Influence of 2′-O-Alkylation on the Structure of Single-Stranded Polynucleotides and the Stability of 2′-O-Alkylated Polynucleotide Complexes†

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ABSTRACT: The effect of 2′-O-alkylation on the structure of poly(adenylic acid) was studied as a function of pH. Cooperative transitions in the temperature–absorbance profiles were observed for both poly(2′-*O*-methyladenylic acid) [poly(Am)] and poly(2′-*O*-ethyladenylic acid) [poly(Ae)] at pH 7.0 but were absent in poly(A). All three polymers displayed cooperative transitions at pH 5.7 but the transitions observed with poly(Am) and poly(Ae) occurred at a higher temperature and displayed a greater degree of hyperchromicity. Although 2′-*O*-alkyl groups did not effect the pK_a's of adenine-containing nucleosides and nucleotides, significant alterations in the apparent pK_a values (pH of helix–coil transition) for the polynucleotides were observed. The apparent pK_a values for poly(A), poly(Am), and poly(Ae) were 5.85, 6.20, and 6.50, respectively. T_m values for the cooperative transitions were determined at the apparent pK_a of each polymer and at 1 pH unit above the pK_a. 2′-O-Alkylation also enhanced the structure of these adenine-containing polymers when compared at similar degrees of protonation. Therefore, the ordered self-structure of these polymers as measured by the stability of their cooperative transitions is, poly(Ae) > poly(Am) > poly(A). Increasing levels of 2′-O-methylation in poly(Am,A) heteropolymers resulted in an enhancement of polymer self-structure in proportion to the 2′-*O*-methyl content. The synthesis and structural stability of poly(2′-*O*-methylinosinic acid) are also described. Temperature–absorbance studies on this polymer at neutral pH indicate that the effect of 2′-*O*-methyl groups is consistent with earlier observations on poly(Am), poly(Cm), and poly(Um), *i.e.*, an enhancement in the ordered self-structure of the single-stranded polymer. The stability of 2′-O-alkylated polynucleotide duplexes was determined by measuring their thermal dissociation under several conditions. The presence of 2′-*O*-alkyl groups either slightly destabilized or increased the stability of these complexes, depending on which strand in the complex was alkylated. The role of the phosphodiester backbone in complex stability was studied by examining the T_m values of polynucleotide–nucleoside complexes. The enhancement of complex structure by 2′-*O*-methyl groups in poly(Um) was expressed in a poly(Um)·nucleoside complex, paralleling the effect observed earlier in poly(Um)·polymer complexes. The thermal stability of poly(I)·poly(C) complexes, in which either strand contained progressively higher levels of 2′-*O*-methyl groups, was also determined. Little effect on T_m was noted until the content of Cm reached 30–50% at which point the T_m values were decreased, while the increased stability resulting from 2′-O-methylation of the poly(I) strand roughly paralleled 2′-*O*-methyl content.

Although the physical properties of RNA and DNA molecules have been intensively investigated, a basic understanding of the factors leading to differences in structural stability between these two polynucleotides has not yet been achieved (Felsenfeld and Miles, 1967). One approach to this problem is to utilize synthetic polymers containing identical base residues but differing only in the sugar–phosphate backbone. In this way the contribution of the phosphodiester backbone to intrinsic polymer stability can be at least partially separated from base-pairing and base-stacking effects.

The physical and biological properties of RNA molecules containing 2′-*O*-methyl nucleotides, a modification that occurs extensively in natural RNA (Hall, 1971), can be studied using synthetic model compounds. Polynucleotide phosphorylase has been shown to catalyze the polymerization of 2′-*O*-methyl nucleoside diphosphates, forming homopolymers and heteropolymers with 2′-*O*-methyl nucleotide contents ranging from a few per cent to complete methylation (Rottman and Heinlein, 1968; Rottman and Johnson, 1969; Zmudzka *et al.*, 1969). These 2′-O-methylated polymers have provided a new series of model compounds to further explore the influence of the sugar–phosphate backbone on polynucleotide structure.

The substitution of the 2′-hydroxyl of synthetic RNA molecules with 2′-*O*-methyl groups was found to substantially enhance the ordered structure of these single-stranded polynucleotides (Bobst *et al.*, 1969; Zmudzka and Shugar, 1970). More recently the presence of the bulkier 2′-*O*-ethyl group in an RNA molecule was shown to result in even greater enhancement of single-stranded polymer structure (Khan and Rottman, 1972).

2′-O-Methylated RNA molecules also provide a set of model compounds with which to study the forces promoting complementary strand interaction and the contribution of the phosphodiester backbone to complex stability. Earlier studies by Chamberlin (1965) comparing duplexes formed between ribo- and 2′-deoxyribopolynucleotides indicated that the nature of the sugar moiety in the phosphodiester backbone markedly influences the stability of the resulting duplex. The T_m values of several complexes containing 2′-O-methylated polymers have been reported (Bobst *et al.*, 1969; Zmudzka *et al.*, 1969; Zmudzka and Shugar, 1971) but little has been done to explain the rather dramatic effect 2′-*O*-methyl groups

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have on the stability of the complex. The ultimate effect of this naturally occurring modification on the physical properties and biological activities of RNA molecules will depend to a large degree on the influence of 2′-*O*-methyl groups on base pairing and the ability of these polynucleotides to form double-stranded complementary complexes.

Single-stranded 2′-O-methylated RNA molecules have recently been shown to be active in several biological systems. Poly(2′-*O*-methylcytidylic acid) [poly(Cm)][1] is a specific template for viral-directed RNA-dependent DNA polymerase; none of the normal DNA polymerases present in animal cells respond to this polymer (Gerard *et al.*, 1974). Poly(Cm) could by virtue of its stability to enzymatic degradation and selective template recognition thus serve as an efficient probe for the detection of "Reverse Transcriptase" activity. In other studies, poly(2′-*O*-methyladenylic acid) [poly(Am)] has been reported to be an effective inhibitor of leukemia virus replication in mouse embryo cells (Tennant *et al.*, 1972).

Polynucleotide complexes in which one or both strands are 2′-O-methylated have been used as templates for DNA-dependent RNA polymerase (Gerard *et al.*, 1972) and as inducers of interferon. A poly(I)·poly(Cm) duplex in which the pyrimidine strand was completely 2′-O-methylated was reported to lack interferon inducing capacity (DeClercq *et al.*, 1972). However, recent experiments using poly(I)·poly(C) molecules in which either strand was partially 2′-O-methylated indicated that these polymers efficiently induced interferon (Merigan and Rottman, 1974). Further information on the structure and stability of these 2′-O-alkylated polynucleotides will be required to provide a molecular explanation for their altered biological properties.

In this publication we wish to report the results of several experiments designed to elucidate the contribution of 2′-*O*-alkyl groups to polynucleotide structure. With single-stranded RNA molecules we were particularly concerned with the enhanced self-structure observed with 2′-O-alkylated polymers at neutral pH. Our results indicate that 2′-O-alkylation of single-stranded adenine-containing polynucleotides caused the adenine moieties to be more easily protonated, thereby favoring the acid helix. However, under conditions of comparable protonation, single-stranded adenine polymers still exhibited enhanced ordered structure upon 2′-O-alkylation. In other studies, the effects of 2′-*O*-alkyl groups on the stability of complementary polynucleotide complexes are described and the involvement of the phosphodiester backbone in this interaction is examined through the use of polynucleotide–nucleoside complexes.

