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Association of Complementary Oligoribonucleotides in Aqueous Solution*

S. R. Jaskunas, Charles R. Cantor,† and I. Tinoco, Jr.

ABSTRACT: Oligoribonucleotide complexes are plausible models for codon–anticodon interactions and the double-strand regions in transfer ribonucleic acid. Several pairs of antiparallel complementary oligoribonucleotides have been mixed in a 1:1 mole ratio under conditions favorable for intermolecular association. The conditions were 0.01 M total residue concentration, 0.01 M $MgCl_2$, or 0.5 M NaCl, pH 7, and 1°. Optical rotatory dispersion has been used to detect the existence of a complex. Interaction between GpGpC and GpCpC has been observed.

The complex probably contains two molecules of GpGpC for every molecule of GpCpC. However, there are more than three trinucleoside disphosphates per complex. Other pairs of complementary oligo ribonucleotides were mixed under similar conditions, but no interaction was observed. These include ApC and GpU, ApCpU and ApGpU, ApGpC and GpCpU, and ApApApA and UpUpUpU. Self-aggregation has been observed with ApGpC and GpGpC. Aggregates of the latter compound contain up to 40 trinucleoside diphosphates/complex. Our results suggest that the ribosome or transfer ribonucleic acid structure must help, in some way, to stabilize complexes between the anticodon and messenger ribonucleic acid. Calculations of the stability of triple-strand regions in transfer ribonucleic acid like $(GpGpC)_2$:GpCpC indicate that such structures could exist.

A base-pairing arrangement resembling a cloverleaf has been suggested as a possible secondary structure for tRNA molecules (Holley *et al.*, 1965). In this model the polynucleotide chain folds back upon itself forming loops that are held together by A–U and G–C base pairs. Each molecule contains several loops, separated by short double-stranded regions.

The existence of base pairs in tRNA is supported by several different types of evidence (Englander and Englander, 1965; Felsenfeld and Sandeen, 1962; Cantor *et al.*, 1966). It is also known that the base pairs are organized into more than one distinct helical region (Fresco *et al.*, 1963; Felsenfeld and Cantoni, 1964). Recent small-angle X-ray (Lake and Beeman, 1967) and chemical studies (Brostoff and Ingram, 1967; Nelson *et al.*, 1967) are in general consistent with the cloverleaf model for tRNA. Perhaps the best evidence for this cloverleaf pattern of base pairs is the fact that this model can be constructed for every one of the tRNAs of known

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base sequence (Holley *et al.*, 1965; RajBhandary *et al.*, 1967; Madison *et al.*, 1966; Zachau *et al.*, 1966; Baev *et al.*, 1967). However, it is possible to draw other structures that are also similar for all tRNA molecules. In particular the dihydrouracil region and the base paring in the stem can be changed.

We are interested in finding more direct evidence for the existence of loops that are held together by the formation of only a few base pairs. In particular, are these short regions of base pairs sufficiently stable to endow the tRNA molecule with a unique secondary structure? This question could be tentatively answered if we knew the equilibrium constant for the association of complementary oligonucleotides. The stability of a loop can then be estimated by taking into account the effect of tying the oligomers together.

We are also interested in developing a method to determine precisely which residues are base paired to which other residues. Previous work has shown that the optical rotatory dispersion of molecules containing base pairs is sensitive to the number and sequence of residues that are base paired (Cantor *et al.*, 1966). This present work was undertaken with the intention of determining quantitatively the optical rotatory dispersion of base-paired RNA residues as a function of sequence.

The influence of sequence on the optical rotatory dispersion of single-strand residues depends mainly upon adjacent residues (Cantor *et al.*, 1966; Holcomb and Tinoco, 1965; Bush and Scheraga, 1967; Cantor and Tinoco, 1965). Thus, the optical rotatory dispersion of ApApU is the sum of the optical rotatory dispersion of ApA and ApU (Cantor and Tinoco, 1965). The optical rotatory dispersion of single-stranded RNA in solutions of low ionic strength has also been found to be the same as the sum of the optical rotatory dispersion of the dinucleoside phosphates (dimers) (Cantor *et al.*, 1966). We also expect mainly nearest-neighbor effects to be important for base-paired residues. The optical rotatory dispersion of a short helical region will thus be the sum of the optical rotatory dispersion of dimers of base pairs. In order to calculate quantitatively the optical rotatory dispersion of a double-strand region we must measure the optical rotatory dispersion of base-paired complexes of dinucleoside phosphates or longer complementary molecules of known sequence.

We have attempted to study complexes formed in aqueous solution containing dimers, trinucleoside diphosphates (trimers), or tetranucleoside triphosphates (tetramers). Regions of base pairs in tRNA in general are presumed to contain only Watson–Crick base pairs, A–U and G–C, which form between antiparallel chains (Fresco *et al.*, 1963; Holley *et al.*, 1965; RajBhandary *et al.*, 1967; Madison *et al.*, 1966; Zachau *et al.*, 1966). Thus, at present we have directed our attention to complexes of this type.

Complexes of trimers are especially interesting because of their close analogy to the source of specificity for the interaction of tRNA with the mRNA–ribosome complex. The specificity is believed to be the result of the base pairs that form between a specific triplet of the tRNA and a complementary triplet on the mRNA (Crick, 1966). If the ribosome does not in any way affect the binding of the tRNA to its codon then the equilibrium constant for a complex of complementary trimers should be the same as for the interaction of tRNA and mRNA. A site for the nonspecific binding of tRNA molecules to the ribosome has been frequently suggested (Kalafofsky and Nakamoto, 1966). Such binding would increase the equilibrium constant for the interaction of codon and anticodon as compared with trimers that are free in solution. However, it would not affect the specificity.

Optical rotatory dispersion has been used to detect the existence of complexes of complementary oligomers. The optical rotatory dispersion of each of the oligomers is first measured separately under identical conditions. The oligomers are mixed in a 1:1 mole ratio, and the optical rotatory dispersion of the mixture is compared with the sum of each measured separately. The change that will accompany base-pair formation can be estimated from the published optical rotatory dispersion of polyribonucleotide complexes (Cantor *et al.*, 1966). In general, the formation of base pairs will shift the peak; trough, and crossover to shorter wavelengths; for trimers this shift may be 5–10 mμ. The magnitude of the rotation at the peak should also increase. From these estimates we can expect to be able to detect 10% complex formation in a mixture of trimers.

Conditions have been chosen that will favor complex formation. These include low temperature, high concentration of nucleotide, and high ionic strength. Magnesium ions frequently result in the formation of triple strands (Lipsett *et al.*, 1961; Bautz and Bautz, 1964). Double strands are more likely to form in the absence of magnesium. The experiments to be described have generally been done in both the presence and absence of magnesium ions.

These same conditions are also optimum for self-aggregation. It is important to detect aggregates because they may prevent the formation of the complementary complex. Aggregates themselves are also interesting because they may be indicative of the formation of non-Watson–Crick base pairs. Self-aggregation can be detected in the usual way by studying the optical rotation of the oligomers as a function of concentration. More simply, we can compare the measured optical rotatory dispersion with the semiempirical calculation for an unaggregated oligomer (Cantor and Tinoco, 1965). Both methods have been employed.

Materials and Methods

Oligomers. The tetramers ApApApA and UpUpUpU were purchased from Miles Laboratories. A two-dimensional map, as described below, indicated that the compounds were homogeneous so they were used without further purification.

The dimers ApC and GpU that were used in optical studies were gifts from Dr. M. M. Warshaw.

A. Preparation from RNA by enzymatic hydrolysis. ApGpU was prepared as described previously (Cantor and Tinoco, 1965). GpGpC was prepared by a similar procedure that resulted in higher yields and greater convenience. This modified procedure is described below.

Escherichia coli RNA was prepared as described by Tissières *et al.* (1959). The RNA was primarily rRNA, but it contained some 4S RNA (S. Mandeles, personal communication). In a typical preparation, 300 mg of *E. coli* RNA (4 mg/ml) was incubated with 30 mg of Worthington pancreatic ribonuclease (code R) at 40°. The pH was adjusted to 7.5, and the reaction was followed to completion by the addition of 0.5 M KOH using a Radiometer TTT-1 titrimeter. At the completion of the reaction, 6 mg of Worthington *E. coli* alkaline phosphatase (code BAPC) was added. Incubation was continued for 3 hr at 37°. The solution was then lyophilized to dryness, redissolved in a small volume of 7 M urea–0.01 M Tris-Cl (pH 7.5), and then applied to a DEAE-Sephadex A-25 column (2.5 × 90 cm). The oligomers were separated according to chain length (Tomlinson and Tener, 1963) by elution with a gradient from 7 M urea–0.01 M Tris-Cl (pH 7.5) to 7 M urea–0.05 NaCl–0.01 M Tris-Cl (pH 7.5) in 15 l. The trimer fraction was diluted to five times its original volume with distilled water and washed onto a Dowex 1-X2 (Ag) column (0.5 × 80 cm). This column was washed with several column volumes of 10^{-3} M HCl. Then a pH gradient was run from 10^{-3} to 10^{-2} M HCl in 1 l. ApApC came off at the end of the gradient. The other trimers came off in a subsequent salt gradient from 10^{-2} to 10^{-2} M HCl–0.4 M NaCl, in 2 l. The order of appearance was the same as for the chromatography of trinucleotides by Rushizky and Sober (1964) on DEAE-cellulose. The trimers ApGpC and GpApC are not resolved but ApGpU and GpApU are.

