Poly(8-Bromoadenylic Acid): Synthesis and Characterization of an All-syn Polynucleotide

(Received for publication, August 20, 1974)

FRANK B. HOWARD, JOE FRAZIER, AND H. TODD MILES

From the Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Poly(8-bromoadenylic acid) (poly(8-BrA)) has been synthesized by polymerization of 8-BrADP with polynucleotide phosphorylase in the presence of oligonucleotide primers. In the absence of oligonucleotides, significant (i.e. >1%) polymerization does not occur. Oligo(I) primer was removed selectively from the polymer with ribonuclease T1 to yield the homopolymer, poly(8-BrA).

End group analysis, based on quantitative infrared measurement of the (Ip),I-(8-BrA)n and of polyadenylic acid (poly(A)) suggest a significantly higher population of C3' endo conformation of ribose residues in the primed polymer than in poly(A) at 81°C.

Rotation of the sugar residues of nucleosides and nucleotides about the glycosidic bond gives rise to conformationally isomeric structures, syn and anti, defined in terms of the angle of rotation about this C—N bond (1–4). The common purine nucleosides have been observed in crystal studies in both syn and anti conformations, but with anti predominating (2, 5, 6). The common pyrimidine nucleosides, however, have been observed in the solid state only with anti conformations (5). Although there has been intensive investigation of syn and anti rotamers of nucleosides and nucleotides in recent years (e.g. Refs. 1–22, and references cited therein), there has been little information on the effect of the less common syn conformation on the properties and structure of polynucleotides. Several authors have suggested that syn conformations would prevent two strand complementary helix formation (7, 14, 17) as well as stacking (17) of single-stranded polymers. Semiempirical energy calculations have been used to predict that syn polymers in general and poly(8-bromoadenyl acid), as a specific member of the class, would be incapable of helix formation or stacking (17). This study also concluded that the C2' endo configuration should be highly favored in a syn purine polynucleotide and that the characteristic ratio should be considerably larger than that found for polyadenylic acid.

The major impediment to establishing the properties of syn polymers has been the lack of all-syn polynucleotides for experimental study. The common polynucleotides are generally considered to have predominantly or exclusively anti nucleotide residues. Though the common purine nucleotide residues in polymers may be capable of undergoing syn-anti interconversions, there has been no known way of causing such a conversion to occur and no unambiguous method of establishing whether it had occurred. A bulky residue in position 8 of a purine nucleoside or nucleotide is capable of forcing the sugar into a syn conformation (7, 12, 13), but there has heretofore been an impasse at the next step of polymer synthesis. Nucleoside diphosphates with bulky substituents in position 8 have been found not to be substrates for homopolymerization with polynucleotide phosphorylase (12, 14, 15), as discussed in greater detail in later paragraphs.

The direct method of chemical modification of a preformed polymer is one possible approach to synthesis of a syn homopolynucleotide but one that has serious disadvantages. The principal objection to such an approach is the general one that few, if any, organic reactions have only a single product. There are usually one or more side reactions, and these often compete seriously with the desired reaction. Although undesired reactions also occur with monomeric nucleotides, the by-products can be removed from the nucleotide by chromatography. When the chemical modification is carried out at the polymer stage, however, the undesired reaction products are trapped in the polynucleotide chain and cannot be removed. The problem tends to become progressively more serious as one attempts to obtain complete reaction of all residues in the chain by use of excess reagent or forcing conditions. In the later stages of a reaction the undesired by-products will have accumulated in the chain, rendering further addition of reagent futile, or even destructive of those desired residues which are already present. Chemical modification of preformed

...
polymers is more likely to be useful at lower levels of substitution, but even here it is necessary to degrade the polymer to the nucleoside or nucleotide level and examine the product with sufficient care to demonstrate clearly the presence or absence of expected (and unexpected) contaminants.

We present here the first report of synthesis and characterization of an all-syn homopolymer nucleotide. Some of the physical properties of the polymer have been reported previously (23).

**EXPERIMENTAL PROCEDURE**

**Synthesis and Purification of 8-Bromoadenosine 5'-Diphosphate**

Adenosine 5'-diphosphate was brominated according to the procedure of Behara and Uesugi (26), in which bromine is added to a solution of the nucleoside diphosphate in aqueous acetic buffer at pH 4. Though the conversion is high, a trace of unchanged ADP remaining in some preparations (e.g., 1 to 2%) is enough to cause major changes in the apparent substrate properties of 8-BrADP. The following chromatographic procedure gave excellent resolution of the two nucleoside diphosphates, though apparently minor modifications of the procedure led to lack of separation.

No nucleoside diphosphate (0.14 mmol) obtained from bromination of ADP was applied to an anion exchange column (Bio-Rad AG 1-X8) with resin bed dimensions of 0.9 cm diameter and 5.5 cm height. After the column was washed with 50 ml of water it was developed at a flow rate of 2.5 ml/min with a linear gradient consisting of 1.5 liters of water in the mixing flask and 1.5 liters of 0.05 M sodium chloride-0.003 M hydrochloric acid in the reservoir. The effluent was monitored automatically by a uv detector and the absence of ADP in 18 ml fractions. 8-BrADP was eluted in tubes 84 to 145 and was recovered from tubes 95 to 140 (807 ml, 0.12 mmol), with maximum concentration in tube 112, and ADP was eluted in tubes 49 to 59, with maximum concentration in tube 55.

