baldwin, 1964). The procedure for preparation of 5-bromo-dCTP (dBrCTP) was similar to that of Bessman *et al.* (1958). [γ^{32}-P]-rATP was prepared according to Weiss *et al.* (1968b).

DNA polymers were prepared and purified as previously described (Grant *et al.*, 1968; Wells *et al.*, 1970; Burd and Wells, 1970; Wells and Larson, 1970). The purity of polymers was checked by analytical cesium chloride density gradient centrifugation and the molecular weights were 10^5 or more as judged by alkaline sedimentation velocity analyses. $(rI\text{-}rC)_n\cdot(rI\text{-}rC)_n$ was synthesized as described (De Clercq *et al.*, 1971).

Enzymes. Micrococcus luteus DNA polymerase (Harwood *et al.*, 1970b) (gift of S. J. Harwood and P. F. Schendel) was used at two stages of purification, specific activities 41 and 750 ($(dA\text{-}dT)_n\cdot(dA\text{-}dT)_n$ assay). Two fractions of *E. coli* DNA polymerase were used. They were fraction VII in the purification procedure of Richardson *et al.* (1964) (specific activity 145) and fraction 6 in the purification procedure of Jovin *et al.* (1969) (specific activity 1790) (gift of R. W. Sweet) [$(dA\text{-}dT)_n\cdot(dA\text{-}dT)_n$ assay]. *Escherichia coli* RNA polymerase (specific activity 2200) was a gift from Dr. A. R. Morgan (Morgan and Wells, 1968). Spleen phosphodiesterase, bacterial alkaline phosphatase, pancreatic DNase, and pancreatic RNase were purchased from Worthington. The BAP was further purified (Weiss *et al.*, 1968b). The purified bacterial alkaline phosphatase was free of nucleolytic activity as judged by its inability to alter the sedimentation properties of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. Micrococcal nuclease was purchased from Mann (specific activity 5000). Exonuclease I (Lehman, 1966) was purified through fraction VI (specific activity 100). The preparation contains virtually no endonuclease (Figure 7). Polynucleotide kinase and DNA ligase were purified from T4 phage infected *E. coli* as previously described (Richardson, 1965; Weiss *et al.*, 1968a). The specific activity of kinase and ligase was 32,000 and 520, respectively.

Standard Polymerization Conditions and Assays. Standard DNA polymerase reactions (0.10 ml) contained the following components: 50 mM Tris-HCl buffer (pH 8.0), 5.0 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol, dGTP (or dITP as indicated), and dCTP each 0.20 mM (including any radioactively labeled triphosphate present), 10–30 μM $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ or $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ as indicated and *M. luteus* DNA polymerase (10–40 units/ml).

Two assays were employed: Assay I was the hypochromicity assay of Schachman *et al.* (1960). Assay II measured the formation of acid-insoluble product (Nishimura *et al.*, 1964).

Nuclease Digestions. Degradation of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ and $(dI)_n\cdot(dC)_n$ by pancreatic DNase was monitored by DEAE-cellulose paper chromatography (Bollum, 1965); the paper was irrigated with 0.80 M NH_4HCO_3. The dried chromatogram was cut into 0.5-in. strips and radioactivity was determined.

Degradation of [^{14}C]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ template was monitored during the synthesis of $(dI\text{-}dBrC)_n\cdot(dI\text{-}dBrC)_n$. The following concentrations were used: 0.54 mM dITP, 0.54 mM dBrCTP, 40 μM [^{14}C]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (1500 cpm/nmole) and 24 units/ml of *M. luteus* DNA polymerase (320 units/mg). All other components were as given for the standard reaction conditions. Susceptibility to pancreatic RNase was examined in a reaction mixtue (0.10 ml) containing 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0), 0.5 mg/ml of pancreatic RNase, and 88 μM [^{14}C]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (1500 cpm/nmole). R17 bacteriophage RNA (0.40 mg/ml) served as the control. At intervals, aliquots were removed and discharged into an equal volume of ice-cold 5% $HClO_4$. After standing several minutes in ice, the samples were centrifuged and the absorbance of the supernatant at 260 nm was determined.

Alkaline Phosphatase and Polynucleotide Kinase Reactions. A typical phosphatase reaction mixture (2.6 ml) contained 1.0 mM DNA (or RNA) polymer, 10 mM Tris-HCl buffer (pH 8.0), 2.0 mM sodium chloride, 10 mM magnesium chloride, and 6 units of alkaline phosphatase. The mixture was incubated for 30 min at 65° and additional phosphatase (6 units) was added at 10- and 20-min incubation. The product was deproteinized as described above.

A typical large scale kinase reaction (2.0 ml) contained 0.25 mM phosphatase-treated DNA polymer, 66 mM Tris-HCl buffer (pH 7.6), 10 mM magnesium chloride, 17 mM β-mercaptoethanol, 7.6 μM [γ-^{32}P]rATP (2.0×10^4 cpm/pmole), and 84 units of polynucleotide kinase. The mixture was incubated for 5 min at 37° and the reaction product was deproteinized as described above. Small scale reactions provided the basis for calculating the chain length of DNA polymers.

DNA Ligase Reactions. A typical DNA ligase reaction mixture (0.2 ml) contained 0.02 mM 5′-[^{32}P]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (2.9×10^4 cpm/nmole, 8.5×10^3 cpm/pmole of 5′-phosphate), 66 mM Tris-HCl (pH 7.6), 10 mM dithiothreitol, 6.6 mM magnesium chloride, 0.066 mM rATP, and 0.26 unit of polynucleotide ligase. The mixture was incubated at 37° and at time intervals, 10-μl aliquots were taken.

Two types of assays were routinely used. Assay I measured the formation of phosphatase resistant, acid-insoluble ^{32}P product; 0.010 ml of the reaction mixture was mixed with 0.10 ml of 0.5 M Tris-HCl (pH 8.0), 0.01 M magnesium chloride, and heated at 100° for 2 min. Alkaline phosphatase (0.3 unit) was added and the mixture was incubated for 40 min at 65°; additional phosphatase (0.3 unit) was added at 20 min. Acid-insoluble ^{32}P was measured as described above. Assay II measured the formation of exonuclease I resistant acid-insoluble ^{32}P product; 0.010 ml of the reaction mixture was mixed with 0.10 ml of 0.08 M glycine buffer (pH 9.5)–0.008 M magnesium chloride, and was heated at 100° for 2 min. β-Mercaptoethanol (final 2 mM) and 0.14 unit of exonuclease I were added and the mixture was incubated for 60 min at 37°. Acid-insoluble ^{32}P was determined. Using assay II, ^{3}H- or ^{14}C-labeled DNA could be used as a substrate for ligase reaction.

