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Studies of Trinucleotide Conformations. Role of Guanine Residues in an Oligonucleotide Chain*

J. Brahms, A. M. Aubertin, G. Dirheimer, and M. Grunberg-Manago

ABSTRACT: A comparison of spectroscopic (circular dichroism) and thermodynamic properties of various trinucleotides studied at different conditions of ionic strength, nucleotide concentration, and temperature, allows one to detect the conformational characteristics of polynucleotide chains, *i.e.*, the unstacked, single-stranded stacked, and double- or multiple-stranded helical associative forms. Several trinucleotides can form, at low temperatures, single-stranded stacked structures, *e.g.*, ApApUp, ApApCp, and GpApUp. Thermodynamic analysis of the thermal "melting" process gives a relatively small value of ΔF° for the guanine-containing trinucleotide. This suggests a tendency of guanine to unstack. The presence of guanine residues probably has a dual role. In trinucleotide sequences like GpGpC or GpGpU, the formation of intermolecular associations is observed under appropriate conditions.

In other sequences, *e.g.*, ApGpU, UpGpA, and GpUpA, there is no evidence for the formation of dissymmetrical stacked base conformation under conditions favoring the formation of single-stranded structures. Our results suggest the existence of *syn* and *anti* conformers whenever unpaired guanosine residues are next to uridine.

The knowledge of various factors contributing to the conformational stability of ribonucleic acid is of a special importance for the determination of its structure in solution. For the past few years the application of optical methods to the studies of simple oligonucleotides has been very fruitful for the understanding of conformation and of forces contributing to conformational stability. Thus, stacking interactions were shown to be the major source of conformational stability of single-stranded helical structures (Van Holde *et al.*, 1965; Poland *et al.*, 1966; Leng and Felsenfeld, 1966; Brahms *et al.*, 1966; Fasman *et al.*, 1964). Studies of natural 3′→5′ dinucleotides showed that essentially similar thermodynamic stacking interaction parameters were found for various sequences (Brahms *et al.*, 1967; Davies and Tinoco, 1968) and only uridylates at low ionic strength may exist in unstacked conformation (Simpkins and Richards, 1967a,b).

Since the trinucleotides already bear some characteristics of the simplest polynucleotide chain, it was of interest to extend our studies to these compounds, using circular dichroism as a method of conformational investigation. In principle, one could expect that the structural features of a polynucleotide chain could already be detected in simple trinucleotides. These include features such as the base pairing and the intermolecular associations in double or multiple helices, and the base stacking in single-stranded helices or formation of loops. The stacking interaction leading to single-stranded helical structures has been previously observed in various trinucleotides (Cantor and Tinoco, 1965; Zavil'gel'skii and Li, 1967; Inoue *et al.*, 1967). It was shown that in single-stranded ordered structures the optical rotary dispersion mainly depends upon the influence of the nearest residue and can be expressed by a simple semiempirical expression (Cantor and Tinoco, 1965, 1967). The present work was done in an attempt to establish the role of factors contributing to the conformational stability of a polynucleotide helical chain using the temperature dependence of circular dichroism. We have been concerned with the characterization of both intermolecular and intramolecular interactions in trinucleotides. The comparison of optical and thermodynamic properties shows that at high ionic strength intermolecular associations are detectable in some simple trinucleotides when guanine residues are present. This was not observed with other trinucleotides. At low ionic strength and in the absence of complementary bases, guanine residues promote unstacking. This tendency to unstack seems to be particularly pronounced whenever nonpaired guanine residues are next to uridine.

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Experimental Methods

Trinucleotides Preparation. The trinucleotides were prepared by enzymatic digestion of yeast rRNA. The standard conditions were as follows. rRNA preparation (160 mg) in 25 ml at pH 7.9 was hydrolyzed in the presence of 4 mg of pancreatic ribonuclease (Worthington) and a few drops of chloroform were added. The reaction was followed using TTT-1 titrimeter Radiometer. The mixture of oligonucleotides thus obtained was separated on a DEAE-cellulose column (1 × 90 cm) in the presence of urea, according to Tomlinson and Tener (1963). The elution of oligonucleotides was first made by applying 150 ml of 0.0025 M Tris (pH 7.8)–7 M urea, followed by a linear gradient to 0.3 M NaCl–0.0025 M Tris (pH 7.8)–7 M urea. The total volume of the gradient was 2 l. The salt was removed from this solution by gel filtration on a Dextran Sephadex G-10 column (3 × 180 cm) at 4°, according to the procedure of Rémy *et al.* (1967). The column was eluted with distilled water. Some assays of desalting procedure were done with Bio-Gel P-2 at room temperature without any success, since the major part of trinucleotides eluted was hydrolyzed to mononucleotides.