Materials and Methods

Adenosine and 2′-deoxyadenosine were purchased from P. L. Laboratories. The 2′- and 3′-O-alkylated derivatives of adenosine were prepared as described previously (Rottman and Heinlein, 1968; Khan and Rottman, 1972). Poly(A), poly(U), poly(C), and poly(I) were purchased from Miles Laboratories, Inc. Concentrations of nucleosides and polyribonucleotides were determined using published extinction coefficients (Ts'o *et al.*, 1962; Bollum, 1966).

[1] Abbreviations used are: poly(Am), poly(2′-*O*-methyladenylic acid); poly(Ae), poly(2′-*O*-ethyladenylic acid); poly(Im), poly(2′-*O*-methylinosinic acid); poly(Cm), poly(2′-*O*-methylcytidylic acid); poly(Um), poly(2′-*O*-methyluridylic acid); poly(Am,A), a random heteropolymer of 2′-*O*-methyladenylic acid and adenylic acid; poly(Im,I), a random heteropolymer of 2′-*O*-methylinosinic acid and inosinic acid; poly(Cm,C), a random heteropolymer of 2′-*O*-methylcytidylic acid and cytidylic acid.

Chromatographic Systems. Descending paper chromatography was carried out on either Whatman No. 1 or No. 40 paper using the following solvent systems: (A) isopropyl alcohol–concentrated NH_4OH–0.1 M boric acid (7:1:2, v/v); (B) 95% ethyl alcohol–1 M ammonium acetate (pH 7.5) (7:3, v/v); (C) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v); and (D) *n*-propyl alcohol–concentrated NH_4OH–H_2O (55:10:35, v/v).

Synthesis of Polynucleotides. The preparations of poly(Am) (Rottman and Heinlein, 1968), poly(Ae) (Khan and Rottman, 1972), poly(Um) (Dunlap *et al.*, 1971), and poly(Cm) (Zmudzka *et al.*, 1969), have been described. Although we have earlier reported a study utilizing poly(Im) (Gerard *et al.*, 1972), its synthesis and characterization were not described. Recently Tazawa *et al.* (1972) have also reported the synthesis of poly(Im). Since this polynucleotide presents some unique problems in isolation its preparation will be briefly outlined.

AmDP (10,000 A_{260} units) was dissolved in 125 ml of 2 N acetic acid and 4 g of $NaNO_2$ was added slowly. After 3 hr at room temperature, the solution was neutralized to pH 7.5 by the addition of sodium hydroxide and the nucleotide was precipitated as the barium salt by adding barium acetate and an equal volume of ethanol. Removal of barium ion was accomplished by passing the dissolved precipitate over a short Dowex 50 H^+ column. The yield of ImDP was 60%, with apparent loss of significant amounts of material during nitrous acid treatment and in the barium precipitation step which was necessary for removal of salt. ImDP was further purified by chromatography on a Bio-Rad AG1-X2-Cl^- column by elution with a linear gradient of 0–0.5 M LiCl in 0.01 M HCl. The recovered ImDP (96% yield) was diluted to 0.025 M LiCl and applied to a DEAE-cellulose column. Elution with a linear gradient of 0–0.3 M ammonium bicarbonate gave ImDP (92% yield) and a small contaminant of ImMP which was eluted earlier. Ammonium bicarbonate was removed by repeated lyophilization after the addition of 2 equiv of NaOH. The homogeneity of the ImDP was checked in solvent systems A, B, and C and was shown in each system to contain a single ultraviolet-absorbing component.

Polymerization of ImDP by *M. luteus* polynucleotide phosphorylase (Type 1, P-L Biochemicals, Inc.) was carried out in a reaction mixture containing the following components in a total volume of 1.0 ml: 2′-*O*-methylinosine diphosphate, 25 mM; MnCl, 5 mM; Tris buffer (pH 9.0), 100 mM; polynucleotide phosphorylase, 2.4 mg. The reaction became very viscous after 25 hr and was terminated after 42 hr. After dilution of the reaction mixture to a total volume of 15 ml it was made 1% in sodium lauryl sulfate. Deproteinization was achieved by adding 5 ml of a 3:1 chloroform–isoamyl alcohol mixture and stirring vigorously on a Vortex mixer. The aqueous layer was removed following centrifugation and the extraction was repeated a total of 16 times. These unusual conditions were necessary to remove traces of protein shown to be present by Lowry analysis. The remaining aqueous phase was dialyzed 24 hr at 4° against 0.2 M NaCl, 0.01 M EDTA, and 0.01 M β-mercaptoethanol and then for 12 hr more against water. The yield of poly(Im) was 89 A_{247} units. The product was shown to be free of monomer by chromatography in solvent D, and possessed a typical inosine spectrum.

Synthesis of the heteropolymers, poly(Am,A), poly(Im,I), and poly(Cm,C) required special consideration due to the possibility of clustered incorporation of 2′-*O*-methyl nucleotides (Rottman and Johnson, 1969). Two modifications of the usual enzymatic polymerization conditions, substitution of Mn^{2+} for Mg^{2+} and inclusion of 30% dimethyl sulfoxide,

facilitated a more random insertion of 2′-*O*-methyl nucleotides.

Characterization of Polynucleotides. The concentrations of 2′-O-alkylated polynucleotides were determined by total phosphate analysis (Ames and Dubin, 1960). The molar extinction coefficients in 0.01 M phosphate (pH 7.4)–0.05 M NaCl calculated from these measurements are as follows: poly(Am), ϵ_{259} 10.2; poly(Ae), ϵ_{259} 10.6; poly(Um), ϵ_{259} 9.4; poly(Cm), ϵ_{270} 4.9; poly(Im), ϵ_{250} 11.0.

The mean value of the sedimentation coefficients for each polynucleotide was determined by centrifugation through sucrose gradients at pH 9.0, and the values are as follows: poly(Am), 16 S; poly(Ae), 14 S; poly(Um), 16 S; poly(Cm), 4 S; poly(Im), 17 S. Sedimentation coefficients for the heteropolymers, poly(Cm,C) and poly(Im,I) are given in the legend to Figure 6. Poly(Im) and heteropolymers of poly(Im,I) containing high levels of 2′-O-methylation tended to form aggregates and often gave multiple peaks on sucrose gradient analysis. When this occurred the sedimentation value reported corresponds to the smallest component observed.

Verification of random distribution of 2′-*O*-methylnucleotides in synthetic heteropolymers was obtained by acid precipitation of the alkaline-stable radioactive oligonucleotides after incorporation of [^{3}H]AmDP (Rottman and Heinlein, 1968) in the presence of ADP. Long runs of 2′-*O*-methyl nucleotides would have remained resistant to alkaline hydrolysis. Degradation of the polymer to acid-soluble oligonucleotides with 0.5 M KOH for 18 hr at 37° was indicative of random incorporation.

Alternatively, nonradioactive polymers were examined for alkaline-stable oligonucleotides using paper chromatography in solvent D. The actual content of 2′-*O*-methyl nucleotide present in each polymer was determined by conversion of the 2′-*O*-methyl groups to methanol and detection by gas chromatography as described previously (Abbate and Rottman, 1972).

pK_a Determinations of Nucleosides, Nucleotides, and Polynucleotides. The pK_a values for adenine-containing nucleosides were determined spectrophotometrically by following the absorbance at 285 nm as a function of pH. Identical aliquots of nucleoside or nucleotide were diluted into buffers which differed by 0.2 pH unit in the region of the pK_a and covered a total range in pH from 1.6 to 5.5. Absorbance measurements were made on a Gilford Model 240 spectrophotometer and pK_a values determined by calculating the inflection point of the absorbance change at 285 nm.