Large-Scale Preparation of Ligase Product of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. The DNA polymerase reaction mixture (7.0 ml) contained 0.027 mM $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ template, 1 mM dITP,

1 mM [^{14}C]dCTP (250 cpm/nmole), 50 mM Tris-HCl (pH 7.6), 5 mM magnesium chloride, 1 mM β-mercaptoethanol, and 50 unit/ml of *M. luteus* DNA polymerase. The mixture was incubated at 37° for 12 hr. Dithiothreitol (final 10 mM), rATP (0.06 mM), and 256 units of ligase were added to this solution (final volume 9.74 ml) followed by further incubation at 37° for 15 min; the ligase reaction was followed by assay II. The product was purified as described above. (dI-dC)$_n$·(dI-dC)$_n$ thus obtained (4.8 μmoles) was digested with 330 units of exonuclease I in 67 mM glycine buffer (pH 9.5), 6.7 mM magnesium chloride, and 1 mM β-mercaptoethanol (final volume 36.8 ml) at 37° for 60 min; the reaction was followed by acid-insoluble radioactivity. The remaining polymer was again purified as described above. The final product (3.9 μmoles) was more than 90% resistant to further exonuclease digestion.

Nearest-Neighbor Analysis of Ligase Product. 5′-[^{32}P]-(dI-dC)$_n$·(dI-dC)$_n$ was treated with DNA ligase followed by alkaline phosphatase to remove any radioactive phosphorus which was not in a diester linkage. The product was deproteinized and dialyzed. The DNA (48 μM, 7.2 × 10^3 cpm/nmole) was treated with pancreatic DNase (100 μg/ml) for 3 hr and then with snake venom phosphodiesterase (100 μg/ml) for 3 hr at 37°; the mixture (0.11 ml) contained 10 mM glycine buffer (pH 8.8) and 10 mM magnesium chloride. Another sample (DNA concentration 27 μM) was treated with micrococcal nuclease (50 μg/ml) for 12 hr at 37° in 0.2 ml of 10 mM Tris-HCl (pH 8.5), 10 mM calcium chloride, and then with spleen phosphodiesterase (0.5 unit/ml) for 4 hr at 37° in 0.21 ml of 30 mM potassium phosphate buffer (pH 6.5), to digest the DNA into 3′-nucleotides. Other details have been described (Wells *et al.*, 1967).

Both digests were analyzed in the following two systems: (A) electrophoresis (2000 V, 1.5 hr) in 0.05 M sodium citrate buffer (pH 3.5) on Whatman No. 3MM paper; (B) descending paper chromatogram, solvent B, described below. No radioactivity was observed at the origins; a small amount (1–10%) of radioactive P_i was always observed.

5′-End Analysis of Pancreatic DNase Digests by Kinase Reaction. (dI-dC)$_n$·(dI-dC)$_n$ (1 mM) was digested with pancreatic DNase (1.4 unit/ml) in Tris-HCl, pH 8.0 (67 mM), and magnesium chloride (10 mM) or manganese chloride (10 mM) at 20° for 30 min (total volume 1.4 ml). The reaction was stopped by heating at 80° for 10 min and the mixture was treated with alkaline phosphatase (45 units) at 70° for 30 min. The preparation was deproteinized and desalted as above. The DNAs were phosphorylated with kinase, deproteinized, and desalted. The 5′-[^{32}P](dI-dC) oligomers thus obtained (1.2 × 10^4 cpm/nmole) were digested with pancreatic DNase and snake venom phosphodiesterase and the radioactivity of 5′-nucleotides was determined in systems A and B.

Preparation of Oligo(dC-dI) with Varying Chain Lengths. The pancreatic DNase reactions were as described above. The mixture was divided into six tubes which were incubated at 20° for 0, 5, 10, 20, 30, and 60 min, respectively. Each reaction was stopped by heating at 85° for 10 min and the mixtures were then treated with alkaline phosphatase (5 units) followed by chloroform extraction and dialysis as described above. The chain lengths of the DNA digests were determined by kinase reactions and total phosphate assays.

Titration of DNA. All pH measurements were performed with a Beckman Zeromatic II pH meter equipped with a ceramic junction combination electrode. Meter drift was less than 0.1 pH unit.

For the determination of ultraviolet absorbance *vs.* pH profiles, a 2.0-ml sample containing 0.1–0.2 μmole of polymer in 0.01 M NaCl–1 × 10^{-4} M EDTA was placed in a 1.0-cm path-length quartz cuvet in a spectrophotometer. The solution was stirred by a stream of helium bubbles from a capillary inserted in the cuvet. HCl or NaOH was added from a 10-μl syringe. No turbidity developed during any determinations as evidenced by readings at 320 nm. All absorbance measurements were corrected for volume changes.

Viscosity Measurements. Viscosity *vs.* temperature profiles for (dI-dC)$_n$·(dI-dC)$_n$ were determined using a Cartesian-diver viscometer (Gill and Thompson, 1967) modified with an automatic timer. A detailed description of the modified apparatus may be obtained from R. D. Wells. The shear rate with water at 15° was 1.0 sec^{-1}.

The DNA solution was degassed *in vacuo* and was centrifuged. The cell was assembled and after allowing sufficient time for the diver to reach equilibrium (no detectable precession), measurements were begun. After each temperature increase, ample time (20 min) was allowed for the sample to reach equilibrium before measurements were resumed. The molecular weight of the DNA as determined by alkaline sedimentation velocity analysis was 1.9 × 10^5 daltons/strand.

Other Techniques. Paper chromatography solvent systems were: solvent A, isobutyric acid–concentrated NH_4OH–water (66:1:33, v/v); solvent B, 1 l. of 0.1 M sodium phosphate (pH 6.8)/600 g of $(NH_4)_2SO_4$/20 ml of 1-propanol. Both were used in descending chromatography on Whatman No. 1 paper.

Analytical sedimentation velocity, buoyant density studies, molar extinction coefficients, and T_M measurements were determined as previously described (Wells and Blair, 1967; Wells *et al.*, 1970). Circular dichroism spectra were determined with a Cary 60 equipped with a CD attachment; we thank Dr. Phillip A. Hart, Department of Pharmacy, University of Wisconsin, for his generous help with these determinations. Other methods were as described (Wells *et al.*, 1970).

Results and Discussion

I. Rereplication of (dI-dC)$_n$·(dI-dC)$_n$

(dI-dC)$_n$·(dI-dC)$_n$ was initially formed as a *de novo* product by the *M. luteus* DNA polymerase (Grant *et al.*, 1968; Burd and Wells, 1970). Further studies indicate that buffer pH has a marked influence on the rate of this reaction. This DNA is replicated approximately five times faster at pH 9.3 than at pH 7.4 (results not shown). However, the final extent of all reactions between pH's 7.4 and 9.3 was about the same after 17.5-hr reaction time.

In general, the *M. luteus* DNA polymerase does not have a high pH optimum for DNA synthesis (Zimmerman, 1966). Indeed, the sequence isomeric DNA, (dI)$_n$·(dC)$_n$, is efficiently and faithfully rereplicated only at lower pH values (pH 7.4) (Grant *et al.*, 1968).

It should be noted that this effect of pH on the rereplication rates of the two polymers is in harmony with the effect of pH on the type of product which is formed in a *de novo* reaction (Burd and Wells, 1970). At low pH values, the *de novo* product is (dI)$_n$·(dC)$_n$, whereas at higher pH values, the *de novo* product is (dI-dC)$_n$·(dI-dC)$_n$.