Trinucleotides of different sequence were isolated by chromatography on DEAE-cellulose column (1 × 90 cm) equilibrated with 7 M urea and 0.1 M formic acid. The column was eluted by applying 4 l. of a linear gradient of NaCl (0–0.1 M) in the formic acid–urea solution, according to Rushizky and Sober (1964). The appearance of various trinucleotide fractions was similar to that obtained by these authors, and by others (see Cantor and Tinoco, 1965). The fractions were analyzed after hydrolysis (either with snake venom phosphodiesterase or with alkali), followed by two-dimensional chromatography (ammonium acetate (pH 5)–ethanol, and isobutyric acid–water–ammonia).

The ultraviolet absorption spectra of all these compounds were determined. The results obtained fully confirmed the order of appearance of elution fractions by chromatography of trinucleotides described by Rushizky and Sober (1964). To completely eliminate the contaminant salt the trinucleotides were dialyzed continuously during 4 hr, using Visking tubes 23–32 against flowing distilled water. The final desalting procedure included chromatography on Sephadex G-10.

In order to obtain the trinucleoside diphosphates, the mixture of trinucleotides was desalted and hydrolyzed by *E. coli* alkaline phosphomonoesterase (Worthington). In order to eliminate contaminating phosphodiesterase the enzyme was purified by chromatography on DEAE-cellulose, following the procedure of Garen and Levinthal (1960). The separation of trinucleoside diphosphates was obtained by chromatography on DEAE-cellulose column in the same conditions as those described by Cantor and Tinoco (1965). The final purification procedure of dephosphorylated trimers was identical with that previously described for trinucleotides. The purified, salt-free trimers thus obtained, ApApUp, ApApCp, ApGpUp, GpGpCp, and GpGpUp, were lyophilized.

Dephosphorylation was carried out to compare the influence of terminal 3′-phosphate on the formation of ordered structure by studying trimers of identical sequences. The results of circular dichroism investigations indicate the similarity of their spectra. However, some differences of band intensity do not allow to reach a definite conclusion from the present studies.

Other trinucleotides studied, GpApCp, ApGpCp, UpGpU, UpUpGp, and GpUpG, were prepared by enzymatic synthesis with primer-dependent *Micrococcus lysodeikticus* polynucleotide phosphorylase (10 phosphorolysis units[1]/ml), the appropriate dinucleotides and nucleotide diphosphates (in a ratio 1:1) until the reaction comes to an equilibrium. The appropriate dinucleotides (Zellstoff Fabrik, Waldhof, Germany) were used as primers. For UpUpGp and GpUpG, the incubation was carried out in the presence of RNase T1 (100 units); for GpApCp and ApGpCp, the incubation was carried out in the presence of pancreatic RNase (20 μg/ml). When necessary, the terminal phosphate was removed by addition of alkaline phosphate (2 μg/ml) at the end of the incubation. The triplets were deproteinized by Sevag procedure (Sevag *et al.*, 1938) and purified by chromatography on Whatman No. 3MM paper in the following solvent *n*-propyl alcohol–ammonia–water (55:10:35, v/v). They were subsequently identified by their chromatographic behavior (R_F) compared with standard samples (dinucleotides, tri-, and diphosphates) and spectroscopic characterization in 0.1 N HCl.[2]

Optical Studies of Absorption Spectra and of Circular Dichroism. The solvent used for dissolving lyophilized compounds was 4.7 M KF–0.01 M Tris (pH 7.4) which was previously used for the low-temperature studies of dinucleotides (Brahms *et al.*, 1967). In order to detect the formation of intermolecular complexes, the solutions at lower ionic strength were used, that is 0.1 M KF–0.01 M Tris (pH 7.4) and also of almost null ionic strength, that is in the absence of added salt in 0.01 M Tris (pH 7.4).