8-BrADP was prepared in the same way and found by measurement of a radioautogram with a Joyce-Loebl densitometer to contain 98% of unchanged ADP. The contaminating ADP was removed by the column procedure described above, and the absence of ADP after chromatography was confirmed by a radioautogram of a thin layer chromatogram (solvent system for nonradioactive material is given below) of a venom digest of the diphosphate, using a high gain setting of the densitometer.

8-Bromoadenosine 5'-diphosphate was desalted by percolating the combined chromatography fractions through a column (1.2 x 8 cm) of activated charcoal (Wako Junzaku Kagyo Co., Ltd., Osaka, Japan). The column was washed with water (1.5 liters) until the pH of the effluent was 6. The nucleotide was eluted with 100 ml of ethanol-water-concentrated ammonium hydroxide (50:48:2). The eluent was concentrated to dryness in vacuo (bath <30°) and 8-BrADP dissolved in a small volume of water. Recovery of nucleotide from the charcoal column was 82%.

A sample of the product on Whatman No. 3MM paper in 0.05 M citrate buffer, pH 5.0, at 2000 volts for 2 hours indicated that 8-BrADP contained only a trace (about 1 to 2%) of 8-BrAMP. Migration rates (relative to AMP) of our preparation of 8-BrADP, 1.52 (trace of 8-BrAMP at 0.96); 8-BrAMP, 0.95.

The identity of the product was established by dephosphorylation with crude venom of Crotalus adamanteus, incubated for 3 days at 37°. Chromatography of a sample of the digest on a silica gel thin layer slab showed a chloroform-soluble fraction which, after diethyl ether precipitation, showed a single, ultraviolet-absorbing component with the same Rf (0.58) as authentic 8-bromoadenosine. No adenosine (Rf 0.31) was detected.

1 Chemical bromination of poly(G) has been reported to produce poly(8-BrG) (13), but the published characterization does not establish that the resulting polymer was chemically homogeneous, containing only 8-bromoguanyclic acid residues. In particular, the known oxidation reactions of guanine and R-bromoguanine derivatives by bromine (24, 25) were not considered in the report or excluded by the published evidence.

2 In one variation of this procedure we employed a hydrochloric acid concentration of 0.005 M instead of 0.005 M, to form the linear gradient, keeping all other factors constant. The resulting polymer was chemically homogeneous, containing only 8-bromoguanylic acid residues. In particular, the known oxidation reactions of guanine and R-bromoguanine derivatives by bromine (24, 25) were not considered in the report or excluded by the published evidence.

Attempts to polymerize purified 8-BrADP with primer-independent nucleotide phosphorylase from Micrococcus luteus under conditions used for synthesis of the common nucleotides (cf., Figs. 1 and 2), failed to yield significant quantities of poly(8-BrA)3. To establish that our enzyme preparation was in fact primer-independent, we tested the ability of the enzyme to catalyze the polymerization of [8-14C]ADP. Under the conditions employed (0.017 M [8-14C]ADP (5.26 x 10^6 dpm/mmol), 0.207 M MgCl2, 2 units/ml (phosphorolysis units as defined in Ref. 27) of enzyme) [8-14C]ADP was polymerized at a linear initial rate of 6.6 x 10^-5 dpm/min (23% in 30 min). No lag period was observed, indicating that no primer was required for polymerization of ADP. Despite this independence of primer for ADP, however, experiments demonstrated the effectiveness of added primer in stimulating 8-BrADP polymerization (Figs. 1 and 2) (see below for experimental conditions).

We should note that under the conditions reported here, minor amounts of ADP are capable of stimulating polymerization of 8-BrADP in the absence of oligonucleotides, though less effectively than the latter. In the presence of added ADP or primer the incorporation of 8-BrADP into acid-precipitable polymer was 1% in 25 hours under conditions of Fig. 2. With 0.08% added ADP (nonradioactive) the incorporation was 7% in 55 hours, and with 0.6% ADP it was 27% in 6 hours. The radioactivity is measured at adequate sensitivity of measurement even at low levels of incorporation.

Some of the reaction variables were examined before we discovered the importance of minor contamination of 8-BrADP with ADP and were not subsequently reinvestigated with highly purified 8-BrADP. A comparison of the effectiveness of manganese and magnesium, for example, (0.015 M 8-BrADP [about 3% contamination with ADP], 0.005 M divalent cation, 30.4 units/ml of enzyme, no primer) showed almost twice as high a yield with Mn^2+ as with Mg^2+ in 24 hours.