II. Additional Properties of (dI-dC)$_n$·(dI-dC)$_n$

(dI-dC)$_n$·(dI-dC)$_n$ is unique since (1) like (dA-dT)$_n$·(dA-dT)$_n$, it possesses a self-complementary sequence of nucleotides, (2) it has a circular dichroism (CD) spectrum which is

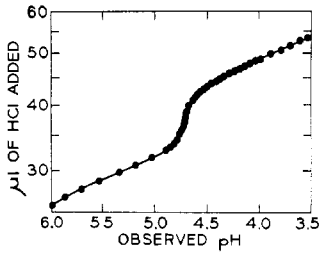


FIGURE 1: Acid titration of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. A sample of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (6.0 μmoles in 2.0 ml of 0.02 M NaCl–2 × 10^{-3} M sodium phosphate–1 × 10^{-5} M EDTA) was titrated with 0.199 N HCl at room temperature. The titration of the buffer has not been subtracted. The apparent pK was 4.7.

markedly different from ten other double-stranded DNA polymers and natural DNA (Wells *et al.*, 1970; Mitsui *et al.*, 1970), and (3) it showed a bizarre X-ray diffraction pattern (Mitsui *et al.*, 1970). The X-ray studies on the sodium salt of the polymer, at 75% relative humidity, indicated that the DNA has eight base pairs per turn of helix (25.0 Å). A number of models were constructed to explain the data and a left-handed model showed at least as good a fit of the calculated intensities to observed intensities as any right-handed models. The "inverted" CD spectrum for this polymer was consistent with this possibility. Further X-ray studies at both 75 and 95% relative humidity have provided patterns with appreciably higher resolution than the reported pattern (Mitsui *et al.*, 1970); these patterns are identical with our published pattern. However, when the humidity was raised to 98%, a typical DNA B pattern was observed (R. Langridge, personal communication), thus suggesting that the structure at 75 and 95% relative humidity is some unusual right-handed helix. The CD results remain unexplained. In an effort to better understand the nature of this unique structure we performed titration studies, enzymatic studies and viscosity studies.

a. Titration of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. We concluded (Mitsui *et al.*, 1970) that this DNA contains normal Watson–Crick base pairs. Thermal stability studies indicated that there were at least two hydrogen bonds per base pair (Wells *et al.*, 1970). Only the normal Watson–Crick base pair does not require that an additional proton be shared by the bases; Hoogsteen, reversed Hoogsteen, and displaced Watson–Crick-type pairs all require protonation.

Titration studies were performed to determine if the base pairs were protonated at neutral pH values (Figure 1). The titration which occurred between pH 6.0 and 3.5 was attributed to the protonation of cytidylic acid moieties in the polymer

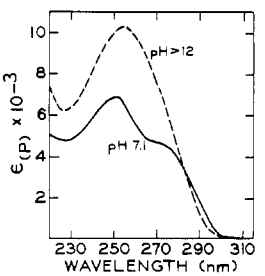


FIGURE 2: Spectra of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ in neutral and alkaline solution. The spectrum of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ in 0.01 M NaCl–1.0 × 10^{-4} M EDTA was determined at pH 7.1 (solid line) and at pH >12 (dashed line).

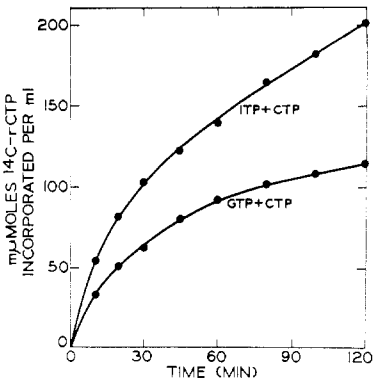
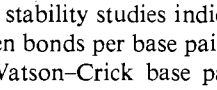


FIGURE 3: $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ as template for RNA polymerase. The 0.10-ml reaction mixtures contained 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 12 mM 2-mercaptoethanol, 0.46 mM CTP, [^{14}C]CTP (1.7 × 10^3 cpm/nmole), either 0.48 mM ITP or 0.53 mM GTP, 40 μM $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$, and 120 units/ml of *E. coli* RNA polymerase. All reactions were at 37°. The formation of acid-insoluble product was monitored by assay II.

based on a comparison of the pK's of cytidine and inosine. That none of the cytidylic acid residues in the polymer were protonated above pH 5.5 was established by the fact that 3.0 μequiv of acid were required to lower the pH from 5.19 to 4.29, the range in which protonation occurred. This amount of acid was equivalent to the total amount of cytidylic acid in the sample. The apparent pK for cytidylic acid moieties in the polymer was 4.7. The titration curve was completely reversible. The ultraviolet absorbance *vs.* pH profile showed the sharp increase in absorbance characteristic of a helix–random coil transition in the pH range 4.9–4.6 (data not shown).

The absorbance *vs.* pH profile in alkaline solution was also determined (data not shown). A highly cooperative, reversible change in absorbance occurred at pH 10.4–10.5. The change in the ultraviolet spectrum coincident with this transition is shown in Figure 2. Since only the inosinic acid groups in this polymer have a functional group which may be titrated in this pH region, this transition was attributed to ionization of the



—NH—C=O group of inosinic acid. The observed pK for $(dI)_n\cdot(dC)_n$ was 9.9 (Inman and Baldwin, 1964). This is consistent with our finding (Wells *et al.*, 1970) that $(dI)_n\cdot(dC)_n$ is less thermostable than $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. These studies proved that the base pairs in $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ do not involve protonated cytidine residues and hence must assume a normal Watson–Crick arrangement.

b. Enzymatic Studies. If, in fact, $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ does exist in an unusual helical configuration, it is conceivable that the structure might not be recognized by some DNA-metabolizing enzymes. Twelve different DNA-metabolizing enzyme and one RNase were tested for activity with this DNA as the substrate or template. All, except pancreatic ribonucleases, were active on this DNA.

1. TRANSCRIPTION. Figure 3 shows the kinetics of synthesis of $(I\text{-}C)_n\cdot(I\text{-}C)_n$ (Krakow and Karstadt, 1967) and $(G\text{-}C)_n\cdot(G\text{-}C)_n$ (Karstadt and Krakow, 1970; De Clercq *et al.*, 1971). The amount of $(I\text{-}C)_n\cdot(I\text{-}C)_n$ formed in 2 hr represented a 10-fold transcription of the DNA template. In the synthesis of $(G\text{-}C)_n\cdot(G\text{-}C)_n$, 5.7-fold transcription of the DNA was observed in two hours. The RNA polymerase of *Azotobacter vinelandii* is also capable of utilizing $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ as a template (Karstadt and Krakow, 1970). This DNA also is a template for DNA polymerase (see below and Burd and Wells, 1970).