Ultraviolet absorption spectra were measured in a Cary 15 or a Cary 14 spectrophotometer, using cells of 0.1- or 0.2-cm optical path length. The concentration of trinucleotides was determined spectrophotometrically. The values of ε were either obtained by alkaline hydrolysis to the monomer, or, in some cases, they were taken from previous measurements of Cantor and Tinoco (1965) and of Inoue *et al.* (1967).

The circular dichroic spectra were measured with a Jasco ORD–UV 5 spectrophotometer equipped with a thermostatically controlled cell holder. A thermocouple was in close contact with the cell, and the temperature was recorded during all measurements. The samples were measured in 0.1- and 0.2-cm path-length cells. In some instances, 0.5-, 1-, 2-, and 5-cm cells were used after diluting. This allowed us to test the influence of concentration and the formation of intermolecular associations.

The calculation of rotational strength was performed as pre viously indicated (Brahms, 1963) by the estimate of the integrated area of the longer wavelength positive band.

Results

The optical properties of several trinucleotides have been studied under different conditions of temperature, ionic strength, and nucleotide concentration. The results allow the division into the following groups. (1) Trinucleotides which exist in stacked, single-stranded form, *e.g.*, ApApU, ApApC, GpApU, under appropriate conditions. (2) Trinucleotides

[1] A phosphorolysis unit is defined as 1 μmole of ADP liberated from poly A during a 15-min phosphorolysis at 37°.

[2] We are grateful to J. Dondon for the preparation of these trinucleotides.

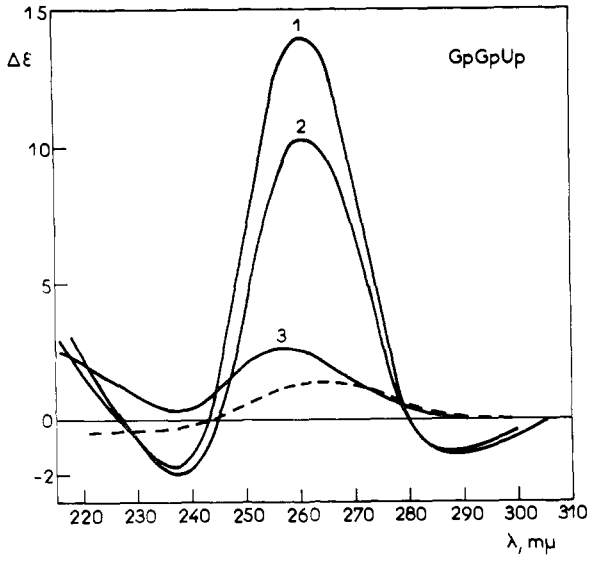

FIGURE 1: Circular dichroic spectra (———) of GpGpUp, at about 2°, in solution of different ionic strength: (1) 4.7 M KF–0.01 M Tris, pH 7.5; nucleotide concentration 1.1×10^{-3} M; (2) 0.1 M KF–0.01 M Tris, pH 7.5; nucleotide concentration 1.5×10^{-3} M; and (3) 0.01 M Tris, pH 7.4; nucleotide concentration 1.4×10^{-3} M. (– – – –) Circular dichroic spectrum of monomer constituent.

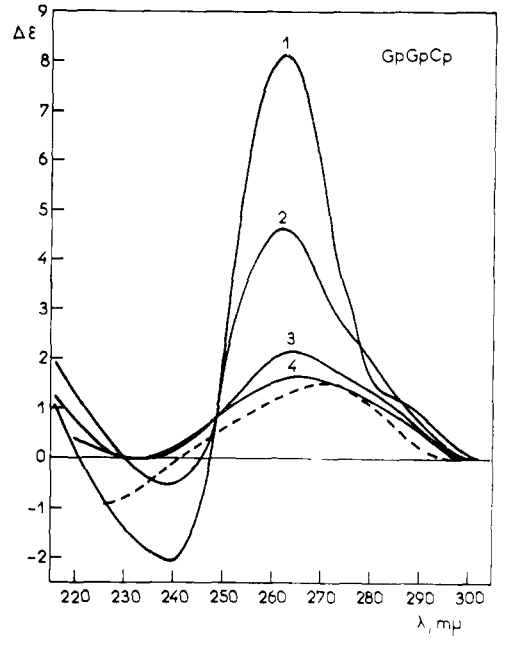

FIGURE 2: Influence of the ionic strength on the circular dichroic spectra of GpGpCp in solutions containing: (1) 4.7 M KF–0.01 M Tris, (pH 7.5) at 2°; nucleotide concentration 1.5×10^{-3} M; (2) 0.1 M KF–0.01 M Tris (pH 7.5) at 0°; nucleotide concentration 1.3×10^{-3} M; (3) 0.01 M Tris (pH 7.4) at 1°; nucleotide concentration 1.5×10^{-3} M; and (4) 0.01 M Tris at 28°; nucleotide concentration 1.5×10^{-3} M.