Due to limiting amounts of 2′-O-alkylated polynucleotides, the apparent pK_a values of polymers were determined spectrophotometrically using a circulating system similar to that described by Hobbs *et al.* (1972b). The term "apparent pK_a" is used in this case since a potentiometric determination could not be performed with the limited material available and the spectrophotometric measurement employed in these studies really measures the pH of the helix–coil transition. As pointed out by Holcomb and Timasheff (1968) this transition may occur with poly(A) at a point other than 50% protonation. A solution of polynucleotide (0.8 A_{260} unit) in 3 ml of sodium citrate which was 0.1 M in sodium ion was circulated from a small reservoir through a flow cell in a Gilford 240 spectrophotometer. The pH of the solution in the reservoir was continuously monitored using a Radiometer Type PHM-4C pH meter, and the absorbance of the solution was determined at 262 and 285 nm as a function of pH. The solution was titrated by addition of small aliquots of concentrated HCl.

Determination of T_m. Samples of single-stranded polynucleotides were prepared for T_m analysis by dialysis for 20 hr against the appropriate buffer. The first dialysis (5 hr) was against chloroform-saturated buffer at 4° to inhibit microbial growth, followed by dialysis against fresh buffer.

Polynucleotide complexes were prepared for T_m analysis as follows: a mixture containing 1 μmol of each polymer, based on nucleotide phosphate, was diluted in a final concentration of 0.01 M sodium phosphate (pH 7.4) and 0.05 M NaCl, to a total volume of 1 ml. This solution was first dialyzed 3 hr against standard buffer containing $CHCl_3$ to inhibit microbial growth and then against fresh standard buffer for 18 hr at 4°. An aliquot of the dialyzed complex (0.025 μmol of each polymer), concentrated standard buffer, and varying concentrations of NaCl or organic solvent were diluted to a total of 500 μl to give the concentration of each component shown in the legends to tables and figures.

Temperature–absorbance transitions (T_m) for polynucleotides were obtained with a Gilford Model 2427 thermal programmer attached to the 240 spectrophotometer in conjunction with a Model 6040 analog multiplexer. An electronically heated and cooled four-sample cuvet containing a thermistor probe (Gilford Instrument Co.) facilitated the automatic monitoring of both absorbance and temperature. Linear temperature increase was programmed at either 1 or 2°/min over a range of 5–95°. With this system it was possible to simultaneously record both absorbance and temperature on the same chart (*cf.* Figures 4 and 5) and complete the entire T_m analysis of four samples in approximately 45 min. The T_m's of adenine- and hypoxanthine-containing polymers were determined at 260 and 247 nm, respectively.

For analysis of T_m for polynucleotide complexes, the samples were rapidly cooled to the starting temperature following the initial determination of T_m and kept at that temperature for 10 min. The samples were then reheated progressively and the T_m was determined a second time. In addition to these repeated determinations on the same sample, additional samples of the same polymer were prepared for duplicate analysis as described in the legends to tables and figures. As a further check that the T_m values were independent of a particular polynucleotide preparation, separate polymer preparations were run for all completely 2′-O-alkylated polymers except poly(Ae). The T_m values reported are generally the average of four separate determinations with a variance of less than 1°. Analyses with duplicate heteropolymers containing only partial methylation were not performed.

For accurate T_m determinations of complexes containing poly(Im), *i.e.*, poly(Im)·poly(C) and poly(Im)·poly(Cm), the solution containing the complex was first heated in the cuvet to 90° and held at that temperature for 10 min. Subsequent temperature–absorbance profiles gave smooth transitions, presumably through prior dissolution of aggregates formed in the presence of poly(Im). It was observed that failure to preheat these samples gave irregular tracings.

The stability of complexes formed between polynucleotides and nucleosides was determined with the Gilford Model 2427 using a method described earlier by Davies and Davidson (1971). Either poly(U) or poly(Um), present at a final concentration of 0.25 mM, was mixed with an adenine-containing nucleoside, final concentration 10 mM, in 0.01 M sodium cacodylate (pH 7.0) and 1.0 M NaCl. The high concentration of monomer in the cuvet (~150 A_{260}/ml) necessitated monitoring at 291 nm rather than at the λ_{max} of either component. Temperature and absorbance were plotted automatically as described previously and the T_m's calculated from the inflection point of the curves obtained in this manner. When temperature scans were initiated below ambient temperature the

cuvet chamber was continuously flushed with dry nitrogen to prevent condensation on the cuvet surface.

Results

Ordered Structure of Single-Stranded Adenine-Containing Polynucleotides. Holcomb and Tinoco (1965) and Witz and Luzzati (1965) on the basis of small-angle X-ray scattering studies concluded that at neutral pH poly(A) has a single-stranded helical structure with stacked bases involving no hydrogen bonding as a stabilizing force. At acidic pH poly(A) forms a well-defined two-stranded helical structure which is stabilized by hydrogen bonding (Rich *et al.*, 1961). Temperature–absorbance profiles indicated that the ordered self-structure of single-stranded adenine-containing polynucleotides increased as follows: poly(adenylic acid) < poly(2′-*O*-methyladenylic acid) < poly(2′-*O*-ethyladenylic acid) (Figure 1). The increased self-structure of the 2′-O-alkylated polymers in comparison to poly(A) is observed both in cooperative transitions, reflecting the acid helix–coil transition, which are destabilized at higher temperatures as well as a higher degree of hyperchromicity accompanying the transition. The most striking characteristic of the 2′-O-alkylated polymers is the cooperative transition in the temperature–absorbance profiles observed at neutral pH; such a transition is absent in poly(A). The 2′-*O*-ethyl group has an even greater effect than 2′-*O*-methyl at both acidic and neutral pH.

According to Feldman (1973) low concentrations of formaldehyde can be used to probe the degree of H-bonding between polynucleotide strands without interfering with base stacking within the strand. Furthermore, those strands already interacting by H-bonding are not affected by formaldehyde at temperatures below the T_m for strand interaction while at temperatures above the T_m, reaction occurs readily.

The effect of formaldehyde on poly(A), poly(Am), and poly(Ae) structure was examined at both acidic and neutral pH and at two concentrations of formaldehyde. After a 10-min incubation with 2% formaldehyde at pH 7.0 and 20° the cooperative transition normally observed for poly(Am) at pH 7.0 and occurring at 13° was completely eliminated (data not shown) while most of the cooperative interaction for poly(Ae) at 29° still remained. However, after heating the samples to 40°, cooling to 5°, and repeating the temperature–absorbance scan, the cooperative transition previously observed with poly(Ae) was absent.