The trinucleotide GpGpCp was prepared by a similar procedure as described above. The alkaline phosphatase treatment was not done. The pH gradient for the Dowex column was eliminated and the salt gradient for this column was done in 7 M urea at pH 3.

B. Desalting procedures. The salt was generally removed from the GpGpC fraction by the charcoal procedure of Mandeles and Kammen (1966). The salt-free solution was lyophilized to dryness and stored at −10° until needed.

On one occasion we used the Amicon ultrafiltration (Diaflo) apparatus with membrane UM-3 to desalt the GpGpC fraction. As judged by the index of refraction, the final solution was more salt free and had a lower ultraviolet blank than when the charcoal procedure was used (see Experimental Section on ultracentrifugation). However, the oligomer was partially hydrolyzed to monomers. The solution used in the ultracentrifugation experiment contained only trimer, however, since the smaller molecules passed through the membrane. Gillam *et al.* (1967) used the Amicon Diaflo apparatus with membrane UM-1 to desalt solution of tRNA. They apparently have no problem with hydrolysis. The difference between membrane UM-1 and membrane UM-3 is that the latter carries a net negative electrical charge while the former is neutral. It is possible that membrane UM-3 catalyzes the hydrolysis.

C. Preparation with polynucleotide phosphorylase. The trinucleoside disphosphates GpCpC, ApCpU, ApGpC, and GpCpU were prepared with primer-dependent polynucleotide phosphorylase from *Micrococcus lysodeikticus*. The enzyme was isolated according to the procedure of Singer and O'Brien (1963) through stage VII. This enzyme catalyzes a reaction between nucleoside diphosphates ppN and dimers LpM to give oligomers of the form $LpM(pN)_n$ (Leder *et al.*, 1965; Thach and Doty, 1966). We have used incubation conditions similar to those suggested by Thach (1966) for the synthesis of tri- and tetraoligomers. The reaction mixture contained 0.2 M glycine buffer (pH 9.3), 0.1 mM $CuSO_4$, 0.4 M NaCl, 10 mM dimer LpM, 10 mM magnesium acetate, 20 mM ribonucleotide diphosphate PPN, 0.02 mg/ml of BSA, and 120 μg/ml of polynucleotide phosphorylase, stage VII. It was incubated at 34 or 37° for 24 hr.

The trimer LpMpN was first separated from unreacted LpM and ppN and oligomers with chain length greater than 3 by paper chromatography on Whatmann No. 3MM paper with a solvent containing equal volumes of 95% ethanol and 1 M ammonium acetate (Thach and Doty, 1966). A total reaction volume of 0.400 ml was applied as a band 15 cm long. Developing times of 20 hr resulted in excellent resolution of all bands up to about the hexamer. Ammonium acetate was removed from the paper by soaking in absolute ethanol followed with absolute ether (Thach and Doty, 1966). After eluting the nucleotide with distilled water the volume was reduced to less than 50 μl by lyophilization.

The oligomer was further purified with a two-dimensional map on Whatmann No. 3MM paper. The first dimension was electrophoresis at 30 V/cm in 0.05 M formic acid at a pH between 2.4 and 3.5 for 2.5 hr. The pH was adjusted with concentrated ammonia to a value that gave maximum resolution of oligomers with various chain lengths. If pH 3.5 was used, the concentration of formate was reduced to 0.025 M to decrease the current and the spreading of spots due to heating. The second dimension was chromatography with a solvent containing *n*-propyl alcohol–water–concentrated ammonia in the volume ratio 55:35:10. The developing time was 20 hr. The trimer spot was eluted with water, lyophilized to dryness, and stored at −10° until needed. Frequently the two-dimensional map is not necessary except to reassure the investigator that he is working with a pure compound. The yield of GpCpC with respect to the amount of GpC used in the reaction mixture was 22%.

Optical Studies. A. Preparations of solutions. Lyophilized oligomers were dissolved with twice-distilled water and the concentration was adjusted so that the absorbance at 260 mμ was 200. To measure the concentration, 5 μl of the stock solution was diluted to 1.00 ml with a 0.1 ionic strength buffer; 5.55×10^{-3} M KH_2PO_4, 4.80×10^{-3} M $NaHPO_4$, and 0.080 M $KClO_4$ (pH 6.8). The concentration of solution was determined spectrophotometrically at room temperature from the following molar residue extinction coefficients at 260 mμ: ApC, 1.05×10^4 (Warshaw, 1965); GpU, 1.06×10^4 (Warshaw, 1965); GpGpC, 9.2×10^3 (Cantor and Tinoco, 1965); ApGpU, 1.13×10^4 (Cantor and Tinoco, 1965); ApCpU, 1.00×10^4; ApApApA, 1.24×10^4; UpUpUpU, 9.75×10^3; GpCpC, 8.10×10^3; ApGpC, 1.03×10^4; and GpCpU, 8.80×10^3. Except for those taken from

the literature, the extinction coefficients were calculated from the appropriate dimer data. In the case of ApApApA we used the ApApA data instead of dimer data (Cantor *et al.*, 1966; Cantor and Tinoco, 1965). We assumed that the extinction coefficient of GpGpCp is the same as for GpGpC.

The solutions for optical studies were prepared by diluting the oligomer stock solution with an equal volume of the appropriate concentrated buffer. The resulting solutions generally contained either 0.5 M NaCl–sodium phosphate buffer (pH 7.0) or 0.01 M $MgCl_2$–sodium phosphate buffer (pH 7.0). These buffers shall be referred to as the NaCl and $MgCl_2$ buffers, respectively. In some instances slightly different buffers have been used. These will be indicated in the text. The pH of the solution containing the oligomers is assumed to be that of a solution prepared by diluting the concentrated buffer with an equal volume of twice-distilled water. The buffering capacity of every buffer is sufficiently high to ensure that this is a good assumption.

The optical density of each buffered nucleotide solution was about 100 at 260 mμ. The optical rotatory dispersion and ultraviolet spectrum of each solution was measured with a 0.125-mm path-length, 10-mm diameter quartz cell obtained from Opticell (Brentwood, Md.) and a specially designed cell holder. The volume of solution needed for each measurement was less than 7 μl. The path length of the cell was determined spectrophotometrically with potassium chromate solution in 0.05 M KOH. Extinction coefficients for dilute solutions were obtained from Meites (1963) and Beer's law was assumed to hold over a 100-fold range of concentration.

B. MEASUREMENTS. All ultraviolet spectra were measured with a Cary Model 14 or 15 spectrophotometer. Optical rotation was measured with a Cary Model 60 spectropolarimeter.

Thermal stability was maintained by circulating an aqueous ethylene glycol solution through a specially designed cell block. Above room temperature, a Haake F bath was used to control the temperature of the circulating solution. A low-temperature bath, designed by Dr. M. M. Warshaw, was used for temperatures below 25°.

Noise in the optical rotation signal was reduced by applying the smoothing procedures described by Savitzky and Golay (1964). The wavelength and pen position of the spectropolarimeter were recorded every 0.5 mμ on paper tape by a Datex analog to digital converter. A least-squares fit to a 25 point cubic function was done with the coefficients of Savitzky and Golay (1964). Recently the Datex has been replaced with a Digital PDP 8/S computer. In this case the computer finds the arithmetic average of 150 points taken every 0.5 mμ. These data points are subsequently used in a Savitzky and Golay 13 point smoothing program for a cubic function. The latter two computer programs were written by Dr. M. Itzkowitz and Mr. B. Tomlinson, respectively.

Optical rotation data are expressed as molar rotation per nucleoside: $[\phi] = 100\ \theta/cl$, where θ is the measured rotation in degrees, l is the path length in centimeters, and c is the concentration in moles of mononucleosides (residues) per liter.

C. ULTRACENTRIFUGATION. All centrifuge experiments were done at 2° with a Spinco Model E ultracentrifuge equipped with an electronic speed controller designed by Hearst and Gray (personal communication, 1967). Schlieren optics were used. Data for equilibrium experiments were taken 24–36 hr after the start of the run. There was generally no difference from data taken 12 hr after the start of the run. Methods I and II of Van Holde and Baldwin (1958) were used to obtain weight-average and z-average molecular weights. The refractive increment was determined from the area of the schlieren pattern which forms at the boundary between buffer and nucleotide solution. A double-sector Kel-F cell was used to establish this artificial boundary.

The refractive increment was determined for a solution of poly A in the NaCl buffer. The salt concentration of the poly A solution was adjusted by dialysis against buffer. There was excellent agreement with the refractive increment of one 2:1 mixture of GpGpC and GpGpC in the same buffer. Other solutions of GpGpC and mixtures of GpGpC and GpGpC had refractive increments that were higher. The GpGpC in the solution with the same refractive increment as poly A had been desalted with the Amicon Diaflo apparatus. The samples of GpGpC in the other solutions had been desalted with the charcoal procedure. The greater refractive increments for these solutions is probably the result of incomplete desalting. The residual salt in these solutions will not effect the equilibrium experiments, however, since the gradient will be negligible.