which associate with themselves in more concentrated solutions, *e.g.*, GpGpC and GpGpU. (3) Trinucleotides containing guanine which do not show evidence for the formation of dissymmetrical "helical" structures, *e.g.*, UpGpA, GpUpA, ApGpU, UpUpG, and GpUpG. The evidence for this qualitative classification will be developed hereafter.

Intermolecular Association. In order to detect intermolecular association between identical trinucleotides, we have studied their circular dichroic spectra under different conditions of ionic strength and nucleotide concentration. The results of these investigations indicate that of all the trinucleotides studied, only GpGpCp and GpGpUp exhibit pronounced changes in the form of the spectra and in the position and intensity of the bands.

This suggests the occurrence of intermolecular associations, which are reflected in the circular dichroism parameters. We will thus examine first the intermolecular trinucleotide complexes, and then the second class of oligomers in the nonassociated form.

INFLUENCE OF IONIC STRENGTH AND NUCLEOTIDE CONCENTRATION. The circular dichroic spectra of GpGpUp were investigated in the following three solutions of different ionic strengths at pH 7.4 with Tris buffer either 4.7 M KF or 0.1 M KF, or without any added salt.

Figure 1 shows the comparison of results of circular dichroism measurements performed at a temperature of about 0–1°. One can clearly see that the intensities of GpGpUp circular dichroic spectra decrease strongly upon changing the ionic strength from 4.7 M KF (curve 1) to 0.1 M KF (curve 2), and, in the absence of added salt even the form of the spectrum is completely changed (curve 3). The GpGpUp spectra at high and moderate ionic strength are composed of two negative bands (287 and 235 mμ) and of a strong positive band (261 mμ). At low ionic strength the spectrum has the profile and intensity of the corresponding monomers (curve 3, Figure 1). We interpret all these drastic spectral changes, such as the disappearance of both negative bands and the shift of the positive band maximum (from 261 to 255 mμ) as reflecting the collapse of the double or multistranded ordered helical structure.

Changes in ionic strength also cause very pronounced spectral modifications of GpGpCp (Figure 2). The "conservative" spectrum of GpGpCp, which is composed of positive and negative bands at high ionic strength (curve 1), shows a sharp decrease in the intensity of both bands at lower ionic strength (curve 2). At very low ionic strength, in the absence of added salt, the circular dichroic spectrum became "nonconservative" (curves 3 and 4), that is, very similar to that of the monomer spectrum (Figure 2, dotted line). Again, these pronounced spectral changes, both in form and intensity, are taken to reflect conformational changes from ordered double- or multiple-stranded associated "helical" form to essentially disordered, random coil.

The nucleotide concentration influences the intensity of circular dichroism bands. At constant temperature and high ionic strength the decrease of trinucleotide concentration from 10^{-3} to 5×10^{-5} M causes a sharp diminution of the intensity of circular dichroic bands. This is also considered as further evidence for the existence of complexes.

INFLUENCE OF TEMPERATURE. The changes of circular dichroic spectra of GpGpCp and GpGpU with temperature are shown in Figures 3 and 4. Figure 5 shows that, in contrast to other trinucleotides studied, this temperature dependence has a plateau at low temperatures (from −20 to 10°) and that at high temperatures there is a sharp decrease. This type of curve, characterized by a sharp slope, is different from gradual, non-