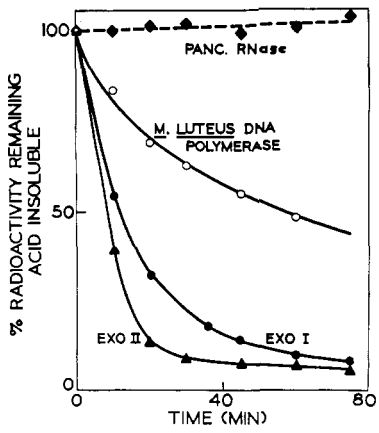


FIGURE 4: Nucleolytic degradation of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. Exonuclease I digestion was carried out in a 0.375-ml reaction mixture containing 66.7 mM sodium glycinate (pH 9.5), 6.67 mM $MgCl_2$, 1.7 mM 2-mercaptoethanol, 38 μM [^{14}C]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (1500 cpm/nmole), and 1 unit of exonuclease I. For exonuclease II digestion the 0.375-ml reaction mixture contained 66.7 mM sodium glycinate (pH 9.5), 6.67 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol, 38 μM [^{14}C]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ and *E. coli* DNA polymerase (fraction 6 of Jovin *et al.*, 1969), 36 units of polymerase activity/ml. The degradation by the nuclease activity accompanying the preparation of *M. luteus* DNA polymerase was measured during synthesis of $(dI\text{-}dBrC)_n\cdot(dI\text{-}dBrC)_n$. All reactions were at 37°. In each case, the loss of acid-insoluble radioactivity was monitored by assay II. Other details are in Materials and Methods.

2. NUCLEASE DIGESTIONS. The ability of micrococcal nuclease and spleen phosphodiesterase to degrade this DNA made possible the successful nearest-neighbor frequency analyses used in establishing its base sequence (Grant *et al.*, 1968). The kinetics of degradation in the presence of purified *E. coli* exonuclease I, *E. coli* exonuclease II (Lehman and Richardson, 1964), pancreatic RNase and the exonucleolytic activity associated with a partially purified preparation of *M. luteus* DNA polymerase (Harwood *et al.*, 1970a; Miller and Wells, 1971) are shown in Figure 4. No detectable degradation occurred in the presence of pancreatic RNase. Under identical conditions, digestion of an R17 bacteriophage RNA control was 91% complete in 10 min. Essentially complete degradation was observed with both exo-I and exo-II when higher enzyme concentrations were used.

$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ was also readily degraded by pancreatic DNase (Figure 5). After 150 min essentially no polymer remained at the origin of the chromatogram and only 11% remained as oligonucleotide with an R_F of less than 0.46.

For the sake of comparison, essentially equal amounts of the sequence isomeric polymer, $(dI)_n\cdot(dC)_n$, were subjected to degradation by exo-I, exo-II, pancreatic DNase and the nucleolytic activity of the *M. luteus* DNA polymerase preparation. In every case, the alternating copolymer was attacked far more readily than the isomeric homopolymer pair (data not shown).

3. LIGASE REACTION WITH $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ AND RELATED POLYMERS. $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ is an effective substrate for DNA ligase from T4-infected *E. coli* (Figure 6). The substrate was labeled at the 5′ terminus with ^{32}P as mediated by polynucleotide kinase (Materials and Methods). The maximum reaction limit was around 60–65%; further reaction was not obtained if experiments were performed at 45 or 27° or if more ligase was added or if the DNA substrate was heat denatured. When ligase was omitted, the substrate showed 15% phosphatase resistance, which conceivably was due to ligase contamination of the kinase preparations.

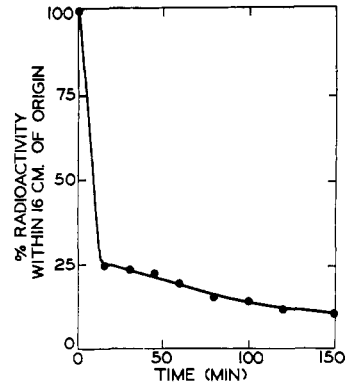


FIGURE 5: Degradation of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ by pancreatic DNase. The 0.10-ml reaction mixture contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.21 mM [^{14}C]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (1500 cpm/nmole), and 8.3 μg/ml of pancreatic DNase. The reaction was incubated at 37°. Degradation was monitored by chromatography as described in Materials and Methods.

It cannot be determined from these results if ligase has joined overlapping linear molecules to give a net increase in polymer molecular weight or if the enzyme has joined hairpin structures (Olivera *et al.*, 1968) to give circular products. Hence, we examined the exonuclease I susceptibility of the ligase product. This exonuclease cannot degrade a circular DNA which has no free ends.

Figure 7 shows that ligase-treated 5′-[^{32}P]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ was resistant to exonuclease I to an extent of approximately 60% which coincided with the limit of phosphatase resistance. By nicking a circular form by an endonuclease, all the radioactivity became sensitive to the action of exonuclease I. Although DNA bearing 3′-phosphate terminii is also resistant to exonuclease I, it is not likely that this is the reason for the insensitivity, because the substrate DNA was pretreated with phosphatase before kinase and ligase reactions. The coincidence of phosphatase and exonuclease resistance of ligase products indicates that most ligase reactions on $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ were accomplished by a circle formation. This fact justifies the use of assay II (exonuclease I resistance) as a general ligase assay, when this polymer is used as a substrate. A similar assay method was recently reported by Modrich and Lehman (1970) using $(dA\text{-}dT)_n\cdot(dA\text{-}dT)_n$. However, if

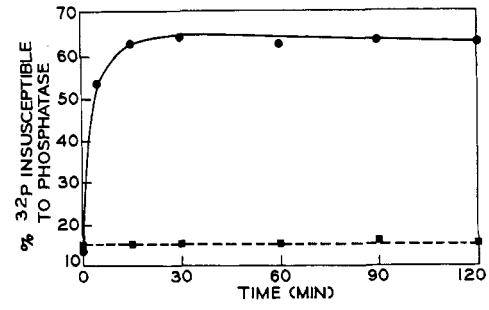


FIGURE 6: Ligase reaction on $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. The reaction mixture (0.045 ml) contained 5′-[^{32}P]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (356 nmole/ml, 4000 cpm/nmole, and 470 cpm/pmole of 5′-phosphate). Other details are described in Materials and Methods. Assay I and the DEAE-cellulose paper assay were used; 100% = 6000 cpm. (■) No ligase added; (●) 20 unit/ml of ligase added.

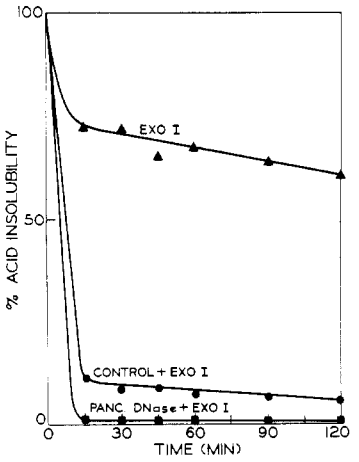


FIGURE 7: Exonuclease I insusceptibility of ligase product of (dI-dC)$_n$·(dI-dC)$_n$. The ligase product was obtained by incubating [5′-^{32}P]polymer with ligase at 37° for 30 min as described in Materials and Methods. The ligase rendered 60% of the isotope resistant to alkaline phosphatase. A control was treated similarly except that no ligase was added. After incubation, both mixtures were kept at 100° for 2 min to terminate the reaction. Both the ligase reaction mixture (▲) and the control reaction mixture (●) were incubated with exonuclease I (1 unit) as described under assay II (total volume 0.86 ml). Another ligase reaction mixture was treated with pancreatic DNase (5 μg) as well as exonuclease I (■). At intervals, samples (0.1 ml) were removed for determination of acid-insoluble radioactivity by filter paper disk method. The zero-time samples (100% acid insolubility) contained 3800–4500 cpm.

an endonuclease is present, such as with Rous sarcoma virus, only assay I can be successfully employed (Mizutani *et al.*, 1971).