Results

Evidence for Association. The following pairs of complementary oligoribonucleotides have been mixed in a 1:1 mole ratio: ApC and GpC, ApCpU and ApGpU, ApGpC and GpCpU, GpGpC and GpCpC, GpGpCp and GpCpC, and ApApApA and UpUpUpU. The total concentration of mononucleoside residues was between 5 and 10 mM in each case. The solutions were buffered at pH 7 and contained either 0.5 M NaCl or 0.01 M $MgCl_2$. The optical rotatory dispersion of each of the solutions was measured at 1 or 2° and compared with the average optical rotatory dispersion of the oligomers measured separately under the same conditions. A significant difference between the two curves was taken as evidence that specific intermolecular association occurs in the mixture. The experiment was also done at 26° in several instances. The results are summarized in Table I. Also included is whether any evidence was found for the self-aggregation of each oligomer.

Specific interaction between GpGpC and GpCpC is observed. There is no evidence of specific interaction between any of the other pairs of complementary oligomers. However, there are indications of self-aggregation in solutions containing GpGpC, GpGpCp, and ApGpC.

The evidence for these qualitative conclusions will be reviewed in greater detail in the following paragraphs. We will then return to the interaction between GpGpC and GpCpC and investigate the nature of the complex.

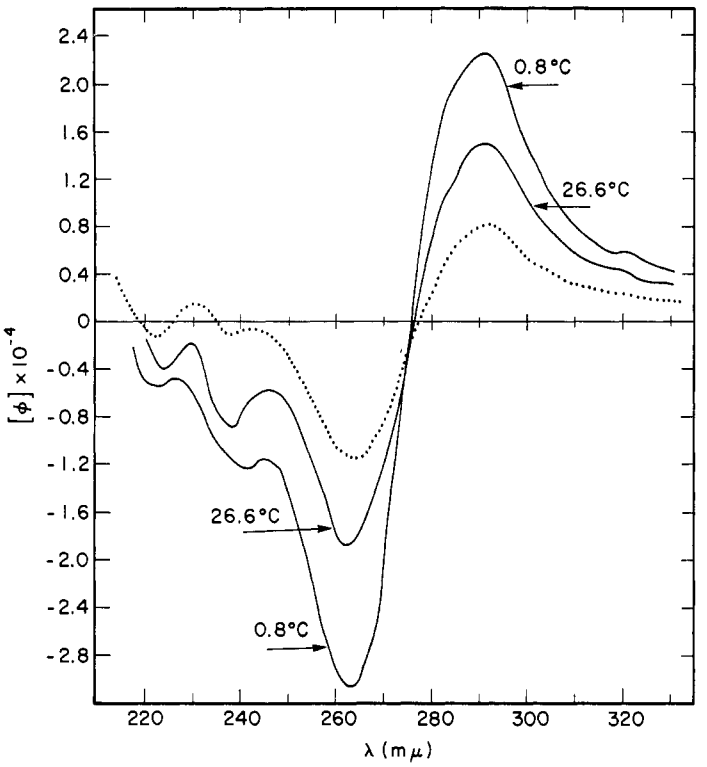


FIGURE 1: Optical rotatory dispersion of ApGpC. (———) Experiment, 0.01 M $MgCl_2$, 0.1 ionic strength sodium phosphate buffer (pH 7.0), 0.8 and 26.6°, 8.23×10^{-3} M mononucleosides. (· · · · · ·) Experimental optical rotatory dispersion of ApGpCp, 0.1 ionic strength sodium phosphate buffer (pH 7.0), 26°, 10^{-4} M mononucleosides (Cantor and Tinoco, 1967).

TABLE 1: Association of Oligoribonucleotides[a] at pH 7.

	0.5 M NaCl		0.01 M $MgCl_2$	
	1°	26°	1°	26°
Dinucleoside Phosphates				
ApC	No	No		
GpU	No	No		
ApC + GpU	No	No	No[b]	
Trinucleoside Diphosphates				
ApCpU	No		No	
ApGpU	No		No	
ApCpU + ApGpU	No		No	
ApGpC			Yes	Yes
GpCpU			No	No
ApGpC + GpCpU			Slight	No
GpGpC	Yes	Yes	Yes	Yes
GpCpC	No	No	No	No
GpGpC + GpCpC	Yes	Yes	Yes	Yes
GpGpCp	Yes	Yes		
GpGpCp + GpCpC	Yes	Slight	Slight	
Tetranucleoside Triphosphates				
ApApApA	No	No	No	No
UpUpUpU	No	No	No	No
ApApApA + UpUpUpU	No	No	No	No

[a] Total concentration of residues between 5 and 10 mM. Yes and No indicate whether any evidence for aggregation was found. A blank indicates that no aggregation was looked for under these conditions. [b] Concentration of $MgCl_2$ in this case was 0.05 M.

A. ApC + GpU. The optical rotatory dispersion and ultraviolet spectra of ApC, GpU, and 1:1 mixtures have been measured at 2° at three different total residue concentrations, 5×10^{-3}, 5×10^{-4}, and 5×10^{-5} M. The ultraviolet spectra of these solutions were also measured at 26°. The optical rotation and absorption spectra that should be observed for the mixture if there is no interaction were calculated from the spectrum of each component measured separately at the same total concentration of mononucleosides. The experimental curves for the mixtures agreed closely with the calculated curve at all concentrations.

Furthermore, the optical rotatory dispersion of each component and each mixture did not show any systematic variation with changes in concentration. However, the residue extinction coefficient at room temperature decreased 3% for GpU, 3.5% for ApC, and 4% for the 1:1 mixture as the concentration was increased from 5×10^{-5} to 5×10^{-3} M nucleoside. This may represent some kind of random aggregation whose contribution to the optical rotatory dispersion is small. Stacked dimers in which there is no preferential orientation of one dimer above another might have a small effect on the optical rotatory dispersion and a significant hypochromic effect. In any case, there is not interaction between ApC and GpC under our experimental conditions that significantly affects the optical rotatory dispersion or ultraviolet spectrum.

If either of the dimers is aggregated throughout the concentration range, then specific interaction may not be possible. It has been shown by Warshaw and Tinoco (1965) that GpC is not aggregated at a mononucleotide concentration of 10^{-4} M. Thus, it seems highly probable that ApC and GpU are not aggregated at this concentration either.

B. ApCpU + ApGpU. There is no indication of interaction between ApCpU and ApGpU at 1° in either the NaCl or $MgCl_2$ buffer. The optical rotatory dispersion of the equimolar mixture is identical, within the experimental uncertainty, to the average of each trimer measured separately.

There is also no indication of any self-aggregation in the solutions of either ApCpU or ApGpU. The optical rotatory dispersion of both of these compounds is the same as has been previously reported for solutions with a concentration of mononucleosides a factor of 100 smaller than what we used (Cantor and Tinoco, 1965, 1967).

C. ApGpC + GpCpU. Some aggregation probably occurs in equimolar mixtures of ApGpC and GpCpU at 1° in the presence of magnesium ions. The crossover in the optical rotatory dispersion of the mixture occurs 2 mμ to the blue of the crossover that is calculated for

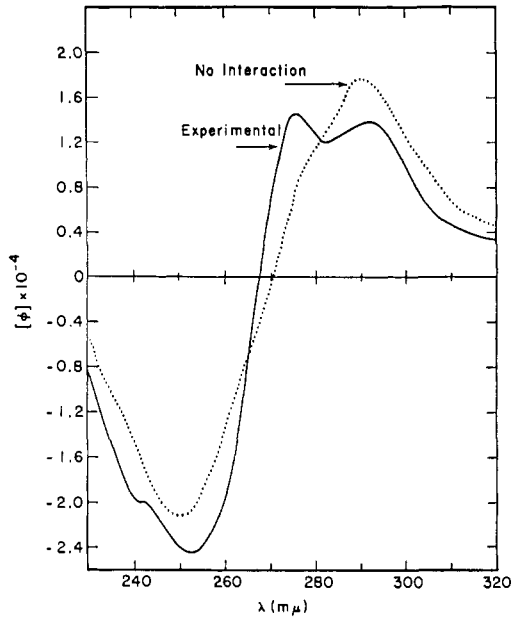


FIGURE 2: Optical rotatory dispersion of a 1:1 mixture of GpGpC and GpCpC. (———) Experiment, 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7.0), 1°, 8.1 × 10^{-3} M mononucleosides. (· · · · · ·) Average optical rotatory dispersion of GpGpC and GpCpC measured separately under the same conditions.

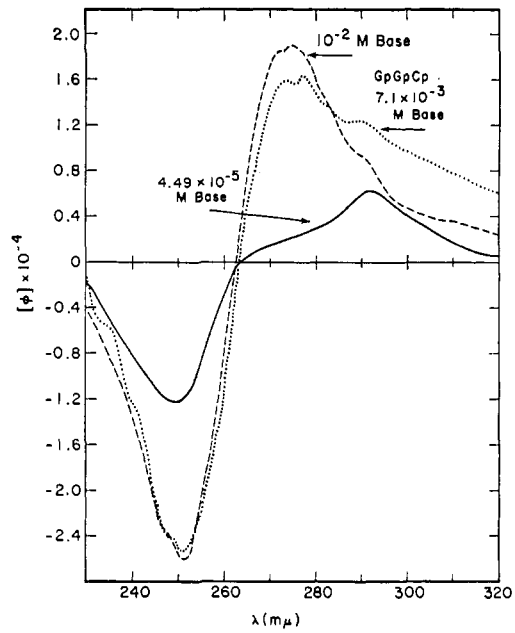


FIGURE 3: Optical rotatory dispersion of GpGpC. (———) 4.49 × 10^{-5} M mononucleosides, 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7.0), 1°. (- - - -) 1.00 × 10^{-2} M mononucleosides, 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7.0), 1°. (· · · · · ·) Optical rotatory dispersion of GpGpCp, 7.1 × 10^{-3} M mononucleosides, 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7.0), 1°

no interaction. This does not happen at 26°. Although the difference between the calculated and experimental curves at 1° is small, it is significant and in the expected direction. However, it is too small to be useful for further studies.