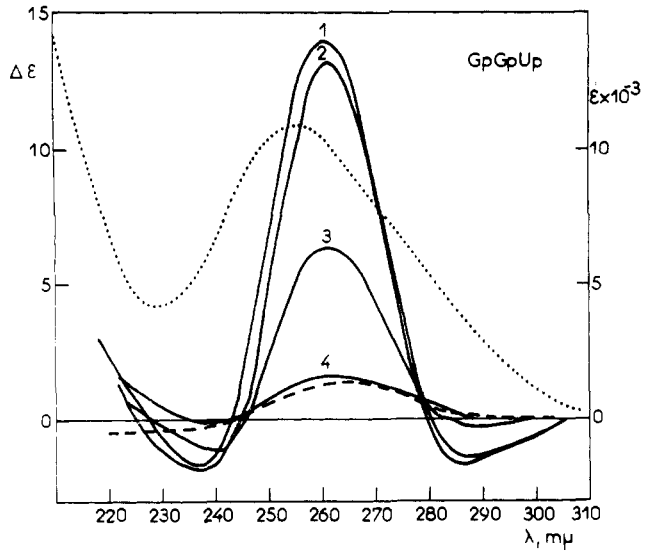

FIGURE 3: Influence of the temperature on the circular dichroic spectra (———) of GpGpUp, nucleotide concentration 1.1×10^{-3} M, in 4.7 M KF–0.01 M Tris (pH 7.5) at: (1) −19, (2) 28, (3) 70, and (4) 81°. (– – – – –) Circular dichroic spectrum of monomer constituents; (· · · ·) absorption spectrum of GpGpUp.

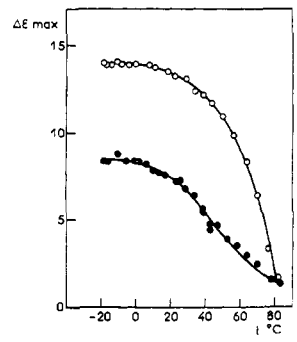

FIGURE 5: Thermal denaturation curve of GpGpUp (○) and GpGpCp (●) in 4.7 M KF–0.01 M Tris, pH 7.5; nucleotide concentration 1.4×10^{-3} and 1.5×10^{-3}, respectively.

cooperative transition of simple nonassociated dinucleotides (Brahms *et al.*, 1967) and trinucleotides (see Figure 11).

The Nonaggregated Trinucleotides. The trinucleotides ApApCp, ApApU, and GpApUp have not shown any evidence of intermolecular association. Measurements of circular dichroism were done in conditions which favor the formation of intermolecular complexes previously described, namely, high ionic strength (4.7 M KF), low temperature (−20°), and at 10^{-3} M nucleotide concentration. The results show that neither the form nor the intensity of the spectra was changed upon 50 times dilution. Thus, the observed circular dichroic spectra are independent of concentration. Essentially similar results were found when the ionic strength of trinucleotide solutions were changed; the solution at pH 7.4 contained either 4.7 M KF–0.01 M Tris or 0.1 M KF–0.01 M Tris, or no added salt (0.01 M Tris). Only relatively small changes in intensity without modification of the form of the spectra were found.

The characteristic circular dichroic spectra of ApApCp, ApApUp, and GpApUp, measured at different temperatures,

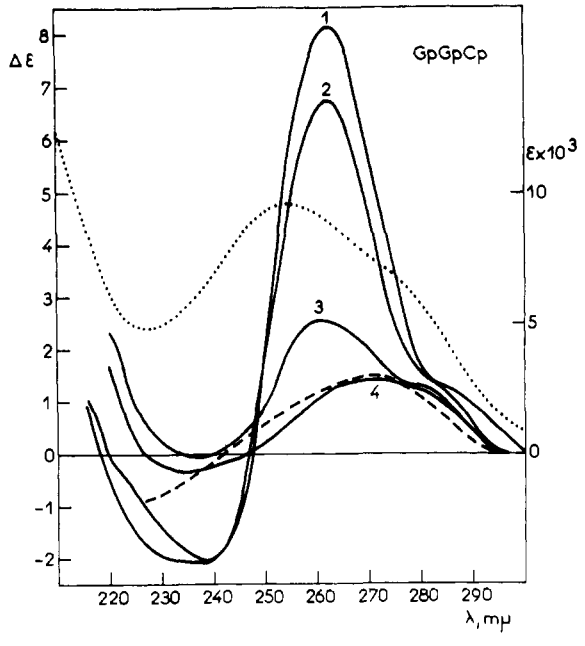

FIGURE 4: Influence of temperature on the circular dichroic spectrum (———) of GGC, nucleotide concentration 1.5×10^{-3} M, in 4.7 M KF–0.01 M Tris (pH 7.4) at: (1) −19, (2) 28, (3) 70, and (4) 78°. (. . . . . .) Absorption spectrum; (– – – – –) circular dichroic spectrum of monomer constituents.