**Dependence of 8-BrADP Polymerization on Oligonucleotides**—The following assay was used to measure the effect of oligonucleotides on polymerization of 8-BrADP. Reaction mixtures (0.5 ml) contained 0.015 M [8-14C]8-BrADP (3.82 x 10^6 dpm/mmol), 4.7 x 10^-5 M manganese chloride, 0.1 M ethanol buffer, pH 9.0, 2 x 10^-5 M ethylene-diaminetetraacetate, 15.2 units/ml (30) of purified polynucleotide phosphorylase (5, 27), and oligonucleotides with a tetrainesic acid primer at approximately 10% (1p)J when examined by paper chromatography (1 8-BrAMP and poly(8-BrA) were the only oligonucleotides tested). Polynucleotide phosphorylase, a and oligonucleotide (expressed as oligomer units) at concentrations indicated in Figs. 1 and 2. In reactions with oligo(I) (but not with oligoA), the reaction mixture also contained 0.01 M dithiothreitol. Aliquots (0.05 ml) were added to 0.45 ml of ice-cold 3.5% perchloric acid. After 10 min, the precipitated polymer was collected on a Millipore HA filter and washed three times with 10 ml volumes of cold 0.35% perchloric acid. Filters were dissolved in 10 ml of scintillation fluid (Aquafluor, New England Nuclear) and counted in a liquid scintillation counter (Nuclear-Chicago). In some experiments polymerization was followed by release of inorganic phosphate. In this case, the precipitated polymer was collected by centrifugation and an aliquot (0.3 ml) of the supernatant was assayed for inorganic phosphate (28).

**Synthesis of Primed Polymer Precipitator Solution**—The reaction mixture contained the following components at the indicated concentrations in 22 ml total volume: 8-BrADP, sodium salt, 0.015 M; MnCl2, 4.68 x 10^-4 M; (Ip)J, 3.7 x 10^-4 M (expressed as molarity of tetramer); 3 dithiothreitol, 0.01 M; EDTA, 2 x 10^-4 M; Triton buffer, pH 9.0, 0.1 M; poly(8-BrA) for phosphorylase. 220 units (27). After 11 hours, 70% of the substrate was polymerized, as measured by P1 release. The polymer containing incorporated (Ip)J was purified and freeze-dried as described previously (28). Expression of the enzyme showed that the isolated polymer contained 57% of the nucleoside phosphate.

The amount of oligo(I) primer incorporated into the polymer was determined by quantitative infrared spectroscopy (30) on the intact polymer and by ultraviolet spectroscopy of an acid supernatant after T1 nuclease treatment. The infrared analysis takes advantage of the wide difference between the strong carbonyl band of the intact acid residues (30) and any of the bands of 8-bromoadenine acid (23).

In some experiments polymerization was followed by release of inorganic phosphate. In this case, the precipitated polymer was collected by centrifugation and an aliquot (0.3 ml) of the supernatant was assayed for inorganic phosphate (28).

1 Chemical bromination of poly(G) has been reported to produce poly(8-BrG) (13), but the published characterization does not establish that the resulting polymer was chemically homogeneous, containing only 8-bromoguanylic acid residues. In particular, the known oxidation reactions of guanine and 8-bromoguanine derivatives by bromine (24, 25) were not considered in the report or excluded by the published evidence.

2 In one variation of this procedure we employed a hydrochloric acid concentration of 0.005 M instead of 0.005 M, to form the linear gradient, keeping all other factors constant. The resulting polymer was chemically homogeneous, containing only 8-bromoguanylic acid residues. In particular, the known oxidation reactions of guanine and 8-bromoguanine derivatives by bromine (24, 25) were not considered in the report or excluded by the published evidence.

3 The abbreviations used are: poly(8-BrA), poly(8-bromoadenyl acid); (Ip)J-(8-BrA)n, poly(8-BrA) with a tetrainesic acid primer at approximately 10% (1p)J when examined by paper chromatography (1 M ammonium acetate plus ethanol, 1:1), but was otherwise homogeneous in this system.
spectroscopy were acquired by an on-line computer (31) and normalized to a molar absorbance basis (30) for the purified polymers obtained before and after T1 nuclelease treatment. These are the observed curves in Fig. 3.

A series of spectra was generated by the computer by adding the catalog spectrum of poly(I) in 1 and 2% molar increments to that observed for the purified polymer obtained after T1 nuclelease treatment. The experimental curve for the untreated, primed polymer coincides with that calculated for 5.0% I, 94.2% BrA, and it is evident from Fig. 3 that deviations of less than 1% from the calculated value would be readily evident.

The neutralized material was dissolved in 100 μl of water and applied to a thin layer sheet of cellulose impregnated with polyvinylpyrrolidone, which had previously been washed with 1 M sodium chloride then washed again with water and dried. The chromatogram was developed in 1 M sodium chloride. None of the material from the acid-soluble fraction migrated from the origin. Control samples (0.4 μmol of each, expressed in terms of nucleoside residues) of poly(I), poly(I)·(BrA), and poly(A) had Rf values of 0.68, 0.62, and 0.55, respectively. The failure of the acid-soluble fraction to migrate in this chromatographic system suggests that it is principally composed of oligomers with chain lengths greater than about 10.

Selective Removal of (Ip)A Residues from Primed Polymer—The reaction mixture contained the following materials in a total volume of 10 ml: 1 M sodium pyrophosphate, pH 7.2; 0.1 M sodium chloride at 20°C. Both the primed polymer and homopolymer were polydisperse, the former more so than the latter. We are indebted to Dr. Eugene Achter, of this Institute, for determining the sedimentation coefficients.