The situation is further complicated by the self-aggregation of ApGpC under these conditions. The optical rotatory dispersion of ApGpC in the $MgCl_2$ buffer at +0.8 and 26.6° is compared in Figure 1 with the optical rotatory dispersion of a more dilute solution of ApGpCp at 26° (Cantor and Tinoco, 1967). The concentration of residues in the dilute solution is a factor of 100 smaller than that of the concentrated solution. The peaks, troughs, and crossovers of the three curves occur at nearly the same wavelengths. However, the magnitudes of the rotation at the peak and trough for the concentrated solution are much larger than that of the dilute solution. We did not have enough material to measure the optical rotatory dispersion of ApGpC at dilute concentrations. We do not expect a 3′-terminal phosphate to affect stacking. Therefore, the optical rotatory dispersion of dilute solutions of ApGpCp should be a good measure of the optical rotatory dispersion of unaggregated ApGpC. The concentrated and dilute solutions also differ in that the concentrated solution contains $MgCl_2$. The presence of this salt should not affect the optical rotatory dispersion of unaggregated oligomers except in its ability to promote aggregation. Therefore, the larger magnitudes of rotation for concentrated solutions of ApGpC compared with dilute solutions of ApGpCp must be because of self-aggregation.

In contrast to the case of ApGpC, the optical rotatory dispersion of GpCpU under the same conditions is very similar to the curve calculated from the dimers (Cantor and Tinoco, 1965). There is probably no self-aggregation. The difference between the extent of aggregation of these two trimers may be a reflection of the different degree of stacking of A and U bases in oligomers (Warshaw and Tinoco, 1965).

The extent of specific association in solutions containing both ApGpC and GpCpU may not be accurately reflected in the difference between the experimental and calculated optical rotatory dispersion of the mixture. The difference will be small if the optical rotatory dispersion of the 1:1 complex is similar to the optical rotatory dispersion of the ApGpC aggregate. Furthermore, the concentration of ApGpC in the mixture is one-half the concentration in the solutions used to measure its optical rotatory dispersion. Thus, there may be less aggregation of ApGpC in the mixture.

D. GpGpC + GpCpC. A complex between GpGpC and GpCpC is formed in either the NaCl or $MgCl_2$ buffer at 1°. The optical rotatory dispersion of a 1:1 mixture is compared in Figure 2 with the average optical rotatory dispersion of the two oligomers measured separately in the same buffer. A second long-wavelength peak occurs for the mixture and the crossover is blue shifted 3 mμ. A similar but smaller difference between experiment and calculation is obtained at 26° in each buffer.

The complex between GpGpC and GpCpC forms despite the self-aggregation of GpGpC. Optical rotatory dispersion curves for dilute and concentrated solutions of GpGpC alone are shown in Figure 3. The magnitudes of the rotation at the peak and trough are greater for the more concentrated solution. This is clearly in-

dicative of some sort of association of GpGpC with itself. The aggregation also has a hypochromic effect on the absorption spectrum of GpGpC. The extinction coefficient at 260 mμ increases from 7950 to 9200 at 1° in the $MgCl_2$ buffer as the concentration is decreased from 10^{-2} to 10^{-4} M.

Equilibrium in concentrated GpGpC solution is reached in less than 1 hr after the temperature is changed by as much as 25°. This association and disassociation is faster than found for G oligomers by Lipsett (1964a) and for GpGpGpU and GpGpU by Dr. S. Podder in this laboratory (personal communication).

E. ApApApA + UpUpUpU. There is no evidence that a complex forms between ApApApA and UpUpUpU in either the NaCl or $MgCl_3$ buffer at 1°. The optical rotatory dispersion of a 1:1 mixture, 1.0×10^{-2} M total nucleoside concentration, is identical with the average of each measured separately. Poly U is known to aggregate under these experimental conditions (Lipsett, 1960); but our results show that UpUpUpU does not. Miles *et al.*[1] have obtained infrared evidence for a complex between UpUpUpU and ApApApA and between UpUpU and ApApA. The stoichiometry in both cases is 2U:1A. Their experiments were done at a tenfold higher concentration of nucleosides than described above. They also used a higher concentration $MgCl_2$. These differences presumably account for out inability to detect a complex between UpUpUpU and ApApApA

F. Summary. The aggregation results summarized in Table I apply only to our conditions. At higher concentrations all the oligonucleotides will aggregate. Furthermore, there is some evidence for slight aggregation even for those oligomers which are designated as "NO."

The Nature of the Complex between GpGpC and GpCpC. A. Stoichiometry. The ratio of two species in a complex can be determined by the continuous variation method of Job (1928; Felsenfeld and Rich, 1957). One measures some property of mixtures containing various ratios of the two reactants at a constant total concentration. The property must be proportional to the concentrations of the reactants and product. The difference, Y, between the property of the mixtures and that calculated for no interaction is plotted *vs.* the mole fraction of one of the reactants. A maximum or minimum in Y corresponds to a maximum in the concentration of the complex. As a result, Job (1928) has shown that the ratio of the reactants in the complex can be determined solely from the mole fraction at which there is an extremum.

The continuous variation method was only justified by Job (1928) for the case where there is a single reaction between two compounds. He did not consider the possibility that the reactants self-associate as well as react to form a complex. In our system, there is aggregation of GpGpC with itself as well as specific interaction between GpGpC and GpCpC. It will be shown later that GpCpC also interacts with itself in our system. The extent of this self-association is much less, however, than for GpGpC. In view of these self-association phenomena it is not obvious that the continuous variation method can be used to determine the ratio, r, of GpGpC to GpCpC in the complex. In order to use the method we must find conditions where an extremum in Y corresponds to a maximum concentration of the complex. Then the relation between r and the mole fraction at which the extremum occurs can be derived.

Given solutions of GpCpC and GpGpC, both at a concentration of C moles of residue per liter, we shall mix x liters of GpCpC and $(1 - x)$ liters of GpGpC, where $x < 1$, and measure the optical rotation of the mixtures. Y shall be the difference between the measured molar rotation per residue and that calculated for no interaction. The condition for an extremum in Y is that $dY/dx = 0$. The following relation can be derived (Vosburgh and Cooper, 1941).

$$\frac{dY}{dx} = \frac{1}{C}\left\{\frac{dC_m}{dx}([\phi](GGC)_m - [\phi](GGC)) + \frac{dC_n}{dx}([\phi](GCC)_n - [\phi](GCC)) + \frac{dC_r}{dx}\left([\phi](rGGC:GCC) - r\frac{[\phi](GCC)}{r+1} - \frac{[\phi](GGC)}{r+1}\right)\right\}$$

The molar rotation per residue of free GpGpC, free GpCpC, the complex, the self-association product of GpGpC, and the self-association product of GpCpC are $[\phi]$ (GGC), $[\phi]$ (GCC), $[\phi]$ (rGGC:GCC), $[\phi](GGC)_m$, and $[\phi]$ $(GCC)_n$. The concentrations C_r, C_m, and C_n refer to the complex, the self-association of GpGpC, and the self-association of GpCpC.

The optical rotation of the GpCpC solutions did not reveal the aggregation, therefore $[\phi](GCC)_n = [\phi](GCC)$. Thus, the extremum in Y will occur at the maximum in the concentration of the complex if $[\phi](GGC)_m = [\phi]$-(GGC). Alternatively, if a wavelength can be found where $[\phi](GGC)_m - [\phi](GGC) = [\phi](rGGC:GCC) - (r/(r+1))[\phi](GCC) - (1/(r+1))[\phi](GCC)$, then the condition for a Y extremum is $0 = (dC_r/dx) + (dC_m/dx)$. An extremum in Y will correspond approximately to a maximum concentration of a strong complex where C_m and dC_m/dx are small.

We must now find the relationship between r and the value of x where there is an extremum in Y. Knowing that $dC_r/dx = 0$, it can be shown (Vosburgh and Cooper, 1941) that

$$r = \frac{C(1-x) + C_m(m-1)}{Cx + C_n(n-1)}$$

The number of molecules of GpGpC and GpCpC in their respective aggregates is m and n. The extent of aggregation in GpCpC solutions is much less than for GpGpC solutions. We shall assume the major effect of the self-association phenomena on r will come from the aggregation of GpGpC and that $C_n = 0$. Thus

$$r = \frac{(1-x)}{x} + \frac{C_m(m-1)}{Cx}$$

If $C_m = 0$, we have the relationship found by Job (1928).

[1] This is quoted in Felsenfeld and Miles (1967).