The ability of ligase to join related polymers was also investigated. 5′-[^{32}P](dG-dC)$_n$·(dG-dC)$_n$ (60,000 cpm/nmole; 7000 cpm/pmole of 5′-phosphate) was a poor substrate for ligase compared to (dI-dC)$_n$·(dI-dC)$_n$; the maximum limit of phosphatase resistance was 20%. Change of incubation temperature (45, 55°), heat treatment (100°, 10 min) of the substrate polymer (in 10^{-4} M EDTA, pH 7.5) or the use of organic solvent (ethylene glycol, formamide, dimethyl sulfoxide, glycerol) to lower the polymer T_m did not produce more efficient joining. For the ligase reaction with this DNA, only assay I was successfully used, because (dG-dC)$_n$·(dG-dC)$_n$ was completely resistant to exonuclease I even without ligase treatment (see below). 5′-[^{32}P](rI-rC)$_n$·(rI-rC)$_n$ (72,000 cpm/nmole; 5000 cpm/pmole of 5′-phosphate) was also tested as a substrate for T4 ligase; manganese chloride and magnesium chloride were individually tested as metal salts. No phosphatase-resistant ^{32}P was detectable above background.

TABLE I: Products of Enzymic Degradation of Ligase Products of (dI-dC)$_n$·(dI-dC)$_n$.[a]

Analysis System	% [^{32}P]5′-dIMP	% [^{32}P]5′-dCMP	Total Radioactivity (cpm)
I. Pancreatic DNase and Snake Venom Phosphodiesterase Digestion			
A	83.5	16.5	7,200
B	82.5	17.5	4,200
II. Micrococcal Nuclease and Spleen Phosphodiesterase Digestion			
	% [^{32}P]3′-dIMP	% [^{32}P]3′-dCMP	
A	27.5	72.5	11,800
B	28.2	71.8	7,200

[a] The percentage radioactivities are ±0.5–1%. Other details are cited in Materials and Methods.

TABLE II: T_m and Molecular Weight Determination on Ligase Products of (dI-dC)$_n$·(dI-dC)$_n$.

Prepn	T_m[a] (°C) Ligase Treated	T_m[a] (°C) Control
1	48.8	47.5
2	48.2	47.4
	Mol Wt	
1	4.4×10^5	2.2×10^5
2	3.9×10^5	2.9×10^5

[a] Each T_m value is the average of several determinations on a given preparation in 0.05 M sodium chloride–1 mM potassium phosphate buffer–10^{-5} M EDTA (pH 7.0). Molecular weights were determined by sedimentation analyses in alkaline solution. The control (dI-dC)$_n$·(dI-dC)$_n$ was taken from the same incubation mixture of DNA replication and was incubated further, without ligase, for the same period as the ligase-treated sample. Other details are given in Materials and Methods.

4. PROPERTIES OF CIRCULAR (dI-dC)$_n$·(dI-dC)$_n$. Conclusive evidence for DNA joining was obtained by nearest-neighbor frequency studies on ligase-treated 5′-^{32}P-labeled (dI-dC)$_n$·(dI-dC)$_n$ (Table I). Digestion of the product to 5′-mononucleotides (Table I, part I) showed that most of the isotope was in d-pI whereas digestion of the product to 3′-mononucleotides (Table I, part II) showed that most of the isotope was transferred to d-Cp.

That the product of the joining reaction on (dI-dC)$_n$·(dI-dC)$_n$ is circular is clearly demonstrated by the exonuclease I susceptibility studies (see above). The T_m and molecular weight studies in Table II are consistent with this finding. The T_m of the ligase-treated polymer was slightly higher than the control value; although this increment was small, it was readily reproducible. This change in T_m is quite small compared to the increment found for circular (dA-dT)$_n$·(dA-dT)$_n$ (Olivera *et al.*, 1968); however, our DNA (chain length ~1000) is approximately 25 times as large as the sample studied (chain length ~42) by these workers. The weight-average molecular weight values (Table II) suggest that the primary mode of joining is to form circles and not to join staggered polymer chains (Burd and Wells, 1970).

c. Viscosity Studies on (dI-dC)$_n$·(dI-dC)$_n$. Ultraviolet absorbance *vs.* temperature profiles of this DNA showed that the width of the helix–coil transition region increased with increasing sodium ion concentration in the range 0.02–1.0 M Na^+ (Wells *et al.*, 1970). Inman and Baldwin (1962) reported a similar behavior for the alternating copolymer

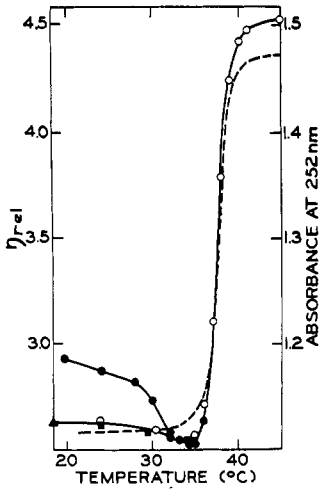


FIGURE 8: Viscosity of $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ in 0.01 M NaCl solution as a function of temperature. The relative viscosity of a $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ sample (15.8 mM) in 0.01 M NaCl–10^{-4} M sodium phosphate–1 × 10^{-5} M EDTA (pH 6.4) was monitored at intervals from 18 to 45°. Initial heating to 36° (●); cooling from 36 to 24° (■); heating from 24 to 45° (○). The sample was cooled from 45 to 18° and held at that temperature for 30 hr (▲, single point). For comparison the absorbance–temperature profile of the identical DNA sample was determined in the above solution (dashed line). The T_m was 37.9°. Additional details are described in Materials and Methods.

$(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$. On the basis of a variety of studies (Inman and Baldwin, 1964; Spatz and Baldwin, 1965; Scheffler *et al.*, 1968; Elson *et al.*, 1970), these workers concluded that this DNA undergoes an intramolecular rearrangement at temperatures below the helix–coil transition region. This rearrangement, made possible by the self-complementary sequence of the strands, resulted in a more compact, highly branched molecule containing shorter helical segments. The extent of the refolding process was determined by the salt concentration.

Since $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ also has a self-complementary structure, viscosity *vs.* temperature profiles were determined for this polymer at two salt concentrations. Figure 8 shows the result in 0.01 M NaCl solution (pH 6.4). The relative viscosity of the solution decreased markedly above 28°, passing through a minimum at 33–35°. The very sharp increase in viscosity in the range 35–40° corresponded to the helix–random coil transition as was shown by the absorbance *vs.* temperature profile of the same sample, also shown in this Figure. The T_m of the sample was 37.9°. Upon cooling of the sample below 33° the viscosity did not return to its initial value. Continued incubation of the sample at 18° for 30 hr gave no further detectable change in viscosity. A second heating followed the lower curve in Figure 8. The reduced viscosity for the helix at 20° was 4.4 dl/g before heating and 3.7 dl/g after cooling; the reduced viscosity of the coil at 45° was 8.1 dl/g.