Because only 3 to 5% of the average chain would be cleaved by the T1 enzyme if complete specificity for I residues is maintained, the observed reduction in sedimentation coefficient may appear to suggest some nonspecific BrA chain cleavage. Acid precipitation of the polymer labeled with [3H]BrA showed a covalent chain length, whereas the acid-insoluble fraction after T1 treatment, indicating that neither monomers nor oligomers shorter than about 10 residues (see above) had been produced by T1 nuclelease.

Both the primed polymer and homopolymer are probably double-stranded (23) with intermolecular hydrogen bonding and the sedimentation coefficients may depend more on end-to-end aggregation (see below) than on covalent chain length. For this reason we conclude that the reduction in sedimentation coefficient produced on treatment of the acid-soluble fraction after T1 treatment, indicating that neither monomers nor oligomers shorter than about 10 residues (see above) had been produced by T1 nuclelease.

Ultraviolet spectra were measured with a Cary 118 spectrometer and infrared spectra with a Beckman IR7 spectrometer, as described previously (30).

NMR spectra were measured with a Varian HR220 spectrometer operated in CW mode, using TSP as an internal standard in D2O solution. Signals were enhanced with a Varian C1024 computer of average transients. We are grateful to Mr. Robert Bradley, of this Institute, for the NMR measurements.

RESULTS AND DISCUSSION

Previous attempts to prepare syn homopolymers by enzymatic polymerization of 8-substituted nucleoside diphosphates or triphosphates have led to uniformly negative results (12, 15, 16). Ikehara et al. (12), however, found that co-polymers could be formed by the action of polynucleotide phosphorylase on mixtures of normal nucleotide diphosphates and analog diphosphates with bulky 8 substituents including 8-BrGDP and 8-BrADP. Both rate of polymerization and yield of polymer decreased as the fraction of analog diphosphate in the reaction mixture increased.

The composition of the acid-soluble and acid-insoluble fractions was determined by infrared spectroscopy in a separate experiment. To 5.4 × 10⁻⁴ mmol of primed polymer in 0.460 ml of water at 0°C we added 0.041 ml of concentrated HCl. The mixture was stirred in an ice bath and centrifuged at 0°C. The precipitate and acid supernatant were separated by gel filtration and lyophilized (liquid nitrogen traps) to remove most of the acid and neutralized with phosphate buffer, pH 7.4, lyophilized, and dissolved in D2O for spectroscopic examination (30). The spectrum of the acid-insoluble fraction exhibited an intense doublet at 1632 cm⁻¹, 1618 cm⁻¹ (35), characteristic of the helical form of poly(8-BrA) (29), with a weak BrA band at 1575 cm⁻¹ and a very weak I carbonyl band at 1623 cm⁻¹ (35). Infrared analysis of the oligo(I) content of the polymer by the method shown in Fig. 3 indicated an I content of about 4.9%.

The polymer underwent reversible melting, observed in the infrared, with a Tm of 60°C in 1.2 M NaCl (Fig. 6). The infrared spectra of the acid-soluble material was quite different from that described above. The oligo(I) carbonyl band at 1672 cm⁻¹ was much stronger, and the intense BrA band was not a doublet but had a peak at 1623 cm⁻¹, characteristic of the monomer (Fig. 4), or melted polymer (5, 23). An infrared analysis (using 10 μmol for 8-BrADP rather than for poly(8-BrA)) showed 36% oligo(I), 64% Br. Since the polymer had been repeatedly precipitated and extensively dialyzed before acid treatment and since thin layer chromatography showed no monomers or short oligomers (see above) the BrA residues must be present as oligomeric extensions of the 3' end of (Ip)I rather than as monomers.

The sedimentation coefficients, measured with a Beckman model E ultracentrifuge equipped with ultraviolet scanner, were 8.1 for the primed polymer and 4.7 for the homopolymer, both measured in 0.01 M sodium pyrophosphate, pH 7.2, 0.1 M sodium chloride at 20°C. Both the primed polymer and homopolymer were polydisperse, the former more so than the latter.
8-BrGMP is capable of forming a stable two-stranded complementary helix with poly(BrC), indicating that such monomer-polymer base pairing is not chemically or sterically prohibited. Formation of similar pairs during RNA synthesis would be consistent with this observation.

Kapuler and Reich (16) proposed that, although nucleoside diphosphates can bind to the enzyme while in a syn conformation, successful completion of any polymerase phosphorylation reaction requires that the product be capable of assuming an anti conformation.

Kapuler and Reich made a similar interpretation for RNA polymerase (16), suggesting that a change in conformation at the glycosyl bond of the substrate could be coupled to one or more of the later steps in enzyme action, perhaps to formation of the diester bond. The fact that 8-BrADP is a reasonably good substrate for polymerase phosphorylation indicates that there is no requirement with this enzyme that a nucleoside diphosphate be able to assume an anti conformation in the ground state. In the transition state bond angles and distances near the bonds being formed and broken are necessarily distorted from their normal values. For a syn - anti conversion to be coupled to the catalytic step in the present instance, however, it would appear to require concerted distortion of many of the sugar bond angles, rotation of the base, a catalyzed displacement reaction, and return to a syn conformation in the ground state. While we have no direct evidence on the subject, this complex reaction path appears to us both unlikely and unnecessary.