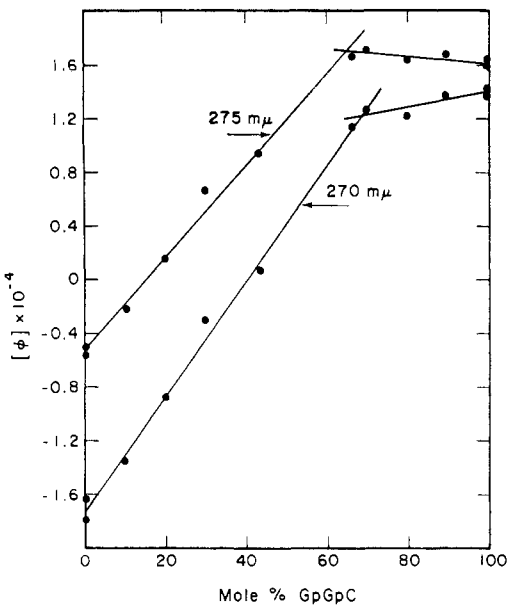


FIGURE 4: Continuous variation experiment for mixtures of GpGpC and GpCpC. The optical rotation spectra of mixtures of GpGpC and GpCpC were measured at a constant total mononucleoside concentration, 8.9×10^{-3} M, but varying ratios of the two trimers. 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7), 1°.

The mixing experiment has been performed in each buffer at 1° and a constant total mononucleoside concentration of approximately 10^{-2} M. The results are identical. At 262.5 mμ, $[\phi](GGC)_m = [\phi](GGC)$. Y is so small at this wavelength, however, that it is not possible to determine an x at which there is an extremum.

At 275 mμ, $[\phi](GCC) = 0$ and $[\phi](r GGC{:}GCC) \simeq [\phi](GGC)_m$. The latter approximate equality can be seen in Figure 4 where molar rotation at 270 and 275 mμ is plotted as a function of the mole fraction of GpGpC. Separate lines are drawn through points for solutions containing an excess of each of the components. The lines intersect at points that should correspond to the rotation of the pure complex at that wavelength. The molar rotation of the complex at 275 mμ is the same as for a solution that only contains GpGpC. Therefore, an extremum in Y at this wavelength will correspond approximately to a maximum concentration of the complex if it is sufficiently strong so that C_m and dC_m/dx are small.

For the experiment shown in Figure 4 a maximum in Y occurs for 275 mμ at 63 ± 4% mole fraction GpGpC. Data for wavelengths 270 and 277 mμ also indicate the maximum occurs between 59 and 67% GpGpC. The position of the maximum when the experiment is done with the $MgCl_2$ buffer is the same. The actual rotation of a solution containing 2:1 ratio of GpGpC to GpCpC is close to that expected for 100% complex formation. The scatter in the data is such, however, that we cannot say there is pure complex formed under these conditions. We estimate that 75 ± 20% of the trimers is in the GpGpC:GpCpC complex for both the NaCl and $MgCl_2$ buffer. The molecular weight determinations, to be described later, also indicate the concentration of the GpGpC aggregate is significantly reduced under conditions yielding a maximum in Y. Therefore, we shall assume that a maximum in the concentration of the complex occurs approximately at 63% mole fraction GpGpC.

To estimate r we need to know x, C, C_m, and m for the conditions yielding a maximum in Y. The total concentration for the experiment, shown in Figure 4, was 8.9×10^{-3} M mononucleosides and $x = 0.37$. The initial concentration of GpGpC resulting in a maximum in Y was 0.63 times the total concentration and only one-fourth of that was not in the complex with GpCpC. The fraction of that actually complexed with itself can be estimated using an equilibrium constant for the aggregation of GpGpC. The model for calculating the equilibrium constant will be described in the Discussion. A measure of the extent of aggregation is the parameter, $p (p \leq 1)$, which is defined in the Discussion. It can be shown (Flory, 1953) that the fraction of GpGpC molecules aggregated is $(2p - p^2)$. Using a p calculated from eq 5 of the Discussion and an equilibrium constant for the self-aggregation of GpGpC in the NaCl buffer, we estimate that 82% of GpGpC not complexed with GpCpC is aggregated with itself. The number-average degree of polymerization calculated from this number is 2.4. Thus, we have taken 2.4 for the value of m. The value of r calculated from these numbers is 2.2. An equilibrium constant for the self-aggregation of GpGpC in the $MgCl_2$ buffer can be calculated in a similar fashion to that described in the Discussion for the NaCl buffer. If we use that equilibrium constant and take 75% for the fraction of trimers in the GpGpC:GpCpC complex then r is 3.0. It seems reasonable to expect, however, that the fraction of trimers in the GpGpC:GpCpC complex is greater for the $MgCl_2$ buffer than the NaCl buffer. If the fraction is 90% instead of 75% then r is 2.0. Therefore, the specific complex between GpGpC and GpCpC in either buffer probably contains 2 moles of GpGpC for every mole of GpCpC.

B. MOLECULAR WEIGHT. The number of trimers in the complex of GpGpC and GpCpC can be determined from its molecular weight. The weight- and z-average molecular weights of the species present in a 2:1 mixture of GpGpC and GpCpC in both the NaCl and $MgCl_2$ buffers at 2° have been determined by equilibrium sedimentation. The same molecular weight averages have been determined for the GpGpC aggregate in both buffers and for GpCpC in the NaCl buffer. The results are given in Table II.

The z- and weight-average molecular weights of unaggregated GpCpC should be 891. For the pure GpCpC solutions, they are close to this. Some aggregation does occur, however. Neither the absorption spectrum nor the optical rotatory dispersion shows any evidence of aggregation. Thus, the aggregation is probably nonspecific.

Solutions of GpGpC in the two buffers are optically quite similar. However, magnesium ions more effectively stabilize the aggregation as seen by the higher molecular weights in the magnesium buffer. The molecular weight of the trimer is 931. Thus, there are aggregates of more than 40 trimers in the solution containing magnesium.

TABLE II: Molecular Weights[a] by Sedimentation Equilibrium.

		0.5 M NaCl			0.01 M $MgCl_2$		
		0.2 Ionic Strength Phosphate Buffer (pH 7.0)			0.1 Ionic Strength Phosphate Buffer (pH 7.0)		
	Sum of Atomic Wt	Bulk Residue Concn	M_z	M_w	Bulk Residue Concn	M_z	M_w
GpCpC	891	0.96×10^{-2}	2,400	1,200			
GpGpC	931	1.27×10^{-2}	14,200	10,200	0.93×10^{-2}	40,000	15,000
GpGpC + GpCpC (2:1)	2,753	1.09×10^{-2}	9,500	6,500	0.85×10^{-2}	16,700	8,700

[a] The estimated uncertainty is ±5%.

TABLE III: Molecular Weight of the Complex 2GpGpC:1GpCpC.

Fraction of Oligomers in Complex (f)	0.5 M NaCl–0.1 M PO_4^{3-} (pH 7.0)		0.1 M $MgCl_2$–0.05 M PO_4^{3-} (pH 7.0)	
	Mol Wt of Complex	n^a	Mol Wt of Complex	n^a
3/4	6200	6.8	8100	8.8
3/4[b]	7100	7.7		
0.33–0.95	5000–6500	5.4–7.1	5100–8700	5.6–9.4

[a] Numbers of trimers in complex. [b] Taking dilution of GpGpC into account (see text).

The molecular weights of the aggregates in the solutions containing 2:1 mixtures of GpGpC and GpCpC are less than those for the pure GpGpC solutions. However, they are greater than 2750, which is the molecular weight of a of a complex containing two molecules of GpGpC and one molecule of GpCpC. This is not surprising since the mixing experiments indicate that the fraction of trimers in the 2:1 complex is less than 1. The species present in these solutions include a 2:1 complex between GpGpC and GpCpC, GpGpC aggregate, and free GpCpC. Thus, the molecular weight of the mixture is the average for these different species.

From the known weight-average molecular weights of GpCpC, GpGpC, and the mixture of all three species, we can calculate the molecular weight of the 2:1 complex. We shall use the weight-average molecular weights given in Table II. The weight-average molecular weight of GpCpC was only determined for the NaCl buffer. There is no optical evidence for more aggregation in the $MgCl_2$ buffer, so we shall assume the same weight-average molecular weight for this buffer. In the mixture of GpGpC and GpCpC, the weight-average molecular weight of the GpGpC aggregate is smaller than for pure GpGpC solutions because the concentration of molecules capable of aggregating is decreased. Therefore, the molecular weight we will obtain for the 2:1 complex will be a lower limit.

The weight fraction, W_i, of a particular species can be calculated from the mole fraction, X_i, of trimer in that species, $W_i = X_i(M_i/M)$. X_i is the number of trimers in the ith species divided by the total number of trimers. The molecular weight of the ith species per trimer is M_i and the number-average molecular weight of a trimer in the solution is M. In the 2:1 mixture of GpGpC and GpCpC the molecular weight of the average trimer and the molecular weight of the 2:1 complex per trimer is 918. The fraction of molecules in the 2:1 complex was estimated from the continuous variation experiment to be 0.75 ± 0.20. The fractions of GpGpC and GpCpC trimers not complexed with one another are two-thirds and one-third of 0.25. The ratio M_i/M is 1.0, 0.97, and 1.01 for the 2:1 complex, GpCpC, and GpGpC. Therefore, the weight fractions are 0.75, 0.17, and 0.08, respectively.

The molecular weight of the 2:1 complex has been calculated with these values of the weight fractions for both buffers. The results are shown in Table III. From these molecular weights and the molecular weight of the average trimer in the 2:1 complex, 918, we can calculate the number of trimers in the complex. These numbers are also given in Table III. There are more than three trimers per complex in either buffer. However, the numbers are not a multiple of three. Therefore, the complex is not a discrete entity, and the molecular weights given in Table II are weight-average molecular weights.