Similar studies were performed with 0.5% formaldehyde and also at pH 5.7 where poly(A), poly(Am), and poly(Ae) have cooperative transitions at higher temperatures (*cf.* Figure 1). The results of these studies (data not shown) indicated that the presence of formaldehyde did not shift the T_m of the cooperative transition obtained during the first melt, but after reaching temperatures above their respective T_m's, subsequent temperature–absorbance scans indicated a complete absence of detectable cooperative transition above 5° in poly(A), poly(Am), and poly(Ae).

pK_a Determination of Nucleosides, Nucleotides, and Polynucleotides. It seemed quite likely that the highly cooperative portion of the transition observed with 2′-O-alkylated polymers might at least partially result from a double-stranded helical "acid form" of the polymer (Rich *et al.*, 1961). If this were true one would expect to see an increased tendency for protonation of the adenine moiety attached to a 2′-*O*-alkyl ribose. Therefore the pK_a values for several 2′- and 3′-substituted nucleosides and nucleotides were determined spectrophotometrically as described in Materials and Methods. Significant changes in pK_a values of nucleosides are noted upon elimination of either of the ribose hydroxyls, as seen for 2′- and 3′-deoxyadenosine, but no significant alteration in pK_a is observed when the 2′-hydroxyl is replaced by either 2′-*O*-methyl or 2′-*O*-ethyl (Table I). Similarly in the nucleotide series, the presence of a 5′-monophosphate shifts the pK_a in comparison to the nucleoside, but 2′-*O*-alkyl substitution had little effect on the pK_a of the nucleotide.

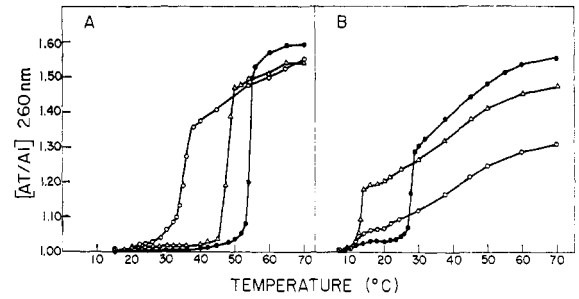

FIGURE 1: Temperature–absorbance profiles of poly(A) (○), poly(Am) (△) and poly(Ae) (●) in (A) 0.01 M sodium cacodylate (pH 5.7)–0.15 M NaCl and (B) 0.01 M sodium cacodylate (pH 7.0)–0.15 M NaCl. AT/AI is the ratio of absorbance at temperature T over initial temperature.

Although no alteration of pK_a values was observed upon 2′-*O*-alkylation of the monomer units it could not be assumed that the presence of similar groups in a polynucleotide would not affect the pK_a of the adenine moiety. The results in Figure 2 show a spectrophotometric determination of the apparent pK_a values for poly(A), poly(Am), and poly(Ae). Absorbance was monitored in a flow cell at both 262 and 285 nm as a function of pH. Under these conditions of ionic strength and temperature (0.1 M sodium ion at 23°) a substantial shift in the pH-dependent helix–coil transition is noted accompanying 2′-O-alkylation. The apparent pK_a values are 5.85, 6.20, and 6.50 for poly(A), poly(Am), and poly(Ae), respectively. The pH-dependent increase in absorbancy at 262 nm is in each case accompanied by a corresponding decrease at 285 nm. Therefore it is likely that the 2′-O-alkylated polymers are more easily protonated at neutral pH and would consequently form the double-stranded acid helix more readily.

To compare each of the adenine-containing polynucleotides under conditions of comparable base protonation, the T_m values were determined at the apparent pK_a and 1 pH unit

TABLE I: pK_a Values for Adenine-Containing Nucleosides and Nucleotides.[a]

Compound	pK_a
Adenosine	3.65
2′-Deoxyadenosine	3.73
3′-Deoxyadenosine	3.83
2′-*O*-Methyladenosine	3.62
3′-*O*-Methyladenosine	3.67
2′-*O*-Ethyladenosine	3.66
Adenosine 5′-phosphate	3.80
2′-*O*-Methyladenosine 5′-phosphate	3.83
2′-*O*-Ethyladenosine 5′-phosphate	3.80

[a] Samples of each nucleoside or nucleotide were diluted into buffer and absorbance determined at 285 nm as a function of pH. The pK_a values were calculated as described in Materials and Methods.

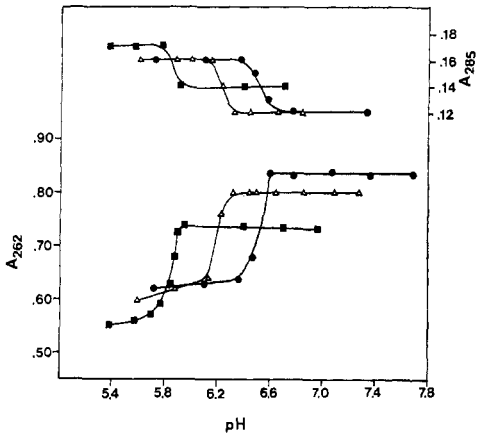

FIGURE 2: Apparent pK_a determinations for poly(A) (■), poly(Am) (△), and poly(Ae) (●). The pH of a solution of each polynucleotide was monitored and absorbance determined by circulation through a flow cell as described in Materials and Methods

above the pK_a for each polynucleotide. These conditions should minimize differences in ordered structure due merely to enhanced protonation and make possible an assessment of the contribution of 2′-*O*-alkyl groups to polymer structure apart from the effect due to shifted pK_a's. As can be seen from the results in Table II, 2′-O-alkylation still elevated the T_m of cooperative transitions when polymers were compared at their apparent pK_a's or pK_a + 1 pH unit. Unfortunately the T_m values obtained at pK_a − 1 pH unit were not consistent, perhaps due to partial precipitation of polynucleotide at these lower pH values.

Self-Structure of Partially 2′-O-Methylated Single-Strand Poly(A) Molecules. Since the level of 2′-O-methylation occurring in natural RNA molecules is generally only a few per cent, it was of interest to examine the effect of partial 2′-O-methylation on the self-structure of RNA molecules. As shown in Table III, increasing levels of 2′-*O*-methyl groups in poly(A) increased the temperature of the T_m's observed with these polymers at pH 5.7. The temperature–absorbance profiles were also examined at pH 7.0, with only the completely methylated polymer, poly(Am), showing a cooperative transition (data not shown).

Single-Strand Structure of Poly(2′-O-methylinosinic acid). Since the presence of 2′-*O*-alkyl groups in other polynucleotides (poly(Am), poly(Ae), poly(Cm), poly(Um)) enhanced the self-structure of those polymers, it was of interest to study the analogous situation with 2′-*O*-methylinosine.

The temperature–absorbance profile for poly(Im) was determined at pH 7.0 and a rather sharp cooperative transition was observed (Figure 3). However, poly(I) did not show a cooperative transition under identical conditions and displayed only a gradual increase in absorbance, typical of the incremental disruption of a random coil.

Effect of 2′-O-Alkylation on the Stability of Polynucleotide Complexes. Although the presence of 2′-*O*-alkyl groups stabilizes the structure of single-stranded polymers, their effect on the stability of a complex has been only partially described (Bobst *et al.*, 1969; Zmudzka *et al.*, 1969; Zmudzka and Shugar, 1971). The determination of a typical set of T_m values using the procedure described earlier is shown in Figure 4. The noticeably sharp transitions occurring at the T_m seem to be accentuated in complexes containing 2′-O-methylated polymers. The presence of 2′-*O*-methyl groups in the poly(Am)·poly(U) complex slightly destabilizes the helix, in comparison to poly(A)·poly(U) (Table IV), while methylation of the pyrimidine-containing strand in poly(A)·poly(Um) dra-

TABLE II: T_m Values for Poly(A), Poly(Am), and Poly(Ae) as a Function of pH.[a]

Polynucleotide	pH	T_m, °C
	(pK_a)	
Poly(A)	5.85	17
Poly(Am)	6.20	27
Poly(Ae)	6.50	34
	(pK_a + 1)	
Poly(A)	6.85	N.D.[b]
Poly(Am)	7.20	~2[b]
Poly(Ae)	7.50	22

[a] T_m's were determined as described in Materials and Methods in 0.15 M sodium chloride and 0.01 M sodium cacodylate, at the pH indicated. N.D., not detectable under these conditions. [b] For these experiments, T_m determinations were initiated at 0°.