The enzyme we have employed is primer-independent for ADP and for most modified nucleoside diphosphates. It thus appears that dependence on an oligonucleotide primer for initiation of significant polymerization is a function of substrate as well as of enzyme preparation.

We found in early experiments that the ability of the enzyme to catalyze polymerization of 8-BrADP in the presence of primer declined with time after a stock solution of the enzyme had been prepared. Dithiothreitol prevented this change, and all later experiments were carried out in the presence of this reagent. Klee and Singer (33) and Klee (34) found that proteolytic cleavage could convert polynucleotide phosphorylase from a primer-independent (for ADP polymerization) to a primer-dependent condition but that dithiothreitol will enable the latter form of the enzyme to catalyze polymerization in the absence of primer. It is possible that a similar process of proteolytic cleavage was occurring in our early experiments, but we have not investigated this point. As we have noted, our preparation of the enzyme did not require primer, permitting synthesis of reasonable amounts of homopolymer without added primer when ADP was the substrate.

From our results (cf. Figs. 1 and 2) it appears that failure of efficient chain initiation accounts for lack of homopolymerization of 8-BrADP under normal conditions. The subsequent step of chain elongation of the homopolymer occurs reasonably well under appropriate conditions, but at a much slower rate than with normal substrates such as ADP. While we have not made explicit kinetic studies, our observations would be consistent with a lower $V_{max}$ for the chain elongation step with 8-BrADP than with ADP. $K_{cat}$ could be either higher or about the same for 8-BrADP as for normal substrates. The effect of small amounts of ADP in permitting polymerization of 8-BrADP is presumably due to synthesis of enough oligo(A) to serve as primer for chain elongation with 8-BrADP. Even in the absence of primer or ADP there is a finite though very small (≤1%) amount of [8-14C]8-BrADP polymerization, as measured by enzyme-dependent formation of acid-precipitable material. It is not clear why this unprimed polymer does not itself serve as primer, permitting synthesis of reasonable amounts of homopolymer after a long lag period. It is possible that the 80-hour duration of the experiment was simply not long enough to reveal such behavior.

Composition and Properties of Primed Polymer—Since oligonucleotide primers function as chain initiators (35, 36), the non-BrA residues occur only at the beginning of the chain in a primed synthesis, in contrast to random co-polymers prepared with mixtures of nucleoside diphosphates. In addition to its role in permitting the polymer to be synthesized, the priming oligomer can serve an invaluable second function in forming the basis of an end group analysis for determining average chain length of the primed polymer (Fig. 3). In the present case we have employed infrared spectroscopy to analyze for oligo(I) in the polymer, taking advantage of the wide frequency separation of the I carbonyl vibration from the BrA ring vibration (Figs. 3 to 5). Oligo(I) of chain length 4 constituted 5.8% of the...
dialyzed polymer (Fig. 3), corresponding to an average chain length of about 69 residues, of which 65 are BrA. As noted below, this preparation includes a small amount of oligomeric material which is soluble in acid and has a quite different composition.

Acid precipitation of the dialyzed, primed polymer prepared with [$^3$H]-BrADP showed that 4% of the preparation was acid-soluble. In a parallel experiment with nonradioactive polymer both acid-soluble and insoluble fractions were investigated by infrared spectroscopy. The acid-soluble fraction contained 36% inosine and 64% 8-bromoadenosine residues. Of the total I residues in the purified, dialyzed polymer preparation, roughly 30% are in the acid-soluble fraction. For the reasons noted above, the BrA residues must be present as oligomeric extensions of the (Ip)$_n$I primer. The (Ip)$_n$I content of this oligomeric material would correspond to an average chain length of about 11, or 7 BrA residues. The fact that the infrared doublet (v$_{max}$ 1632 cm$^{-1}$, 1617 cm$^{-1}$) observed in the helical form of poly(A-BrA) (23) is not observed in the acid-soluble fraction is consistent with the absence of longer chains and suggests the existence of a lower limit of chain length required for helix formation.

The fraction of the polymer preparation precipitated by acid is quite different from that which remains in solution. The (Ip)$_n$I content of the precipitate (comprising 96% of the polymer preparation) is about 4.3%, corresponding to an average chain length of about 93 residues. Though there may have been a minor amount of acid degradation, the BrA peak is a doublet (Fig. 5), indicative of the poly(8-BrA) helix (23), and the sigmoid melting curve has $T_m$ = 60$^\circ$ in ~1.2 M Na$^+$ (Fig. 6).

From these results it appears that nearly one-third of the chains which are initiated under the conditions of our experiments are terminated rather early before long chains have been synthesized. There is no evidence, however, of a significant amount of chain termination after addition of 1 or 2 BrA residues. The majority of chains, nevertheless, can proceed to a reasonable average length.