The calculations have also been done for other values of f. For the NaCl buffer, the number of trimers in the complex varies from 5.4 to 7.1 for f between 0.33 and

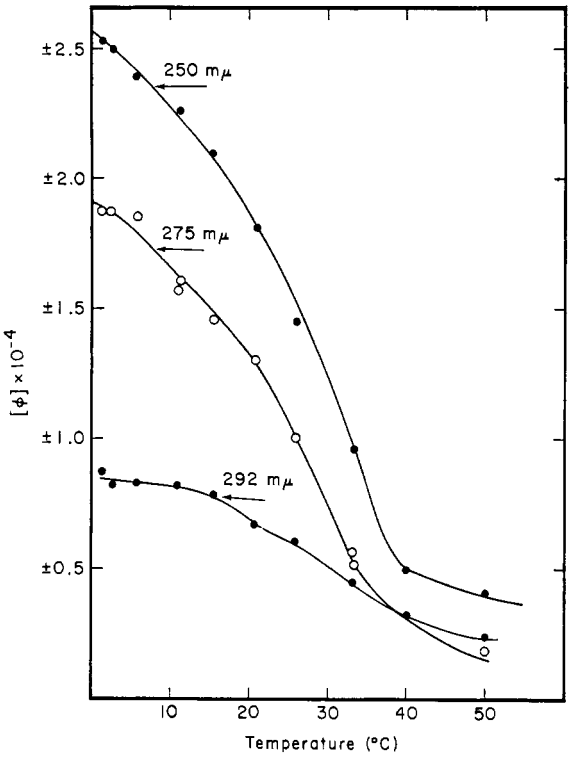


FIGURE 5: Molar rotation of GpGpC, 1.04 M mononucleosides, at 250, 275, and 292 mμ as a function of temperature. 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7), 1°.

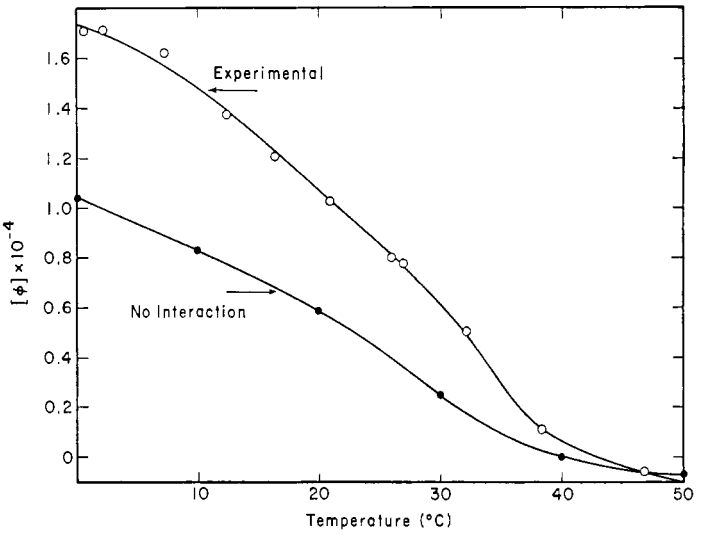


FIGURE 6: Molar rotation of 2:1 mixture of GpGpC and GpCpC, 1.14×10^{-2} M, at 275 mμ as function of temperature and (2:1) average molar rotation at 275 mμ of GpGpC and GpCpC measured separately as a function of temperature under the same conditions. 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7), λ 275 mμ.

0.95. Comparable results are obtained for the $MgCl_2$ buffer for the same range of f. This range of f is greater than the uncertainty in the determination of f. Thus, there does not seem to be any doubt that the complex contains more than two molecules of GpGpC and one molecule of GpCpC.

We have calculated the molecular weight of the 2:1 complex taking the dilution of GpGpC molecules capable of aggregating into account through the use of an approximate equilibrium constant for the self-aggregation of GpGpC. The determination of the equilibrium constant will be described in the Discussion. As expected, the molecular weight of the complex is greater than when the dilution is not considered. The results are given in Table III.

C. TEMPERATURE DEPENDENCE. The extent of aggregation of GpGpC and in solutions containing the 2:1 complex decreases with increasing temperature. The optical rotatory dispersion of GpGpC corresponds to that found in dilute solutions only above 40°. The rotation at several wavelengths is plotted in Figure 5 as a function of temperature. The hypochromic effect of the aggregation is also still pronounced at 26°.

The molar rotation at 275 mμ of the 2:1 complex in the NaCl buffer is shown in Figure 6 as a function of temperature. Also shown is the temperature dependence of the appropriate average of the molar rotation of GpGpC and GpCpC measured separately in the same buffer. The complex is completely melted out only at 50°. Similar curves are obtained at 270 mμ. There is no evidence of a biphasic transition in the 2:1 mixture of GpGpC and GpCpC at either 275 or 270 mμ.

D. pH DEPENDENCE. The rotation of a 2:1 mixture of GpGpC and GpCpC in the NaCl buffer has been followed as a function of pH between 5.8 and 8.0. No change occurs between 7.0 and 8.0. Below 7.0 the rotation at 275 and 270 mμ decreases. The optical rotary dispersions of GpGpC and especially GpCpC are sensitive to pH changes in this region. The change in the calculated optical rotatory dispersion for no interaction partially parallels the changes for the 2:1 complex. However, the fraction of trimers in the complex decreases from 0.75 at pH 7.0 to 0.31 at pH 5.8.

The cytosine residues are probably titrating in this pH region; the pK_a of Cp is 4.2 (Steiner and Beers, 1961). This pK_a is shifted up to 5.7 in poly C due to the stable double-stranded helix that forms when one-half the residues are protonated (Hartman and Rich, 1965). A similar phenomenon might occur for GpCpC. However, there is no indication that a complex is formed between pH 5.8 and 8.0. The fraction of Cp protonated at pH 5.8 is only 5%. The fraction protonated in GpCpC solutions appears to be greater. The pK_a of C in GpCpC is apparently intermediate between that for Cp and poly C. The important observation from the pH studies is that the 2:1 complex is destabilized by decreasing the pH.

E. IONIC STRENGTH DEPENDENCE. The optical rotatory dispersion of a 2:1 mixture of GpGpC and GpCpC was measured at 1° in a buffer that contained only a 0.1 M phosphate buffer (pH 7.0) and no additional salt. As measured by the magnitude of the rotation at the peak, the aggregation of GpGpC is less in this buffer than in a solution containing 0.5 M NaCl or 0.01 M $MgCl_2$. The fraction of trimers in the GpGpC:GpCpC complex is also less than for a solution that contains salt. However, the positions of the peaks, troughs, and crossovers in

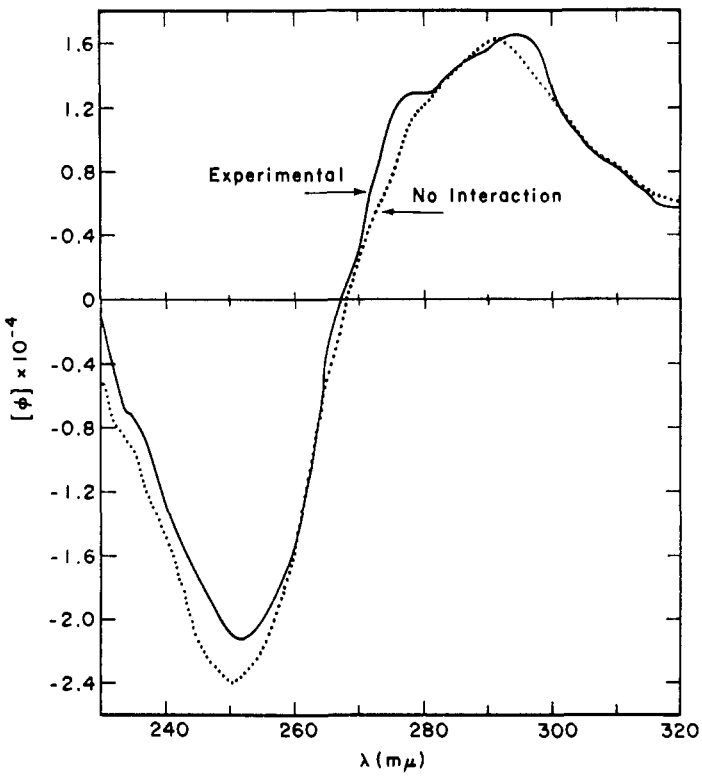


FIGURE 7: Optical rotatory dispersion of 2:1 mixture of GpGpCp and GpCpC. (———) Experiment, 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7.0), 1°, 7.5 × 10^{-3} M mononucleosides. (· · · · · ·) Average optical rotatory dispersion of GpGpCp and GpCpC measured separately under the same conditions.

the optical rotatory dispersion curves are the same Thus, we infer that the 2:1 complex is present at this lower ionic strength, but the fraction of oligomer in the complex is less.

F. EFFECT OF SUBSTITUTING GpGpCp FOR GpGpC. The presence of a 3′-phosphate on GpGpC decreases the fraction of trimers in the 2:1 complex to less than one-half of what it is with the dephosphorylated compound. The optical rotatory dispersion of a 2:1 mixture of GpGpCp and GpCpC is compared in Figure 7 with the calculated curve for no interaction. The shoulder at 275 mμ for the mixture implies that some complex is present. The fraction of trimers in the complex is 30% if it is the same 2:1 complex as found previously, *i.e.*, the same rotation for 100% complex applies here. However, the complex might contain a 1:1 ratio of GpGpCp to GpCpC. Unfortunately the difference between the two curves at any wavelengths in Figure 7 is too small to be useful in a mixing experiment.