The profile in 1.0 M NaCl solution is shown in Figure 9. The viscosity again decreased markedly at temperature well below the onset of absorbance changes. Heating from 29 to 52° caused a 17% decrease in the relative viscosity with no measurable absorbance changes. The T_m, from the absorbance *vs.* temperature profile on the identical sample, was 61.9°. The minimum in the viscosity curve appeared at, or slightly above, this temperature. Cooling of the sample brought about a partial reversal of the viscosity changes. The shape of the cooling curve was found to be time dependent as Inman and

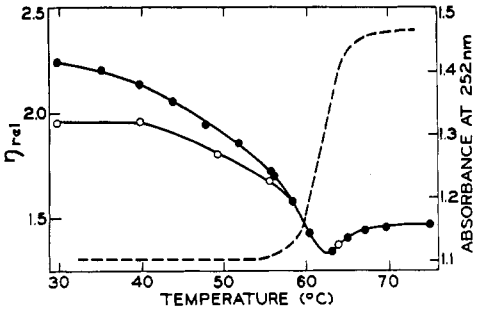


FIGURE 9: Viscosity of $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ in 1.0 M NaCl solution as a function of temperature. The relative viscosity of a $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ sample (15.8 mM) in 1.0 M NaCl–0.01 M sodium phosphate–1 × 10^{-4} M EDTA (pH 7.1) was monitored at temperatures in the region 29–75°. Initial heating (●); cooling (○). The absorbance *vs.* temperature profile of the identical sample in the above solution (dashed line) gave a T_m of 61.9°. Additional details are described in Materials and Methods.

Baldwin (1962) reported for $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$. No attempt was made to allow a new equilibrium to be established at each temperature during the cooling period. The reduced viscosity of the helix at 29.5° was 2.8 dl/g before heating and 2.1 dl/g after cooling; the reduced viscosity of the coil at 75° was 1.0 dl/g.

The shapes of these two profiles are qualitatively similar to those reported for $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$ (Inman and Baldwin, 1962; Spatz and Baldwin, 1965) and indicate that $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ also undergoes an intramolecular rearrangement of the self-complementary strands prior to the major helix–coil transition. The fact that the coil form of the polymer has a higher reduced viscosity than the helix in 0.01 M NaCl and a lower value than the helix in 1.0 M NaCl is consistent with previous observations (Inman and Baldwin, 1962; Eigner and Doty, 1965).

d. Oligo(dC-dI)·Oligo(dC-dI). The base specificity of the pancreatic DNase reaction on $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ was investigated by 5′-nucleotide analyses as mediated by polynucleotide kinase. The polymer substrate, prior to DNase digestion, contained predominantly dI residues (>80%) at the 5′ terminii (Table III). However, after DNase digestion, in either $MgCl_2$ or $MnCl_2$ solution, greater than 90% of the 5′ terminii were dC residues. From comparison of chain lengths before and after pancreatic DNase digestion, approximately one of ten 5′ ends should be a dI residue which came from the

TABLE III: 5′-Nucleotide Analysis of DNase Digests of $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ by Kinase Reaction.[a]

DNA Treatment (Chain Length by Kinase Reaction)	Analysis System	% [^{32}P]5′-dIMP	% [^{32}P]5′-dCMP	Total Radioactivity (cpm)
None (337)	A	82.7	17.3	12,600
	B	82.8	17.2	20,100
DNase in Mg^{2+} (28)	A	9.7	90.3	10,100
	B	8.5	91.5	6,100
DNase in Mn^{2+} (32)	A	9.3	90.7	26,300
	B	6.4	93.6	15,700

[a] Other details are given in Materials and Methods.

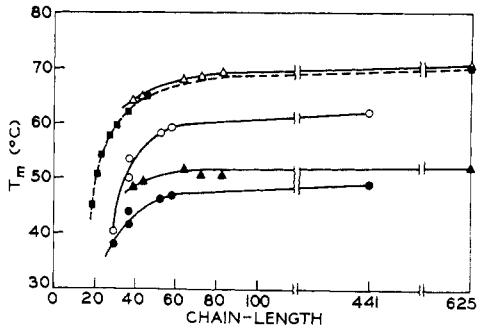


FIGURE 10: Effect of chain length on T_m of oligomers of (dC-dI) and (dT-dA). Oligomers were prepared and characterized as described in Materials and Methods. (●) Oligo(dC-dI) in A; (○) oligo(dC-dI) in B; (▲) oligo(dT-dA) in part A; (△) oligo(dT-dA) in part B; (■) oligo(dT-dA) in part B, data from Scheffler *et al.* (1968). (A) 0.05 M sodium chloride–0.5 mM potassium phosphate buffer (pH 7.0); (B) 0.5 M sodium chloride–5 mM potassium phosphate buffer (pH 7.0).

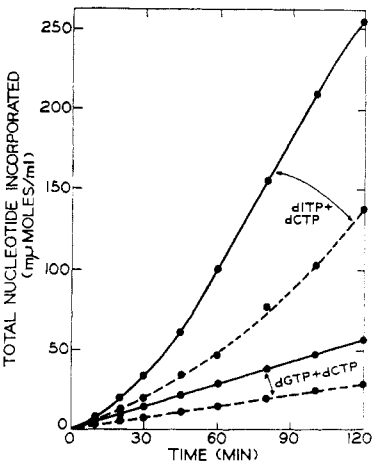


FIGURE 11: $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ as a template for DNA polymerase. The 0.10-ml reaction mixtures contained 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.20 mM dCTP, 0.20 mM dITP or dGTP, 13 μM $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$, and 30 units/ml of either *M. luteus* or the *E. coli* DNA polymerase. The reactions involving dITP contained [^{14}C]dCTP (3.2×10^3 cpm/nmole). The reactions involving dGTP contained [α-^{32}P]dGTP (1.0×10^4 cpm/nmole). All reactions were at 37°. The formation of acid-insoluble product was measured by assay II. Solid lines indicate reactions containing *M. luteus* DNA polymerase. Broken lines indicate reactions containing *E. coli* DNA polymerase.

original DNA. Thus pancreatic DNase cleaves this DNA only at the IpC bond. This is in accord with the finding (Scheffler *et al.*, 1968) that this DNase cleaves $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$ only at the ApT bond.

The effect of oligomer chain length on melting properties and circular dichroism spectra were studied to determine the length of the shortest chain which still retained the properties of the polymer. Figure 10 shows the effect of chain length on T_m at two salt concentrations; oligo(dT-dA)·oligo(dT-dA) is included as a control. A chain length of at least 50 nucleotides is necessary to show a T_m comparable to the polymer.