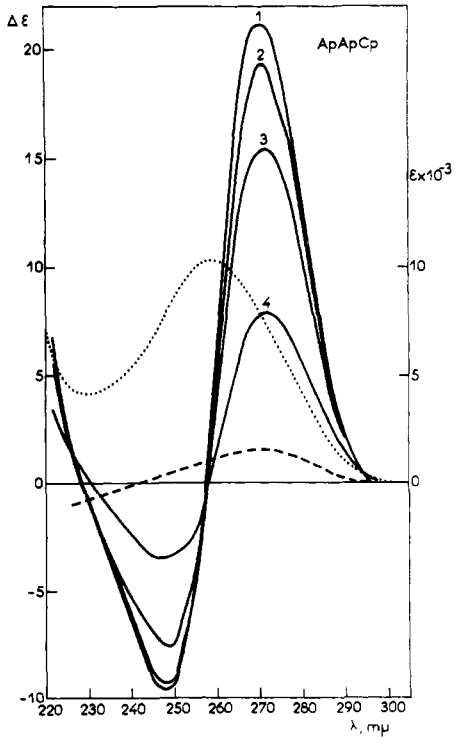

FIGURE 6: Circular dichroic spectrum of ApApCp (———) in 4.7 M KF–0.01 M Tris (pH 7.4) measured at various temperatures (1) −19, (2) 1, (3) 29, and (4) 82°. (· · · · ·) Absorption spectrum; (– – –) circular dichroic spectrum of monomer constituents.

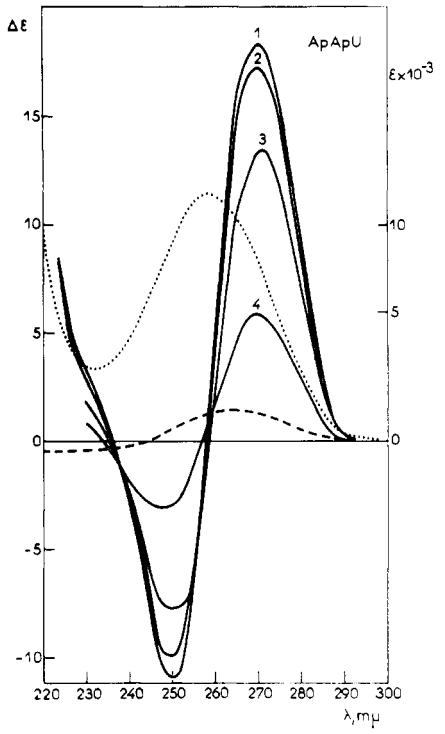

FIGURE 7: Circular dichroic spectrum of ApApU (———) in 4.7 M KF measured at different temperatures: (1) −19, (2) +2, (3) 25, and (4) 75°. (· · · · ·) Absorption spectrum; (– – –) represents circular dichroic spectrum of the monomer constituents.

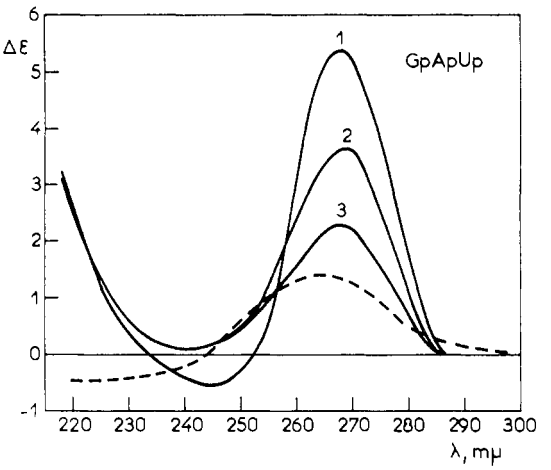

FIGURE 8: Circular dichroic spectrum of GpApUp (———) in 0.01 M Tris (pH 7.4) at various temperatures (1) 0, (2) 25, and (3) 65°. (. . . . . .) Circular dichroic spectrum of corresponding monomers.

are shown in Figures 6–8. The following observations indicate that there is no evidence of intermolecular associative interaction and that the dissymmetrical oligonucleotide structure is due to base stacking.