TABLE III: Effect of Partial 2′-O-Methylation on the Stability of Poly(Am,A).[a]

% 2′-*O*-Methyl	T_m of Cooperative Transition, °C
0	35.0
6	36.6
24	39.1
100	48.0

[a] T_m's were determined as described in Materials and Methods in 0.01 M sodium cacodylate (pH 5.7) and 0.15 M sodium chloride.

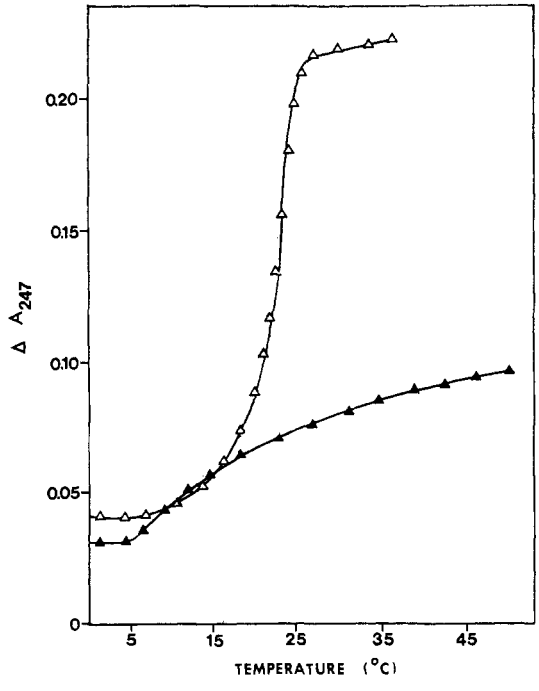

FIGURE 3: Temperature–absorbance profiles of poly(Im) (△) and poly(I) (▲) in 0.01 M sodium cacodylate (pH 7.0)–0.15 M NaCl.

TABLE IV: Effect of Ionic Strength on Stability of 2′-O-Alkylated Complexes.[a]

Complex	T_m Values 7×10^{-2} M Na^+	2.4×10^{-2} M Na^+	5.2×10^{-1} M Na^+
Poly(A)·poly(U)	54.2	44.1	74.6
Poly(Am)·poly(U)	52.4	43.8	69.5
Poly(A)·poly(Um)	63.6	53.9	80.0
Poly(Am)·poly(Um)	64.4	53.6	83.2
Poly(Ae)·poly(U)	49.8	40.1	67.0
Poly(Ae)·poly(Um)	60.1	48.8	79.2
Poly(I)·poly(C)	57.9	49.6	71.1
Poly(Im)·poly(C)	62.9	53.1	78.2
Poly(I)·poly(Cm)	52.7	44.5	71.9
Poly(Im)·poly(Cm)	57.1	47.9	80.0

[a] Polynucleotide complexes were formed and T_m values determined as described in Materials and Methods. Complexes containing poly(Im) were first heated to 90° to eliminate aggregation as discussed in Materials and Methods. The concentration of buffer was 0.01 M sodium phosphate (pH 7.4). The final concentration of sodium ion was adjusted to the value listed in the table by the addition of NaCl.

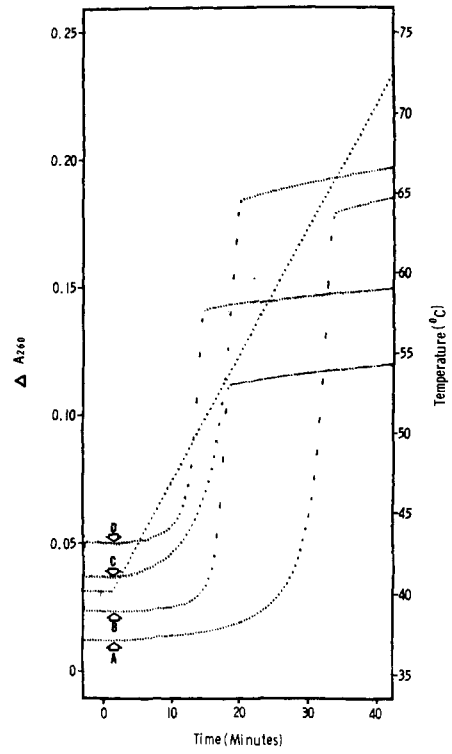

FIGURE 4: T_m determination for 2′-O-alkylated polynucleotide complexes. Polynucleotide complexes were prepared in 0.01 M sodium phosphate (pH 7.4)–0.05 M NaCl as described in Materials and Methods. The actual recording obtained with the instrument described in Materials and Methods is presented in this figure. Absorbancy readings are listed as Δ values since different concentrations of polymers were present in each cell. The linear advance of temperature is recorded by the dotted diagonal line, starting at 40°. Temperature advance, at a rate of 1°/min, was started at min 1. The absorbance profile for each complex is as follows: (A) poly(A)·poly(Um); (B) poly(A)·poly(U); (C) poly(Am)·poly(U); (D) poly(Ae)·poly(U). T_m values were calculated by extending the stable absorbance lines before and after the transition, measuring the midpoint between them, and extrapolating vertically to the temperature line recorded on the chart.

matically increases stability by nearly 10° (Zmudzka and Shugar, 1971). Methylation of both strands in poly(Am)·poly(Um) leads to a further increase. Ethylation of the adenine-containing strand in poly(Ae)·poly(U) destabilizes the complex to an even larger extent than methylation, while the complex of poly(Ae)·poly(Um) is of intermediate stability.

In comparing the poly(I)·poly(C) series with the poly(A)·poly(U) series the opposite effect of 2′-*O*-methylation is observed, *i.e.*, methylation of the purine-containing strand stabilizes the complex whereas methylation of the pyrimidine strand results in less stability (Table IV). Thus the T_m of poly(Im)·poly(C) is 5° higher than the unmodified complex and poly(I)·poly(Cm) is 5.2° lower. The presence of 2′-*O*-methyl groups in both strands gives a complex of intermediate stability, close to that of poly(I)·poly(C).

Effect of Ionic Strength on Polynucleotide Complex Stability. The standard buffer used in these studies contained 7×10^{-2} M sodium ion. Decreasing the sodium ion concentration to 2.4×10^{-2} M decreased the stability of each complex by approximately 10° (Table IV). Under the same decrease in salt concentration, complexes containing 2′-*O*-alkyl substitutions were destabilized to the same extent. Alternatively, an increase in sodium ion to 5.2×10^{-1} M increased the T_m of complexes by approximately 19°. The dependence of T_m on salt concentration has been studied by Record (1967) who demonstrated that the transition from double helix to coil is described by $dT_m/d \log [Na^+]$. For double-stranded RNA polymers this dependence is 17–19°. Therefore one would predict a decrease in T_m at 2.4×10^{-2} M of 9° and an increase in T_m at 5.2×10^{-1} M of 17°, both of which are close to the experimental values reported in Table IV, irrespective of the presence or absence of 2′-*O*-alkyl groups.