The rotation of GpGpCp is slightly less than for GpGpC. An optical rotatory dispersion curve for GpGpCp in the NaCl buffer is included in Figure 3. The mononucleoside concentration of the GpGpCp solution is less than for the more concentrated GpGpC solution whose optical rotatory dispersion is shown in the figure. However, the optical rotatory dispersion of GpGpC for the conditions shown is essentially invariant down to the nucleoside concentration used in the GpGpCp solution. The magnitude of the rotation of GpGpC in the absence of added salt is still smaller than for GpGpCp in the presence of 0.5 M NaCl.

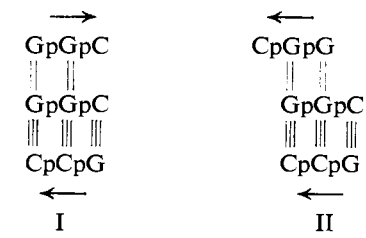
G. BASE PAIRING. The optical rotatory dispersion of the solution containing the aggregate of GpGpC and GpCpC is shifted to the blue relative to the optical rotatory dispersion of the uncomplexed molecules. This also happens when poly G and poly C form a complex. A calculation of the change in optical rotatory dispersion due to formation of the 2GpGpC:1GpCpC complex gives a curve very similar in shape to the difference curve for poly (G:C) minus the single strands. We thus deduce that the 2GpGpC:1GpCpC complex contains C≡C base pairs stacked on top of each other as in the poly (G:C) structure.

Discussion

We shall first discuss the nature of the complexes that have been observed. Then we shall consider what the results reveal about the structure of tRNA and the specificity of the interaction of tRNA with the mRNA–ribosome complex.

The structure of the complex between GpGpC and GpCpC is not known. The following experimental observations should be kept in mind in considering possible structures. (1) The ratio of GpGpC to GpCpC in the complex is approximately 2:1. (2) There are more than three trimers per complex. (3) The complex becomes less stable in the absence of salt, at pH values less than 7, and if GpGpC carries a 3′-terminal phosphate.

The structure of the complex may be similar to the 2G:1C (Lipsett, 1964a; Pochon and Michelson, 1965; Fresco, 1963) aggregate that forms between G oligomers and poly C. In this case, the complex might consist of one GpGpC and one GpCpC hydrogen bonded to each other as they are in the RNA double-stranded helix (Arnott *et al.*, 1966; Fuller *et al.*, 1967). The second GpGpC molecule could be parallel to the first GpGpC molecule and bonded to it by hydrogen bonds between the guanosine residues. This possible 2:1 complex is given as structure I. There would be two G_9:C



base triplets of the type proposed by Lipsett (1964a) for the 2:1 complex of G oligomers and poly C. It is not obvious how the C residue of the second GpGpC molecule would fit into such a complex. The observation that there are more than three trimers per complex can be understood if the triple-strand complexes aggregate end to end. This is reasonable since they have large hydrophobic areas on either end.

A second possible structure again starts with an antiparallel Watson–Crick GpGpC–GpCpC double strand.

The second GpGpC is added antiparallel to the other GpGpC and hydrogen bonded to it by two G–G base pairs. This complex is structure II. It could also aggregate end to end.

All of our experimental observations can be explained by the structures that have just been outlined. However, there are probably other structures that are consistent with the experimental results.

In particular the conformation about glycosidic bonds for all of the residues in the above structures is *anti*. In solution, nuclear magnetic resonance studies suggest that all of the four normal nucleosides have an *anti* conformation (Schweizer *et al.*, 1968). However, several crystal structures are known in which the base is found in the *syn* conformation (Haschemeyer and Rich, 1967; Watenpaugh *et al.*, 1968). Recent calculations by Haschemeyer and Rich (1967) and by Davis (1967) indicate that there may only be a small energy difference between the *syn* and *anti* conformations of guanosine. Therefore, structures of the complex might be considered in which all or some of the G residues have a *syn* conformation.

The natures of the self-association products of GpGpC and ApGpC are quite obscure. Oligomers (Lipsett, 1964b) and polymers (Pochon and Michelson, 1965) rich in G residues are known to readily aggregate. Poly G forms multistranded complexes that can only be dissociated with the most extreme conditions (Pochon and Michelson, 1965). Guanylic acid forms gels at sufficiently high concentration. X-Ray studies (Davies, 1967; Gellert *et al.*, 1962) of these gels indicate that the 5′-nucleotide forms a super helix with 3.75 bases/helix turn. The 3′-nucleotides form stacks of planar structures (Gellert *et al.*, 1962). Each layer contains four guanylic acid residues hydrogen bonded to each other.

The GpGpC aggregate probably involves some vertical stacking as well as intermolecular hydrogen bonding. If the aggregates are only vertical stacks of trimer molecules, we might expect them to have the same conformation as they would in a single-stranded polynucleotide of alternating triplet sequence. We have calculated the optical rotatory dispersion of the infinite polymer $(GpGpCp)_n$ from the experimental optical rotatory dispersion of GpGpC at 1° in dilute nucleoside solutions and the optical rotatory dispersion of CpG at 1°. This is a possible model for the structure of the end-to-end aggregate of GpGpC. The result is compared in Figure 8 with the experimental optical rotatory dispersion of the GpGpC aggregate. The peak and trough of the calculated curve occur about 20 mμ to the red of those for the aggregate. In general we can say that the optical rotatory dispersion of the GpGpC aggregate does not resemble at all what would be expected if the aggregate were only vertical stacks of GpGpC molecules. It is more likely that the aggregate involves some hydrogen bonds between guanylic acid residues and between G and C residues. The optical rotatory dispersion of poly (G:C) (Sarker and Yang, 1965) and the optical rotatory dispersion of poly G (Ulbricht *et al.*, 1966) are also shown in Figure 8. Poly G is aggregated under the conditions used for the measurement (Pochon and Michelson, 1965). Either of these curves is qualitatively

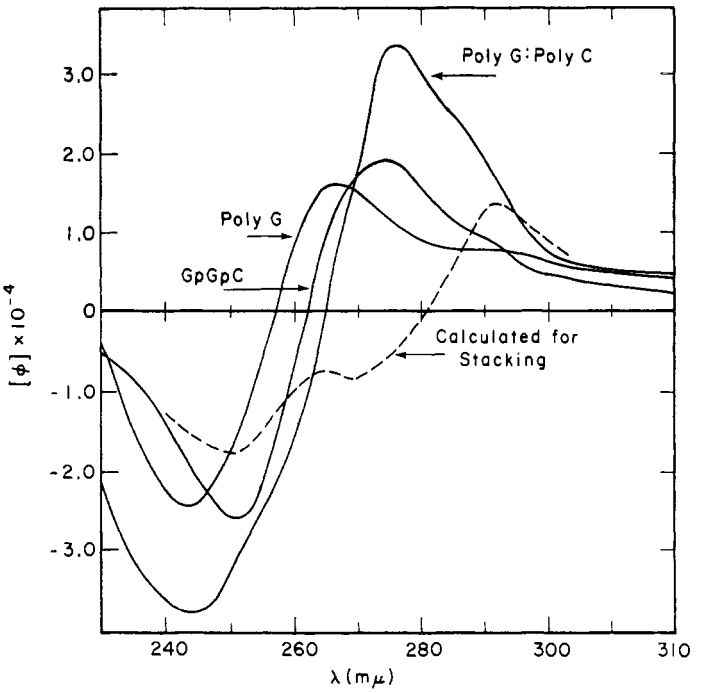


FIGURE 8: Optical rotatory dispersion of GpGpC, 1.0×10^{-2} M mononucleosides, poly G (Ulbricht *et al.*, 1966), poly (G:C) (Sarker and Yang, 1965), and nearest-neighbor calculated optical rotatory dispersion of poly (GpGpCp). 0.5 M NaCl, 0.2 ionic strength sodium phosphate buffer (pH 7), 1°.

a better approximation of the optical rotatory dispersion of the GpGpC aggregate than the optical rotatory dispersion of single-stranded stacks. Thus, we feel the aggregation in concentrated solutions of GpGpC involves hydrogen-bonded base pairs.

The aggregation of GpGpC may be envisioned to occur by the steps given in eq 1. For the purpose of cal-

$$\begin{aligned} M_1 + M_1 &\overset{K_2}{\rightleftharpoons} M_2 \\ M_1 + M_1 &\overset{K_3}{\rightleftharpoons} M_3 \\ &\cdot \\ &\cdot \\ &\cdot \\ M_1 + M_{n-1} &\overset{K_n}{\rightleftharpoons} M_n \end{aligned} \qquad (1)$$

culating an over-all equilibrium constant, K, we assume $K_2 = K_n = K$. Furthermore, we assume the aggregation is similar to the polymerization of bifunctional monomer units. That is, each monomer unit GpGpC has two functional parts capable of reacting with another monomer unit. The fraction of functional parts, p, that has reacted can be calculated from the weight- and z-average molecular weights of the heterogeneous solution (Flory, 1953). For the NaCl buffer p is 0.83 ± 0.01.