(1) The circular dichroic spectra are essentially composed of positive and negative bands having the intersection point at the maximum of the absorption band (Table I). This essentially conservative type of spectra (see Bush and Brahms, 1967; Tinoco, 1968) is in good agreement with coupled dipole theory. Furthermore, the calculations based solely on the vertical nearest-neighbor stacking interactions (Cantor and Tinoco, 1965) predict quite well the form of the spectra. While some differences between calculated and observed curves were noticed in the intensity of the bands, there was no substantial difference in the position of the extreme and of the intersection points. For this calculation the contributions of oligonucleotides were taken from the circular dichroism results on dinucleotides (Brahms *et al.*, 1967).

(2) The circular dichroic spectra measured at different temperatures are of similar shape and decreasing band intensity. It is thus possible to plot the rotational strength or the intensity of the main band as a function of temperature. This allows one to construct the "melting" curves which are of sigmoidal form, gradually decreasing in a fashion similar to other single-stranded oligonucleotides (Brahms *et al.*, 1966, 1967; Poland *et al.*, 1966; Leng and Felsenfeld, 1966; Applequist and Damle, 1966). Furthermore, such a gradual change suggests a low value for the enthalpy of the process. It also suggests that the conformational change is an essentially noncooperative process. The reaction of the breakdown of an ordered trinucleotide structure (at low temperatures) to a disordered form (high temperatures) can be defined by an apparent equilibrium constant. At low temperatures, the circular dichroism temperature dependence approaches a plateau which is not as pronounced as in the case of the aggregated forms; however, it is detectable (mainly because of the use of solvents which lower the freezing temperature to −20°). The limiting values of circular dichroism at low temperatures were obtained by two procedures similar to the methods applied previously (see Brahms *et al.*, 1966, 1967). First, by the extrapolation of the "plateau" of the sigmoidal melting curve to a limiting value of circular dichroism at low temperatures (R_0). Second, by making use of the inflection point R_T($R_T = R_0/2$). Recently, the procedure of estimation of R_T, the midpoint of the transition, was perfected by Richards (1968). At higher temperatures, the available part of the curve facilitates the estimation of the midpoint of the transition, and allows the extrapolation to the limit characteristic of the disordered form. The limits of uncertainty are estimated to be smaller than 20%.

This allows one to construct a Van't Hoff graph (see Brahms *et al.*, 1967) by plotting the apparent equilibrium constant *vs.* $1/T$ (Figure 9). As shown in Figure 9, straight Van't Hoff graphs are obtained, and the lines are parallel,

TABLE I: Thermodynamic Parameters for Thermal Unstacking of Trinucleotides (at Neutral pH).

	In 4.7 M KF–0.01 M Tris			In 0.01 M Tris		
	$\Delta H°$ (kcal/mole)	$\Delta S°$ (eu)	$\Delta F°$ at 0° (kcal/mole)	$\Delta H°$ (kcal/mole)	$\Delta S°$ (eu)	$\Delta F°$ at 0° (kcal/mole)
AAC	6.4	20	1	5.8	19	0.4
AAU	8	25	1	7.2	25	0.4
GAU	6	20	0.5	5.9	22	0