Similarly, CD studies (not shown) demonstrated that oligomers with chain lengths less than 60 had drastically different spectra than the polymer (Mitsui *et al.*, 1970; Wells *et al.*, 1970). As the chain length of the (dC-dI) oligomers decreased, the negative long-wavelength band at 282 nm approached zero whereas the positive band at 238 nm changed from positive to negative values. The CD spectrum of $(dC\text{-}dI)_{15}$ was superposable with the spectrum of heat-denatured $(dI\text{-}dC)_{200}$ (Mitsui *et al.*, 1970). These results demonstrate that at least three to four turns of helix are necessary for an oligomer to have comparable properties to the polymer and suggest that previously unrecognized long-range structural forces may be important for DNA.

e. Concluding Remarks. Though $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ was found to possess unique physical properties, the 13 DNA metabolizing enzymes tested were found to utilize it readily as a template or substrate. These findings suggest that variations in the physical properties of a DNA have little effect on the ability of enzymes to act upon the molecule. At the same time, variations in the secondary structure of a DNA resulting from unique base sequences may still serve as recognition signals for special functions such as initiation and termination of transcription and replication *in vivo* (Szybalski *et al.*, 1969; Wells *et al.*, 1970). The ability of $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ and $(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$ to undergo refolding resulting in the formation of short hairpin helices may have implications for biological systems, since they might serve as recognition sites.

III. Synthesis of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$

a. Kinetics of Synthesis. $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ serves as a template for the synthesis of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ (Figure 11). Both the *M. luteus* DNA polymerase and *E. coli* DNA polymerase were capable of catalyzing the synthesis of the desired copolymer. Two features are immediately apparent: (i) synthesis of $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ proceeded autocatalytically with both enzymes, and (ii) $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ synthesis was linear throughout the 2-hr time period with both enzymes. In the rereplication of $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$, a 19-fold increase over the initial template concentration was seen with the *M. luteus* polymerase after 2 hr; during the same time period a 10-fold increase was seen with the *E. coli* polymerase. In contrast, the amount of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ present after 2 hr corresponded to a 4.2-fold synthesis in the *M. luteus* polymerase reaction and only 2.1-fold in the reaction catalyzed by *E. coli* polymerase. Autocatalytic kinetics have been observed in the synthesis of virtually all DNA polymers studied to date, other than $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ (see Wells *et al.*, 1970, and the references cited therein).

$(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ serves as a template for synthesis of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ (Figure 12). The two template preparations had the following molecular weights: $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$, 1.9×10^5/strand (from alkaline sedimentation velocity), and $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$, $1.8 (\pm 0.3) \times 10^5$/strand (average of two alkaline sedimentation velocity determinations) or 1.7×10^5/strand (determined from kinase experiments). The synthesis of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ proceeded linearly with either template. The rate of the reactions containing $(dI\text{-}dC)_n \cdot (dI\text{-}dC)_n$ as template was 5.2 times that of the reactions containing the $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ template. That the rate was not limited by the enzyme concentration was shown by the fact that two enzyme concentrations gave exactly the same rate with each template preparation.

Linear kinetics in the synthesis of $(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$ seemed to indicate that a constant number of "growing points" was present throughout much of the time course of a reaction. Evidence supporting this interpretation is shown in Figure 13. The addition of 21.5 units/ml of enzyme at 2 hr did not alter the rate of the reaction. In contrast, when 29 nmoles/ml of template was added after 2 hr (an amount essentially equal to the initial template concentration), a 1.7-fold increase

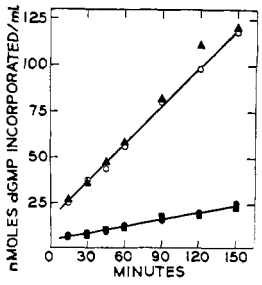


FIGURE 12: Kinetics of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ synthesis from $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ and $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ templates. The 0.10-ml reactions contained 50 mM Tris-HCl (pH 8.3), 5.0 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol, 0.20 mM dCTP, and 0.20 mM dGTP, [^{14}C]dGTP (3.5×10^3 cpm/nmole), either 32 μM $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ or 30 μM $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$, and 30 units/ml or 60 units/ml of *M. luteus* DNA polymerase. All reactions were at 37°. The formation of acid-insoluble product was followed by assay II. The following enzyme concentrations were used: $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ template with 30 units/ml of enzyme (○) and 60 units/ml of enzyme (▲), $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ as template with 30 units/ml of enzyme (●) and 60 units/ml of enzyme (■).

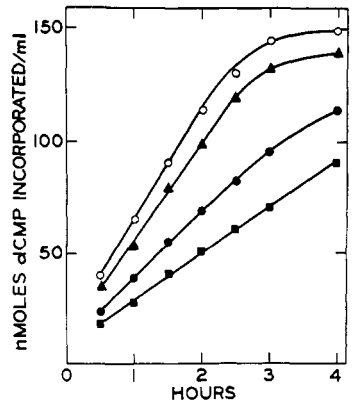


FIGURE 14: Kinetics of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ synthesis from $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ templates. The 0.10-ml reaction mixtures were prepared as described in Materials and Methods. Each contained [^{14}C]dCTP (1.8×10^3 cpm/nmole) and 25 units/ml of *M. luteus* DNA polymerase. The molecular weights and concentrations of the templates were as follows: 6.8×10^4/strand, 13 μM (■) and 40 μM (○); 1.6×10^5/strand, 19 μM (●) and 28 μM (▲). All reactions were at 37°. The formation of acid-insoluble product was followed using assay II.

in rate was noted. The time of addition of enzyme or template did not alter the result.

Because an autocatalytic reaction would have reduced considerably the time required for synthesis of large quantities of polymer, a substantial effort was made to find conditions under which autocatalytic synthesis would occur. Among the variables examined were pH, buffer, temperature, DNase treatment of template, and the divalent metal ion requirement. In addition, *N*-Ac-dGTP (Lefler and Bollum, 1969), a gift from F. J. Bollum, was substituted for dGTP. Variations in the rate of synthesis were observed during these experiments but autocatalytic kinetics were never observed.

Because this result was not expected on the basis of the end addition mechanism, further experiments were performed to examine the role of 3′-OH's in governing the rate of the reaction. Reactions were performed using three separate preparations of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ as templates. The molecular weights of the three preparations were determined from alkaline sedimentation velocity measurements. By varying the concentration of each template it was possible to vary by a factor of 200 the number of 3′-OH's initially present in a reaction (Figure 14). Controls were performed to ensure that none of the reactions was enzyme limiting.

Figure 15a shows the individual rates plotted against the number of 3′-OH's initially present in the reaction. Figure 15b shows the same rate data plotted against the original template concentration expressed in nucleotides per milliliter. The number of sites at which polymerization began was governed by a factor proportional to the template concentration, not by the number of available 3′-OH's. The nature of this additional requirement for initiation of chain elongation remains unknown.

As may be seen in Figure 13, the rate of a reaction containing $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ as template was also dependent

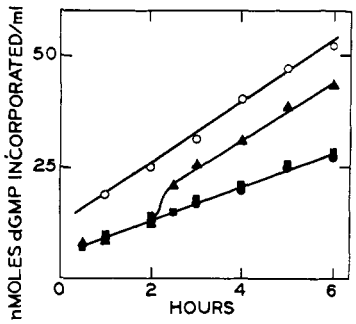


FIGURE 13: Effect of additional enzyme or template on the kinetics of rereplication of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$. The 0.10-ml reactions were prepared as described under Materials and Methods. Each contained [^{14}C]dGTP (3.5×10^3 cpm/nmole) and 30 units/ml of *M. luteus* DNA polymerase. A single reaction contained 60 μM $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ (○). The rest initially contained 30 μM $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$. Additions were as follows: none (●); 21 units/ml of polymerase at 2.0 hr (■); 29 μM $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ at 2.0 hr (▲). All reactions were incubated at 37°. The formation of acid-insoluble product was followed by assay II.