Effect of Organic Solvents on the Stability of Complexes. Organic solvents such as dimethyl sulfoxide and ethylene glycol are known to function as denaturing agents for RNA molecules (Strauss *et al.*, 1968; Helmkamp and Ts'o, 1961). It has been suggested that certain of these solvents, *e.g.*, ethylene glycol and methanol, disrupt hydrophobic interactions (Fasman *et al.*, 1965). In addition these solvents can undoubtedly interfere with hydrogen bonding. The influence of organic solvents on the T_m's of polynucleotide complexes was studied to determine if these solvents had a differential or highly selective effect on 2′-*O*-alkylated complexes. The assumption made in these experiments is that if the stability of a 2′-*O*-alkylated complex is primarily a reflection of hydrophobic interactions between alkyl centers or the ordering or disordering of adjacent water molecules due to alkyl groups, one should be able to interrupt these effects by high concentrations of organic solvents and thereby destabilize the complex. If this were true one would expect to see a much larger solvent effect on the T_m's of 2′-O-alkylated complexes than unmodified complexes.

T_m values for each of the complexes shown in Table IV were determined in the presence of ethylene glycol, dimethyl sulfoxide, methanol, and ethanol. The results (data not shown) can be summarized to indicate that 40% ethylene glycol and 40% dimethyl sulfoxide lowered the T_m's whereas 40% methanol, and particularly 40% ethanol, raised the T_m's of all polynucleotide complexes examined. Of primary importance, however, is the observation that the effect of any one solvent on the stability of comparable normal and 2′-O-alkylated complexes was similar. A selective or exaggerated effect on the alkylated complexes was not observed.

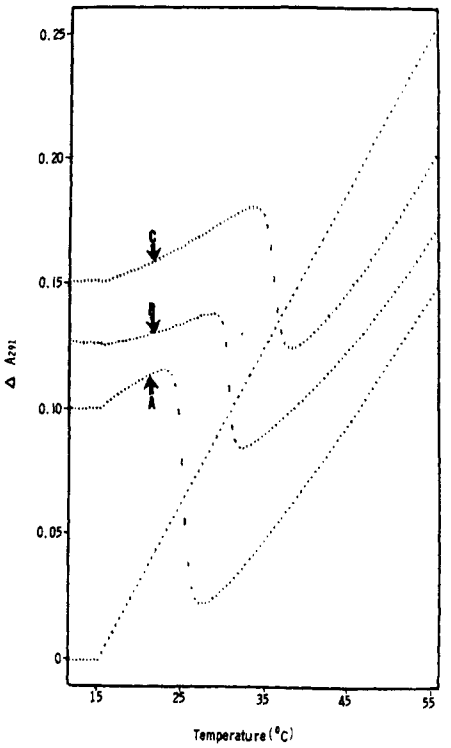

FIGURE 5: T_m determination for polymer–monomer complexes. Mixtures of polynucleotides and nucleosides in 0.01 M sodium cacodylate (pH 7.0)–1.0 M NaCl were heated from 15 to 55°, and the absorbance was monitored at 291 nm. The sharp transition in absorbance was used to calculate T_m values using the procedure described in the legend to Figure 4. Absorbance tracings are shown for the following complexes: (A) poly(U)·2′-*O*-ethyladenosine; (B) poly(U)·3′-*O*-ethyladenosine; (C) poly(Um)·2′-deoxyadenosine.

The Formation of Polynucleotide–Monomer Complexes. Helix formation between polynucleotides and monomers has been reported previously (Howard *et al.*, 1966; Huang and Ts'o, 1966). These studies have shown that polymer–monomer interactions take place in stoichiometric ratios, are strongly cooperative, and are concentration dependent, particularly on the monomer concentration. In an attempt to further analyze the nature of the effect of 2′-*O*-methyl groups on complex stability, the complementary structure formed between a polynucleotide and nucleoside was studied. In this manner one can determine if the effect of 2′-*O*-methyl groups requires the presence of an intact phosphodiester backbone in both strands. Using direct absorbancy measurements as described in Materials and Methods, it was possible to observe the thermal transition for the disruption of the complex (Figure 5). As noted by Davies and Davidson (1971), at this wavelength the transition is accompanied by a hypochromic change, the hyperchromic effect before and after the transition being due to the absorption of the free nucleoside. The T_m's for the interaction between poly(U) and adenosine and between poly(U) and 2′-deoxyadenosine are 27.2 and 27.1°, respectively (Table V). These results are in agreement with those reported by Davies and Davidson of approximately 27° for both nucleosides. The interaction of poly(U) with 2′-*O*-methyladenosine is very similar to that of adenosine while the T_m with 2′-*O*-ethyladenosine is slightly lowered.

When poly(Um) is used in place of poly(U), the T_m's are elevated substantially. This is true not only for poly(Um) plus adenosine but for the other nucleosides tested as well. Thus the T_m of the interaction of adenosine with poly(Um) is about 9° higher than with poly(U).

TABLE V: Stability of Polynucleotide–Nucleoside Complexes.[a]

Polynucleotide	Nucleoside	T_m, °C
Poly(U)	Adenosine	27.2
Poly(U)	2′-Deoxyadenosine	27.1
Poly(U)	2′-*O*-Methyladenosine	28.6
Poly(U)	2′-*O*-Ethyladenosine	25.0
Poly(U)	3′-*O*-Ethyladenosine	32.5
Poly(Um)	Adenosine	36.4
Poly(Um)	2′-Deoxyadenosine	40.0
Poly(Um)	2′-*O*-Methyladenosine	45.2
Poly(Um)	2′-*O*-Ethyladenosine	61.6

[a] Mixtures of polynucleotide and nucleoside in 0.01 M sodium cacodylate (pH 7.0)–1.0 M NaCl were slowly heated and the absorbance was monitored at 291 nm. The T_m values were calculated as described in the legend to Figure 5.

Unfortunately polynucleotide–monomer complexes form only with pyrimidine-containing polynucleotides and purine nucleosides and do not form with purine-containing polymers and pyrimidine nucleosides (Howard *et al.*, 1966; Huang and Ts'o, 1966). Therefore it was not possible to test the corresponding poly(A)–uridine series. Since the T_m of the polymer–polymer interaction between poly(A) and poly(Um) was substantially elevated we did try to form a poly(A)–2′-*O*-methyluridine complex, but without success.

Polynucleotide Complex Formation Using Partially 2′-O-Methylated Heteropolymers. The effect of partial 2′-O-methylation on the stability of a complex was tested in the poly(I)·poly(C) series in which either strand contained 2′-*O*-methyl groups ranging from only several per cent to close to 90%.