The secondary structure of the primed polymer is evidently very similar to that of the homopolymer, poly(8-BrA). Whether (Ap)$_n$A or (Ip)$_n$I has been used as a primer, the polymer has a sigmoid melting curve with $T_m$ values only slightly lower than the homopolymer under comparable conditions (Fig. 6). Infrared spectra of the primed polymer shows splitting of the 1025 cm$^{-1}$ BrA ring vibration (Fig. 5), quite different from that of the monomer 8-BrADP (Fig. 4), but characteristic of the

---

**Fig. 3.** Quantitative infrared spectra of (Ip)$_n$I-(8-BrA)$_n$ and of poly(8-BrA) (solid curves) in D$_2$O solution. The band with $v_{max}$ 1672 cm$^{-1}$ is due to the carbonyl stretching vibration of the oligo(I) residues in the primed polymer. The dashed curves were generated by a computer as the summation of the spectra of poly(8-BrA) and poly(I) in the indicated molar proportions (molarity being expressed in terms of repeating 8-bromoadenosine or inosine units). The ordinate is molar absorbance. The spectra provide a sensitive measure of oligo(I) content at low molar ratios of I because of the wide resolution of the carbonyl band and BrA ring vibrations. For comparison with complete spectra in the double bond region see Fig. 4 (8-BrADP), Ref. 23 (poly(8-BrA)) and Ref. 30 (poly(I)). A relevant discussion of quantitative infrared spectroscopy is given in Ref. 30.

**Fig. 4.** Infrared spectrum of 8-BrADP in D$_2$O, Na$^+$ 2 M, pH 8. This monomer has a single band at 1626 cm$^{-1}$ (30$^\circ$) rather than the doublet observed in poly(8-BrA) (23). There is a slight decrease in $v_{max}$ with temperature, possibly due to base stacking, but little change in intensity. At 30$^\circ$ $v_{max}$ = 1626 cm$^{-1}$ ($v_{max}$ 770, 1577 cm$^{-1}$ ($v_{max}$ 102). Path length 58.3 $\mu$m, scale expansion 2.56-fold. Ordinate index marks are 0.1 absorbance unit apart, uncorrected for path length.

**Fig. 5.** Infrared spectrum of acid-insoluble fraction of (Ip)$_n$I-(8-BrA)$_n$ prepared as described under "Experimental Procedure." This spectrum exhibits the splitting of the 1025 cm$^{-1}$ BrA ring vibration characteristic of the poly(8-BrA) helix (23). The weak band at 1672 cm$^{-1}$ is the carbonyl vibration of the oligo(I) primer, well resolved from the BrA ring vibration. The upper curves are expanded 4-fold; the lower one (87$^\circ$) is unexpanded. Path length 58.3 $\mu$m. The ordinate index marks are 0.1 absorbance unit apart, uncorrected for scale expansion.
ordered structure (23). The oligo(1) carbonyl band is the same in the primed polymer as in (Ip),I and is essentially independent of temperature, suggesting that the oligo(1) end groups fold away from the helix and do not participate in base pairing.

Preliminary NMR observations of primed poly(8-BrA) were made at 220 MHz in D$_2$O solution at several temperatures. Control experiments were carried out with poly(A) and with a mixture of the two polymers in the region of the H2, H8, and H1' protons.

The H2 proton of poly(A) at 19° was observed as a sharp peak at 7.64 ppm, but H1' and H8 were too broad to be detected (Fig. 7; cf. Refs. 37 to 40 for previous NMR observations of poly(A); the spectra in Ref. 39 clearly show differential broadening of H8, H1', and H2). Primed poly(8-BrA), in contrast, gives rise to no H2 proton signal (or any others) under the same conditions, and a mixture of the two polymers showed only the sharp H2 peak of poly(A) (Fig. 8) at 19°.

Observation of a sharp H2 resonance of poly(A) in the mixture indicates that broadening by a paramagnetic impurity in the primed poly(8-BrA) preparation is not responsible for failure to observe the H2 proton signal of the latter polymer.

At a temperature (30°) just below the melting range of (Ip),I-(BrA)$_n$, both H2 and H8 of poly(A) in the mixture of the two polymers gave rise to sharp peaks, at 7.76 ppm and 7.69 ppm, respectively (Fig. 8). Despite signal enhancement by accumulation of 400 CW scans, however, no H2 resonance of (Ip),I-(BrA)$_n$ was observed (Fig. 8).

Poly(A) is a stacked, single-stranded polynucleotide, exhibiting essentially noncooperative melting when observed by a number of different methods (41-45 and other references cited.
The large upfield shifts of the aromatic protons from their monomer values have been observed in poly(A) and poly(dA) by earlier workers (38-40, 46-48) and much smaller shifts are seen in dimers and in more concentrated solutions of monomers (48, 49 and references cited therein). These shifts in the polynucleotides are associated with stacking and presumably arise from magnetic shielding by the ring current of adjacent stacked bases (cf. Refs. 38-40 but note comments in Ref. 39 about susceptibility corrections with external standards in studies of stacking of monomers).

Although all of the moderately flexible, single-stranded polynucleotides have high resolution NMR spectra, long, rigid, base-paired helices such as (rA). -(rU), (rI). -(rC), and native DNA do not (38, 39, 47, 48). Molecules of moderate size, however, such as tRNA (51) and many proteins (52) do give rise to high resolution NMR spectra.