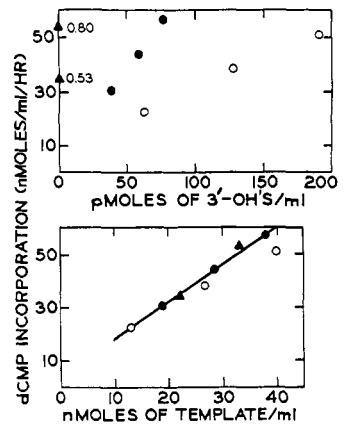


FIGURE 15: (a) Effect of template molecular weight on the rate of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ synthesis. Reactions were performed as described in Figure 14. The rate was determined between 0.5 and 1.5 hr. The molecular weights of the three preparations of $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ used as templates were: 13.5×10^6/strand (▲), 1.6×10^5/strand (●) and 6.8×10^4/strand (○). The two lowest 3′-OH concentrations are given in the figure for clarity. (b) Effect of template concentration on the rate of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ synthesis. The same rate data described in part a are plotted as a function of the initial template concentration (expressed as nmoles of nucleotide per milliliter).

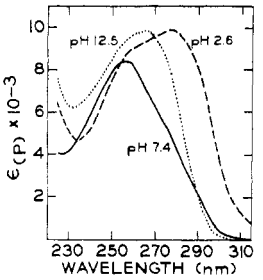


FIGURE 16: Spectra of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$. The spectrum of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ was determined in 0.01 M NaCl–1.0 × 10^{-5} M EDTA before and after titration as described in Materials and Methods. Spectrum at pH 7.4 (—), pH 2.6(---), and pH 12.5 (· · ·).

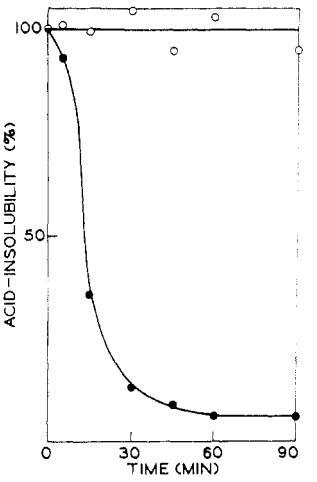


FIGURE 17: Effect of exonuclease I on $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ and $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$. The reaction mixture (0.10 ml for $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$, 0.50 ml for $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$) contained 13 μM [^{3}H]-$(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ (4.4 × 10^4 cpm/nmole) or [^{3}H]$(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (8.2 × 10^3 cpm/nmole), 67 mM glycine buffer (pH 9.5), 6.7 mM magnesium chloride, 1 mM β-mercaptoethanol, and 7 unit/ml of exonuclease I. The mixtures were incubated at 37° and at intervals samples were taken for determination of acid-insoluble radioactivity. 100% radioactivity at 0 min was 6000 cpm for both DNAs. (○) $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$; (●) $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$.

on the initial concentration of template. Increasing the template concentration from 0.03 to 0.06 mM increased the rate of the reaction 1.8-fold. However, a relationship between rate and template concentration which was independent of the molecular weight of the template preparation, such as was found with $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ (Figure 15a,b), was not found using separate preparations of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$. Linear kinetics have been reported in the synthesis of $(dDAP\text{-}dT)_n\cdot(dDAP\text{-}dT)_n$ (Klett *et al.*, 1968).

Three observations can be drawn from the kinetic studies on $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ synthesis. First, the nature of the template does not determine if a reaction will proceed linearly or exponentially. The same $(dI\text{-}dC)_n\cdot(dI\text{-}dC)_n$ which served as a template for the autocatalytic rereplication reaction served as a template for the synthesis of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$, a reaction displaying linear kinetics. Secondly, linear kinetics apparently result when a reaction system fails to produce new chain elongation sites during synthesis (or produces very few). Harwood *et al.* (1970a) reported that the *M. luteus* DNA polymerase used in the present studies did not contain detectable endonucleolytic activity. Therefore, some other activity must give rise to the new 3′-OH oligo- or polynucleotide ends required for autocatalytic synthesis to occur. Finally, it is observed that the presence of a free 3′-OH polynucleotide terminus is not a sufficient condition for initiation of polymerization. An additional feature, proportional to the DNA concentration, is also involved; its nature is unknown. Further study of this reaction system should increase our understanding of the processes which give rise to autocatalytic synthesis.

IV. Additional Properties of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$

This DNA was characterized as the most thermostable DNA yet observed. It is a double-stranded, high molecular weight polymer containing a defined repeating nucleotide sequence as judged by complete nearest-neighbor frequency analyses, analytical sedimentation velocity analyses, analytical buoyant density analyses in both CsCl and Cs_2SO_4 solutions, ultraviolet spectral determinations, T_m studies, and CD studies (Wells *et al.*, 1970).

Ultraviolet absorbance *vs.* pH profiles for $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ were determined by addition of acid or base to a neutral solution of the polymer. A single cooperative transition occurred in the region between pH 12.1 and 12.3; 51% hyperchromicity was observed at 270 nm. The transition was rapidly and completely reversible (no time dependence was observed). The transition in alkaline solution is attributed to ionization of guanosine, since cytidine has no functional groups which may be titrated in alkaline solution. Inman and Baldwin (1964) reported a pK of 11.9 in 0.4 M K^+ for the sequence isomeric polymer $(dG)_n\cdot(dC)_n$.

The ultraviolet spectra of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ are shown in Figure 16. The molar extinction coefficient in neutral solution at 256 nm (λ_{max}) was 8.4 × 10^3 M^{-1} cm^{-1} (±5%) (Wells *et al.*, 1970). Inman and Baldwin (1964) reported a value of 7.4 × 10^3 M^{-1} cm^{-1} for the molar extinction coefficient of the sequence isomeric polymer $(dG)_n\cdot(dC)_n$. These two polymers thus fit the general pattern observed by Wells *et al.* (1970) for sequence isomeric polymers; *i.e.*, the polymers which contained only purines in one strand and pyrimidines in the complementary strand had a lower extinction coefficient than the sequence isomeric polymer in which both purines and pyrimidines occurred in both of the complementary strands.

An additional curious feature of $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ is its insensitivity to degradation by exonuclease I (Figure 17). Polymer which was pretreated with phosphatase or which was heat treated in the presence of formaldehyde showed the same resistance. This is consistent with the finding (Radding *et al.*, 1962) that $(dG)_n\cdot(dC)_n$ is essentially resistant to exonuclease I. However, $(dG\text{-}dC)_n\cdot(dG\text{-}dC)_n$ is degraded by some nucleases since successful nearest-neighbor analyses have been performed (Wells *et al.*, 1970).

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