Polynucleotide complexes were prepared for T_m analysis and the T_m values determined as described in Materials and Methods. As shown in Figure 6, increasing levels of 2′-*O*-methyl groups in the poly(Cm,C) strand of poly(Cm,C)·poly(I) have little effect on complex stability until the concentration of Cm reaches 30–50% where one sees progressive destabilization approaching the T_m of poly(Cm)·poly(I). Alternatively as the level of 2′-*O*-methyl groups increases in the poly(Im,I) strand of poly(Im,I)·poly(C), there is a gradual increase in complex stability as one increases the per cent of Im

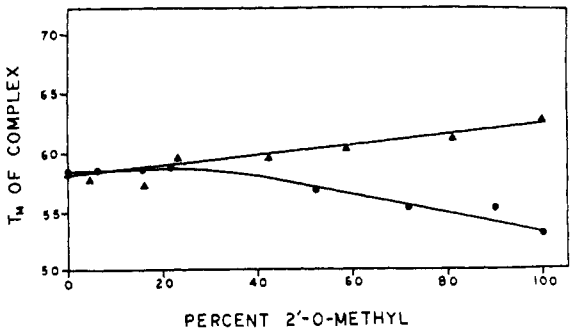

FIGURE 6: Effect of partial 2′-O-methylation on the stability of poly(Im,I)·poly(Cm,C) complexes: poly(Im,I) (▲) and poly(Cm,C) (●). Polynucleotides were synthesized as described in Materials and Methods. The sizes of poly(Im,I) and poly(Cm,C) used in these studies were 5.0–6.6 S and 3.3–5.2 S, respectively. Polynucleotide complexes were prepared in 0.01 M sodium phosphate (pH 7.4)–0.05 M NaCl, and their T_m values were determined as described in Materials and Methods. Reported T_m values were obtained with a single set of polymers.

in the poly(Im,I) strand approaching the T_m of poly(Im)·poly(C). It should be mentioned that small levels of 2′-O-methylation, more closely approximating those in natural RNA molecules, may not influence polymer structure in the same manner as completely methylated polymers where the effect of adjacent alkyl groups could be amplified.

Discussion

Studies on several single-strand 2′-O-alkylated polynucleotides including poly(Am) (Bobst *et al.*, 1969), poly(Um) (Zmudzka and Shugar, 1970), poly(Cm) (Zmudzka *et al.*, 1969), and more recently poly(Ae) (Khan and Rottman, 1972) indicate that in each case the 2′-alkylated polymer has more ordered structure than the analogous ribopolymer. One cannot generalize, however, to say that any 2′ substitution will increase the ordered self-structure of a polymer since 2′-Cl (Hobbs *et al.*, 1972b), 2′-F (Janik *et al.*, 1972), and 2′-NH_2 (Hobbs *et al.*, 1972a) decrease self-structure while 2′-azido (Torrence *et al.*, 1972) leads to an increase.

The mechanism whereby these 2′ substitutions affect intramolecular structure is not immediately apparent. The increasing tendency for a polynucleotide chain to assume a more stable conformation parallels the steric bulk of the 2′ substituent as one follows the progression of polymer stability: 2′-H < 2′-OH < 2′-OCH_3 < 2′-OC_2H_5. In this connection, one of the more striking effects of 2′-O-alkylation is the highly ordered cooperative structure observed in poly(Am) and poly(Ae) at neutral pH.

To explore the possibility that 2′-O-alkylation might affect H-bonding capacity we determined the pK_a values of free monomeric units as nucleosides and nucleotides and also the pH of the polymer helix–coil transition. Although the pK_a values for 2′-*O*-methyl- and 2′-*O*-ethyladenosine and adenylic acid showed no effect of 2′ substitution in comparison to adenosine, the apparent pK_a values of the polynucleotides were substantially different. Such a modification in the apparent pK_a of a polynucleotide by substitutions at the 2′ position has been noted previously (Hobbs *et al.*, 1972b). It appears that although a 2′-*O*-methyl or ethyl group does not affect the pK_a of free monomeric units, the added restriction of the phosphodiester backbone alters the tendency of adenine residues in 2′-O-alkylated polymers to be protonated at a given pH. The occurrence of ordered structure in poly(Am) and poly(Ae) at pH 7.0 and its absence in poly(A) may partially reflect these effects. Furthermore, when one compares the relative stability of the cooperative transitions in T_m at the more acidic pH of 5.7, a striking correspondence to the apparent pK_a values of the respective polymers is noted (*cf.* Figures 1 and 2).

The maintenance of enhanced ordered structure by 2′-*O*-alkyl groups at the apparent pK_a of the polynucleotide and at 1 pH unit above the apparent pK_a (Table II) indicates that 2′-*O*-alkyl groups are exerting an effect on polymer structure in addition to an alteration of apparent pK_a. It should be noted that even if the apparent pK_a's measured spectrophotometrically in these studies are not the true pK_a's of the polymer, the helix–coil transition occurring at this pH differs in thermal stability, depending on the particular 2′ substitution of the polymer.

The effect of formaldehyde on poly(A), poly(Am), and poly(Ae) structure is consistent with the interpretation that the cooperative part of the T_m involves H-bonding interactions and that this cooperative structure is disrupted when H-bonding is prevented.

Several preparations of poly(Im) showed a cooperative transition in T_m at 22° in pH 7.0 buffer, while poly(I) showed only a gradual increase in absorbance under identical conditions. It appears that 2′-*O*-methyl groups stabilize the single-strand structure of poly(Im) as has been noted before for poly(Am), poly(Um), and poly(Cm). The mechanism whereby this is accomplished in the case of poly(Im) is not clear and may in fact be quite different from that of poly(Am).

Increasing levels of 2′-*O*-methylnucleotides in poly(Am,A) heteropolymers indicated that 2′-*O*-methyl groups also affect the stability of the secondary structure of single-stranded polymers when present in only a portion of the nucleotides. The stability of that structure melting in a highly cooperative fashion at pH 5.7, presumably reflecting the double-stranded helical content of the polymer, increases with increasing 2′-*O*-methyl content. None of the heteropolymers studied displayed cooperative transitions at pH 7.0. Perhaps higher levels of 2′-*O*-methyl groups in excess of the 33% used in these studies would produce a heteropolymer with double-stranded structure at pH 7.0.

The rather marked effects that 2′-*O*-alkyl groups have on single-strand polynucleotide structure will have to be taken into account in arriving at a molecular explanation for the contribution of the phosphate–sugar backbone to polymer stability. Enhanced polymer stability might arise from electrostatic effects, hydrophobic interactions, or be due to the steric bulk of the 2′ substituent and the manner in which the size of this substituent, 2′-H, 2′-OH, 2′-OCH_3, or 2′-OC_2H_5, is registered in the base moieties of the polynucleotide.

The effects of 2′-*O*-methyl groups on the structure of these model compounds may have application to naturally occurring RNA molecules, many of which are known to contain 2′-*O*-methyl nucleotides. Although the content of 2′-*O*-methyl nucleotides in natural RNA is usually quite low, recent studies have described "runs" or "clusters" of adjacent 2′-*O*-methyl nucleotides within RNA (Shibata *et al.*, 1973).

The effect of 2′-*O*-methyls on the structure of single-strand RNA molecules may be significantly different from the effect on double-stranded molecules. The presence of 2′-*O*-methyl groups in a polynucleotide modifies the capacity of that polymer to form a complex with a complementary polymer. However, the behavior of a given polynucleotide in a complex cannot be predicted from its properties as a single-stranded polymer. It should be noted that although 2′-O-alkylation uniformly enhances ordered structure in all single-stranded polymers examined it may either increase or decrease the stability of a complex. The tendency for ordered structure within a single-stranded adenine-containing polymer is poly(Ae) > poly(Am) > poly(A) while these same polymers in the presence of poly(U) form complexes with the reverse order of stability. Furthermore the effect of 2′-O-methylation cannot be predicted on the basis of the purine or pyrimidine content of the polymer. 2′-O-Methylation of poly(A) slightly destabilizes the complex while poly(Im) enhances stability. Alternatively, in the case of pyrimidines, poly(Um) increases stability while poly(Cm) leads to a decrease. Thus it appears that the influence of the 2′-*O*-methyl group must be considered in the context of the particular base to which it is attached, and the structure of each complex may be unique. In this connection it is interesting to note that DNA dependent RNA polymerase will use these 2′-O-methylated complexes as templates for RNA synthesis but copies only the pyrimidine-containing strand and not the purine-containing strand (Gerard *et al.*, 1972). The activity of this enzyme in relationship to the rather bulky alkyl groups is intriguing but any molecular explanation

will probably have to await a more careful structural determination of these complexes by X-ray analysis.