Failure of protons in rigid structures to give detectable high resolution spectra is due to wide variation in local magnetic field arising from magnetic dipoles of other nuclei. This variation leads to a wide distribution of resonant frequencies and to spectral lines too broad to be observed (38, 53). If molecular motion is sufficiently rapid, however, a proton experiences the average local field of other nuclei and has a sharp resonance peak.

Poly(8-BrA), in contrast to poly(A), exhibits strongly cooperative melting and has infrared and ultraviolet properties which led us to conclude that it probably has a hydrogen-bonded, helical structure (Ref. 23 and Fig. 9). In view of the relatively short average length of the primed poly(8-BrA) chains (see preceding sections), failure to observe the H2 resonance is consistent with our previous structural conclusion and may further suggest that the BrA-BrA interaction is intermolecular rather than intramolecular. Poly(8-BrA) might undergo base pairing by having each chain loop back on itself in a hairpin structure with antiparallel strands, as in the case of poly(dA-dT) (54), of oligo(dA-dT) (55), or of the poly(U) helical self-structure (56, 57). The maximum length of helix each chain could form in this way would correspond to one-half the length of that chain. Alternatively, the interaction may be intermolecular. Since the chain ends are not co-terminal in polydisperse material, there would presumably be end-to-end aggregation (cf. Ref. 58) in this case, with generation of helices which are longer than the chains composing them. The average chain length of our (Ip)J-(8-BrA)n preparation, determined by infrared analysis of the acid-insoluble fraction (see above), is approximately 35 (the unfractoned polymer would give a value of ~65, but this value is influenced by the large I content of the small fraction which is acid-soluble).

The chain length of tRNA molecules is in the range of 75 to 90 nucleotide residues, or about the same as the (Ip)J-(8-BrA)n preparation used in these experiments. The fact that high resolution NMR spectra are observed with tRNA (51), but not with (Ip)J-(8-BrA)n below its melting range indicates that tRNA can undergo motions which are much more effective in relaxing nuclear dipole broadening than are those of (Ip)J-(8-BrA)n. Since the covalent chain lengths are quite similar in the two cases, the difference in dipolar broadening presumably arises from differences in shape or size of the maximally interacted forms of the 2 molecules. If (Ip)J-(8-BrA)n forms a helix by folding back upon itself, the particle size as well as the chain length would be roughly similar to that of tRNA. The less extended shape of tRNA would presumably lead to a shorter rotational correlation time than a moderately long rod-like helix (formed by intermolecular association), hence to more effective averaging of nuclear dipolar interaction.

With a relatively short helix formed by intramolecular folding of (Ip)J-(8-BrA)n, however, it would appear that the difference (from tRNA) in shape and rate of tumbling should not be too large. If we assume 40 base pairs, a rise per residue of 3.4 A, and a helix diameter of 25 A, for example, the axial ratio would be about 5. This would probably be somewhat more asymmetric and more slowly tumbling than tRNA, but we would not expect the difference to be large enough to account for the observed difference in NMR line broadening. In contrast, intermolecular association with end-to-end aggregation could lead to long, asymmetric rods which would, like (rA). -(rU), tumble too slowly to produce line narrowing by averaging of nuclear dipolar interactions. Although the phenomena involved are complex and the evidence by no means decisive, the foregoing experiments and arguments lead us to the tentative conclusion that the helical structure of (Ip)J-(8-BrA)n is formed by intermolecular rather than intramolecular association.

Above the melting range the spectrum of (Ip)J-(8-BrA)n appears to be normal, with band widths similar to those of poly(A) (Fig. 8). The H2 and H1' resonances are observed at 7.87 ppm and 5.70 ppm, respectively, at 81°C. The helices are dissociated at this temperature (Fig. 6). The particle size is presumably smaller and protons in the chains can undergo averaging through segmental motion.

Chemical shifts occur at slightly higher field for poly(8-BrA) than for poly(A) and peak widths are quite similar for corresponding H2 and H1' protons of both polymers in the mixture at 81°C (Fig. 8). The temperature dependence of chemical shift of the H2 protons is about the same for both polymers in the limited temperature range in which both can be observed. The values for poly(A) are 7.92 ppm (70°C), 7.96 ppm (81°C), and 8.00 ppm (90°C) and for primed poly(8-BrA) 7.82 ppm (70°C), 7.87 ppm (81°C), and 7.90 ppm (90°C). For the monomer 8-BrAMP we find the chemical shift of the H2 proton is 8.12 ppm at 18°C. It thus appears that above its cooperative melting range poly(8-BrA) has residual single strand stacking which is progressively disrupted by further heating. As with poly(A), the chemical shift of H2 is still at an appreciably higher field (i.e. more shielded) at the highest observed temperature (90°C) than it is in the monomer.