Let [M] be the concentration of all species.

$$[M] = [M_1] + [M_2] + [M_3] + \ldots . [M_n] + \ldots . \qquad (2)$$

An expression for the mole fraction of M_1, X_1, in terms

of K can be derived directly from this equation. If there is no limit on the size of the aggregate

$$X_1 = \frac{[M_1]}{[M]} = (1 - K)[M_1] \quad (3)$$

In addition, we have the following equalitites (Flory, 1953)

$$X_1 = \frac{[M_1]}{[M_0](1 - p)} = 1 - p \quad (4)$$

where $[M_0]$ is the initial total concentration of M_1. By appropriate substitution of 4 into 3 we have

$$K = \frac{p}{(1 - p)^2[M_0]} \quad (5)$$

The concentration of GpGpC in the solution for which p is known was 4.2×10^{-3} M. Therefore, the equilibrium constant for aggregation of GpGpC in the NaCl buffer is 6.8×10^3 (moles/l.)$^{-1}$. This number was used to calculate the weight-average molecular weight of GpGpC in a solution containing the complex of GpGpC and GpCpC.

The mechanism for the translation of the genetic code is centered on the association of two complementary triplets, the codon and anticodon (Crick, 1966). Yet the equilibrium constant for the association of two complementary triplets that are divorced from the rest of the translation apparatus is so low that we were unable to observe the complex. It is not clear how large the equilibrium constants must be to account for the translation mechanism. Nevertheless, it seems likely that the equilibrium constant for the interaction of codon and anticodon is greater than for two complementary trimers that are free in solution.

The greater stability of the codon:anticodon complex can be easily rationalized. Part of it is surely due to the unspecific binding of tRNA to the ribosome. But this is probably not all; the stability of the codon–anticodon complex must be sufficiently great to provide specificity. The environment for the interaction of the two complementary triplets on the ribosome is probably different from what is experienced in solution. The interaction may take place in a pocket on the ribosome similar to the cleft found in the crystal structures of lysozyme (Phillips, 1967). The effective water activity and dielectric constant may be lower in such a pocket than in the bulk solution. The phosphate charges of the codon and anticodon may be partially neutralized by positively charged amino acid side chains.

Another possible explanation for the stability of the codon–anticodon complex has been offered by Fuller and Hodgson (1967). Since the anticodon is at the center of a seven-membered loop, they have shown that it is possible to form a structure in which the triplet from the messenger appears to be a part of an almost continuous double-strand helix. This should increase the stability of the complex between codon and anticodon and may be responsible for some of the specificity as well. Högenauer (1967) has demonstrated by chromatography that it is possible to form a weak complex between a trimer and tRNA. The details of this interaction are not yet understood, and the binding constant for timer–tRNA complex cannot be directly obtained from his experiments. Thus, it is not yet possible to verify experimentally whether a loop contributes to the stability of trimer interactions.

Specific 1:1 complexes of complementary trimers are not stable under the conditions we have employed. It is of interest to see whether they could still stabilize loops in tRNA. Let us assume that a 1:1 complex of three G–C base pairs is as stable as the 2:1 complex of GpGpC and GpCpC. Similarly, we assume a 1:1 complex of three A–U base pairs is as stable as the 2:1 complex between ApApA and UpUpU as found by Miles *et al.*[1] Clearly these are the upper limits for the stability of the actual 1:1 complexes. We want to calculate what fraction of loops would be closed by complexes with these stabilities if the complementary oligomers were tied together with a polynucleotide chain.

If we know the equilibrium constant for complex formation between two oligomers and assume that connecting them by a polynucleotide chain only effects their entropy of formation, it is easy to calculate the new equilibrium constant (Wang and Davidson, 1966). The chain simply changes the effective concentration of the oligomers. If the chain is flexible it will always increase the effective concentration and increase the equilibrium constant for chain closure. For a polynucleotide chain containing at least one uracil, our neglect of enthalpy effects is probably reasonable. However, in general one must consider such factors as the enthalpy of stacking in the polynucleotide chain connecting two complementary oligomers.

Jacobson and Stockmayer (1950) have derived an equation for the ratio, j, of two equilibrium constants: one for chain closure by two oligomers and the other for the free association of these two oligomers. If the end-to-end distance of the loop has a Gaussian distribution and the contour length is long compared with the mean end-to-end distance then they find $j = (3/2\pi nb^2)^{3/2}$, where n is the number of units of length, b, in the loop. In the absence of information to the contrary we shall assume that j can be calculated with this equation for chain lengths of 10 or greater. Therefore, the fraction of chain closure by two oligomers, f_1, can be related to the fraction, f_2, bound in solution by

$$\frac{f_1}{1 - f_1} = \frac{548}{n^{3/2}b^3}\left[\frac{f_2}{(1 - f_2)^2 m}\right]$$

where m is the initial concentration of each oligomer (moles of oligomer per liter) that leads to a fraction, f_2, bound in solution (f_2 = (concentration bound at equilibrium)/m). The unit length, b, is measured in angstroms.

For a solution of GpGpC and GpCpC with a concentration of 0.003 M trimer/l. the fraction of GpCpC in the 2:1 complex is 75% at 1° and 9% at 37°. At a tenfold higher concentration of trimers, Miles *et al.*[1] found

that the fraction of ApApA in a U_2:A complex is also 75% at 1°. In analogy with the GpGpC data we shall assume that the fraction complexed at 37° is 9%. Now we shall take these fractions as an upper limit of the fraction of oligomers bound in a double-stranded complex at the same concentration of oligomer. The fraction of loops closed by double-stranded complexes with these hypothetical stabilities has been calculated at 37° for n equal to 10, 20, and 100. The length of a monomer, 7 Å, has been chosen for b. The results are given in Table IV. They indicate that a loop can only be closed by three base pairs at 37° if they are all G–C base pairs. More than three A–U base pairs are needed to close the loop at this temperature.

TABLE IV: Formation of Double- and Triple-Stranded Loops at 37°.

No. of Monomers in Loop (n)	Double-Stranded Loops: Fraction of Loops Closed (f_1) for b = 7 Å	
	m^a = 0.003 M (G–C)	m = 0.03 M (A–U)
10	0.65	0.16
20	0.39	0.06
100	0.06	0.01
No. of Monomers in Each Loop ($n = n'$)	**Triple-Stranded Loops: Fraction of Double Loops Closed (f_3) for $b = b'$ = 7 Å**	
	m = 0.003 M (G–C)	m = 0.03 M (A–U)
10	0.89	0.08
20	0.51	0.01
100	0.01	0.00

[a] m = concentration of trimers in solution required to produce 9% complex formation ($f_2 = f_4 = 0.09$).

These calculations have been for loops closed by double-stranded complexes. However, it appears that triple-stranded complexes of oligomers are more stable than the double-stranded ones. We have been unable to find conditions for a 1:1 complex of GpGpC and GpCpC. Similarly, Pochon and Michelson (1965) were unable to find conditions for a 1:1 complex between oligo G and poly C, although Lipsett (1964a,b) has succeeded. Apparently Miles *et al.*[1] have only observed a 2:1 complex between oligo A and oligo U. On the other hand, conditions have been found where the stoichiometry of the complex between poly G and poly C and between poly A and poly U is 1:1. The one other relevant observation here was given in a recent communication of Cassani and Bollum (1967). They have reported the T_m of complexes between poly d(pA) and oligo d(pT) and between poly d(pT) and oligo d(pA) as a function of the chain length of the oligomer. The stoichiometry of the complex between poly d(pT) and oligo d(pA) is 2T:1A for oligomer chain lengths less than 16 and 1T:1A for chain lengths greater than 16. The complex between poly d(pA) and oligo d(pT) was 1:1 for chain lengths of d(pT) greater than seven. It is possible that the shorter oligomers will give a 2T:1A stoichiometry. In summary, what is common to these observations is that there is a pronounced tendency for short oligomers to form triple-stranded complexes in preference to double-stranded ones. Conversely, longer oligomers are more likely to form double-stranded complexes.

Complementary regions in tRNA are probably rather short. Therefore, we should consider the possibility that loops in tRNA are closed by triple-stranded complexes even at the expense of short double-stranded regions (Guschlbauer, 1966). There are regions in each of the five tRNAs whose sequence is known (Holley *et al.*, 1965; RajBhandary *et al.*, 1967; Madison *et al.*, 1966; Zachau *et al.*, 1966) that can form triple strands instead of some of the double-stranded helical regions that have been proposed. Such triple-stranded helical regions may be important in giving tRNA molecules precise three-dimensional structures.

As in the case of double-stranded loop closure, we must determine what effect the polynucleotide chain has on the equilibrium constant of the triple-stranded complexes. Let f_4 be the fraction of oligomer A in the triple-stranded complex (A:2B) that forms in a solution of m moles of oligomer A and $2m$ moles of its complement. Assuming that closure of each loop is independent of the other, we can relate the fraction f_3 of double loop closed by a triple-stranded complex to f_4 and m by

$$\frac{f_3}{1 - f_3} = \frac{7.49 \times 10^{4+}}{(nn')^{3/2}(bb')^3}\left[\frac{f_4}{(1 - f_4)^3 m^2}\right]$$

The number of units in the different loops are designated by n and n'. The lengths of these units are b and b'.

The fraction, f_3, has been calculated at 37° for $n = n'$ = 10, 20, and 100. The results are included in Table IV. The calculations indicate that double loops can be closed by a triple-stranded complex like 2GpGpC:GpCpC but not by 2UpUpU:ApApA.

Calculation of the fraction of single and double loops closed at 25°, and further discussion of these equations has been presented earlier (Tinoco *et al.*, 1968).

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