Studies dealing with the effect of Na^+ concentration on complex stability as measured by T_m are of dual importance. First, in the absence of a complete characterization of the stoichiometry for each complex, one must be concerned about the interconversion of equilibrium mixtures of double- and triple-stranded species. Apparently in the past the complexity of this phenomena has not been fully appreciated and the unambiguous determination of these structures is very difficult (Howard *et al.*, 1971; Thrierr and Leng, 1972). As shown in Table IV, a change of several orders of magnitude in Na^+ concentration does not alter the relative effect of 2′-*O*-alkyl groups on the T_m's of the complexes. Since one observes a rather uniform effect of salt concentration with all polymers we assume that the general order of complex stability, as determined by their T_m's, is an intrinsic property of each complex rather than a reflection of changes in the stoichiometry of a particular complex due to 2′-*O*-alkyl groups. Secondly, the determination of T_m's as a function of salt concentration is important because the ordered structure of single-stranded polymers resulting from protonation of bases appears to decrease with increasing salt concentration (Steiner and Beers, 1958). Therefore it could be argued that differences in the stability of various complexes merely reflect a displacement of the equilibrium toward single-stranded polymers which by virtue of 2′-*O*-alkyl groups are more or less stable due to protonation at a given salt concentration. Again the maintenance of the relative order of stabilities throughout a large variation in Na^+ concentration argues against this possibility.

Organic solvents do not appear to selectively alter the T_m's of 2′-O-alkylated complexes in comparison to unmodified complexes. Since these solvents do not have a differential effect on 2′-O-alkylated molecules it is unlikely that the primary contribution of the alkyl group to polymer complex stability is through a strong interaction of hydrophobic alkyl centers.

The data on the stability of polynucleotide–nucleoside complexes indicate that the constraint imposed by the phosphodiester backbone is not required in both strands of a complex to see an enhanced interaction in the presence of 2′-*O*-methyl groups. In some fashion, the conformation of poly(Um) is modified by the 2′-*O*-methyl groups so that the uracil residues interact more strongly with adenine residues in both poly(A) and the monomer, adenosine. It is unfortunate that a similar study could not be carried out with poly(Am) and uracil-containing nucleosides but as mentioned earlier, these polymer–monomer complexes do not form. The large elevation in the T_m of the 2′-O-alkylated nucleosides with poly(Um) may result from the self-interaction of these monomers at unusually high concentrations. The integrity of the polymer–monomer complex depends on nucleoside concentration and in these experiments the nucleoside was present at 40 times that of the polymer.

The partial methylation of a polynucleotide influences the stability of poly(I)·poly(C) complexes in a predictable fashion, *i.e.*, the stability is increased or decreased to a degree proportional to the 2′-*O*-methyl content and in the direction observed for the completely methylated polymer. These effects are only seen in these model compounds after a fairly high percentage of the ribose groups have been methylated. Lower levels of methylation more nearly like those found in natural RNA molecules may also affect the structure of these polymers, even though we do not see an alteration in the overall T_m.

These studies on 2′-O-methylated RNA molecules indicate that 2′-*O*-methyl groups can influence the tendency for a polynucleotide to form a complex with a complementary polymer. The means whereby 2′-*O*-methyl groups accomplish this will be of importance in understanding their biological role in natural RNA and the mechanism whereby the highly methylated model compounds function in biological systems as templates for polymerase and inducers of interferon.

A more complete explanation of the effect of 2′-alkylation on polymer structure will be possible when the positions of each of the polynucleotide moieties in three dimensional space are known. This will probably only be possible through X-ray analysis of the polymers and these studies are in progress.

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Conformational Changes of Transfer Ribonucleic Acid. The pH Phase Diagram under Acidic Conditions†

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ABSTRACT: We report measurements of the conformational behavior of $tRNA^{Tyr}$ (*Escherichia coli*) as a function of hydrogen ion concentration below pH 7. Thermal melting curves at 150 mM Na^+, without Mg^{2+}, were measured at several wavelengths, and the rate of conformational change following a sudden pH increase from 4.4 to 7 was measured as a function of temperature. We also measured spectral changes accompanying titration at constant temperature. We conclude that $tRNA^{Tyr}$ undergoes a conformational change centered around pH 5.5. The acid form contains some structural elements which must be dissociated before the native structure can be reformed at neutral pH, and it has greater proton affinity than the neutral form. It is only marginally less stable than the neutral form at pH 7 and therefore cannot be discounted as a possible functional intermediate. Addition of Mg^{2+} at acid pH alters the acid form but does not produce the native form.

Transfer RNA is frequently exposed to mildly acidic pH, one common reason being to stabilize the aminoacyl linkage against base-catalyzed hydrolysis. Little is known about the influence of pH on tRNA conformation, however. Our own interest in this problem began in the course of attempts to compare the melting of aminoacylated and uncharged tRNA, with the hope of detecting conformational or stability differences induced by aminoacylation. Retention of the aminoacyl linkage at elevated temperatures requires that one work at slightly acidic pH, and we soon discovered that there are dramatic differences between the thermal unfolding of tRNA in acid and neutral media.

In order to elucidate the effects of acid pH on tRNA conformation, we carried out a series of experiments closely analogous to those reported earlier (Cole *et al.*, 1972) concerning the influence of cation concentration on tRNA structure. The first step was to measure thermal denaturation profiles at varying pH. These revealed pH-dependent effects that indicated a conformational alteration of $tRNA^{Tyr}$ around pH 5.5. We then performed pH-jump kinetic experiments in which the pH was changed from 4.4 to 7 by mixing, and the optical change followed as a function of time. The results indicate a slow conformational change with substantial activation energy, analogous to the effects observed on increasing salt concentration at neutral pH (Cole *et al.*, 1972). The result of these experiments is a phase diagram that shows the dependence of conformational state on pH at constant counterion concentration.

The experiments reported here refer to $tRNA^{Tyr}$ from *Escherichia coli*, measured at 150 mM Na^+, generally in the absence of Mg^{2+}. We could not study systematically the effect of Mg^{2+} on thermal denaturation at acid pH, because acid catalysis of the destruction of 4-thiouridine (Lipsett, 1965) seems to be accelerated by Mg^{2+} (M. Bina-Stein, unpublished results), thus interfering with our spectral measurements at 335 nm. We did find, however, that tRNA exposed to acid pH in the presence of Mg^{2+} also showed a slow optical change on reneutralization, but the rate and activation energy for this reaction were affected by the presence of Mg^{2+}.

The reconversion of the acid conformation of $tRNA^{Tyr}$ to the native state under charging conditions is sufficiently rapid that we could not determine whether the acid form can be aminoacylated or not. We will later report measurements

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