![Fig. 9. Possible hydrogen bonding scheme of poly(8-BrA) helix (cf. Ref. 23).](image-url)
The spectra in Fig. 8 lead to a further conclusion about ribose conformation in poly(8-BrA). The magnitude of proton-proton vicinal spin-spin coupling constants is a function of the dihedral angle between the two C–H bonds (59), and this relationship has been used extensively in studies of nucleoside conformation (60–64 and references cited therein). The H1' proton is coupled to H2' and appears as a doublet in most ribonucleosides and ribonucleotides (cf. Ref. 49). When there is rapid exchange between different structures the observed coupling constants, like the chemical shifts, are weighted averages of the coupling constants of protons in the different structures, the weights being determined by the population of each structure. A recent paper (62) has estimated for the C2' endo conformation a dihedral angle of 155° and \( J_{1',2'} \approx 8.3 \text{ Hz} \) and for C3' endo an angle of 97° and \( J_{1',2'} \approx 0 \text{ Hz} \) (for earlier, though not widely different, estimates, cf. Refs. 60 and 61).

Observed values of \( J_{1',2'} \) are in the range ~4 to 6 Hz for most monomers and have been interpreted in terms of a rapid equilibrium between different conformations, with the C2' endo and C3' endo conformations making major contributions (62, 63 and references cited therein). We find that H1' of 8-BrAMP is coupled to H2 with a constant of 5.79 Hz and has a chemical shift of 6.10 ppm in D2O at 18°C. Absence of a detectable splitting of the H1' resonance of primed poly(8-BrA) (Fig. 8) indicates a reduction in the dihedral H1'-H2' angle from that in the monomers 8-BrAMP and 8-bromoadenosine (65). This change would be consistent with a predominant C3' endo conformation in the polymer under these conditions. This result is especially interesting since all of the syn nucleosides examined in the solid state have a C2' endo conformation (5). When 8-bromoadenosine is heated from 28°C to 70°C, \( J_{1',2'} \) decreases only slightly (by 0.6 Hz; Ref. 65). When the cyclic 3':5'-monophosphate is formed with either adenosine or 8-bromoadenosine there is a large decrease in \( J_{1',2'} \), and no splitting can be detected (65). The cyclic phosphate ring evidently constrains the ribose ring to a C3' endo conformation.

Hruska and Danylik (64) have reported temperature-dependent changes of \( J_{1',2'} \) in ApA and attributed these to conformational changes of ribose associated with stacking. They found that while \( J_{1',2'} \) of 5 AMP changes very little with temperature (for 3'-AMP \( J_{1',2'} \) decreases by 1 Hz between 4°C and 80°C), a much larger change is observed with the dimer, ApA. At 4°C, the values of \( J_{1',2'} \) are 2.3 Hz and 2.1 Hz for Ap and pAp, at 25°C, 3.1 Hz and 2.8 Hz, and at 71°C, 4.7 Hz and 4.5 Hz, respectively. The authors interpreted these changes as indicating that stacking of the bases favors the C9' endo conformation and that at elevated temperatures, where the bases are unstacked, the preferred conformation is C2' endo. The observed values of the coupling constants could presumably result either from time averaging between stacked and unstacked forms of the dimer (each having a single conformational form of the ribose ring) or between the different conformational forms of ribose within the stacked and unstacked structures, or from some combination of the two possibilities. Whatever the detailed picture may be, however, the authors’ correlation of an increase in the population of the C3' endo conformation with stacking of ApA appears qualitatively reasonable.

Alderfer et al. (40) have recently reported the temperature dependence of chemical shifts and of the H1' coupling constant for several adenine polynucleotides. The H1' coupling constant of poly(rA) exhibited a temperature dependence over the range ~48 to 75°C similar to that observed for ApA at lower temperature. These results suggest for poly(rA) a correlation of base stacking with a C3' endo conformation (or other conformations having a similar dihedral angle between H1' and H2') and an increase in population of the C2' endo form with increasing temperature. A correlation between these structural features, however, need not imply a simple cause and effect relationship.

It appears from these NMR experiments that primed poly(8-BrA) above its cooperative melting range shares to some extent with poly(A) the property of a partially stacked structure which is progressively unstacked by further heating. The ribose conformations in the two polymers appear to be significantly different, with a C3 endo conformation present to a greater extent in primed poly(8-BrA) than in poly(A) at 81°C.

REFERENCES

5. Sundaralingam, M. (1973) in Conformation of Biological Molecules and Polymers (Bergmann, E. D., and Pullman, B., eds) p. 417, Israel Academy of Sciences and Humanities, Jerusalem
17. Olson, W. K. (1973) Biopolymers 12, 1787
18. Pullman, B., and Berthod, H. (1973) in Conformation of Biological Molecules and Polymers (Bergmann, E. D., and Pullman, B., eds) p. 209, Israel Academy of Sciences and Humanities, Jerusalem
44. Holcomb, D. N., and Tinoco, I., Jr. (1965) Biopolymers 3, 121
47. Jardetsky, C. D. (1964) Biopolymers Symp. 1, 501
Poly(8-bromoadenylic acid): synthesis and characterization of an all-syn polynucleotide.
F B Howard, J Frazier and H T Miles


Access the most updated version of this article at [http://www.jbc.org/content/250/10/3951](http://www.jbc.org/content/250/10/3951)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/250/10/3951.full.html#ref-list-1](http://www.jbc.org/content/250/10/3951.full.html#ref-list-1)