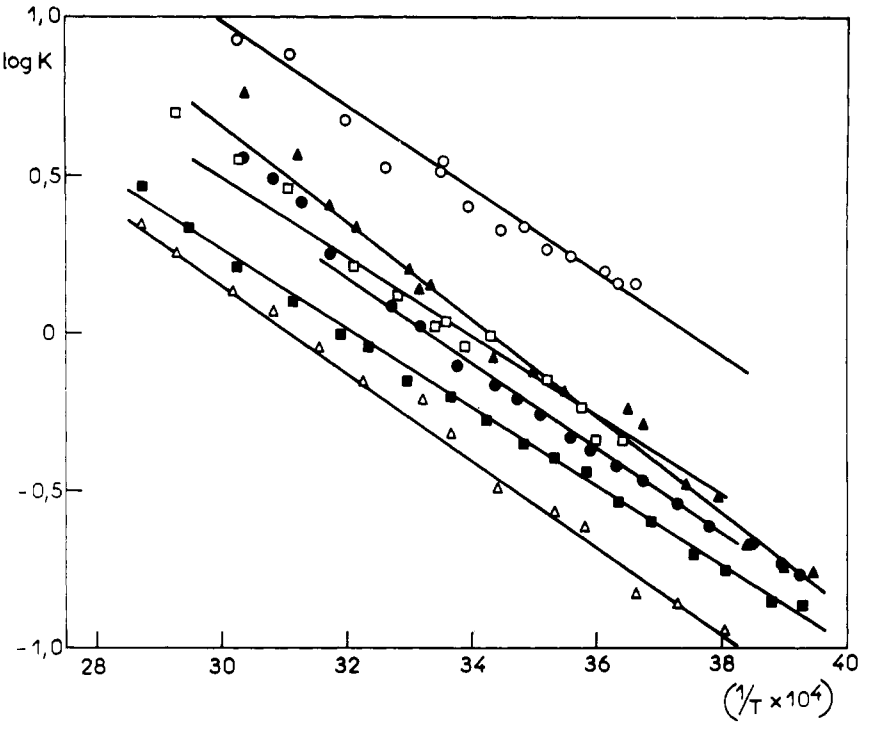

FIGURE 9: A Van'tHoff plot of the thermal denaturation of some 3′→5′ trinucleotides and trinucleosides diphosphates at different ionic strengths. (△) ApApCp in 4.7 M KF–0.01 M Tris (pH 7.4), (■) ApApC in 4.7 M KF–0.01 M Tris (pH 7.4), (□) ApApC in 0.01 M Tris, (●) GpApUp in 4.7 M KF–0.01 M Tris, (○) GpApUp in 0.01 M Tris, and (▲) GpApU in 4.7 M KF–0.01 M Tris.

indicating that the standard state enthalpy change must be nearly the same for all these trinucleotides. The values of $\Delta H°$ for the thermal denaturation of trinucleotides ApApCp, ApApUp, and GpApUp, at two different conditions of ionic strength, are between 6 and 8 kcal per mole (Table I). These values of $\Delta H°$ are very similar to those previously obtained for various dinucleotides (Brahms *et al.*, 1967; Davis and Tinoco, 1967).

One can also conclude that the thermodynamic parameters for the thermal denaturation process indicate the conformational changes of single-stranded, nonassociated "helical" structures.

The comparison of the values of standard free-energy changes (at 0°) indicates that the base-stacking process is favored (Table I). Furthermore, one can also see from Table I that the values of $\Delta F°$ (at 0°) are relatively smaller for the compounds containing guanine, like GpApUp, when compared with two other trinucleotides, determined under similar conditions of low and high ionic strength.

Influence of the Guanine Residue on Base Stacking. The slightly lower values of free-energy changes for a trinucleotide GpApUp suggest that the presence of the guanine residue weakens the stacking interactions. In order to test this assumption a series of trinucleotides containing the guanine residues in various sequences was investigated by means of circular dichroism at various temperatures and at very low ionic strength.

Conditions have been chosen which will favor the existence of single-stranded ordered structures without formation of aggregates. The solution contained no added salt, only 10^{-2} M Tris buffer (pH 7.4). The following trinucleotides containing guanine were investigated at several temperatures: ApGpU, GpUpA, UpUpG, GpUpG, and UpGpAp. Figure 10 shows the comparison of circular dichroic and absorption spectra of three sequence isomers, ApGpUp, UpGpAp, and GpUpAp, measured at low temperatures of about 1° (curve 1). The following observations can be made. (1) In general, the intensity of the circular dichroism bands of these trinucleotides is very low in comparison with various previously considered single-stranded trinucleotides measured under similar conditions. One should notice that the magnitude and the shape of UpGpA and GpUpA circular dichroic spectra are very similar to those of corresponding monomers. (2) The changes with temperature of the circular dichroism bands are very small. Figure 11 shows the comparison of the temperature dependence of circular dichroism of ApGpU and UpGpA when compared with another sequence isomer, GpApU; for comparison, a trinucleotide containing another purine, adenine instead of guanine, is shown. The "melting" behavior of these three-sequence isomers, ApGpUp, UpGpAp, and GpApUp, is quite different. GpApUp melts in a manner similar to other single-stranded stacked structures, whereas UpGpA and ApGpUp exhibit very little changes in circular dichroism as a function of temperature. These observations strongly suggest that differences in conformation must exist at low temperatures between GpApU which is stacked, and UpGpAp for which there is essentially no evidence of stacking. One can conclude that these differences must be related to some particularity of sequences and structure of guanine-containing oligonucleotides.

Figure 12 shows circular dichroic and absorption spectra of two other guanine-containing trinucleotides, UpUpGp and GpUpGp. These two circular dichroic spectra are again of very low intensity, and particularly striking is the absence of

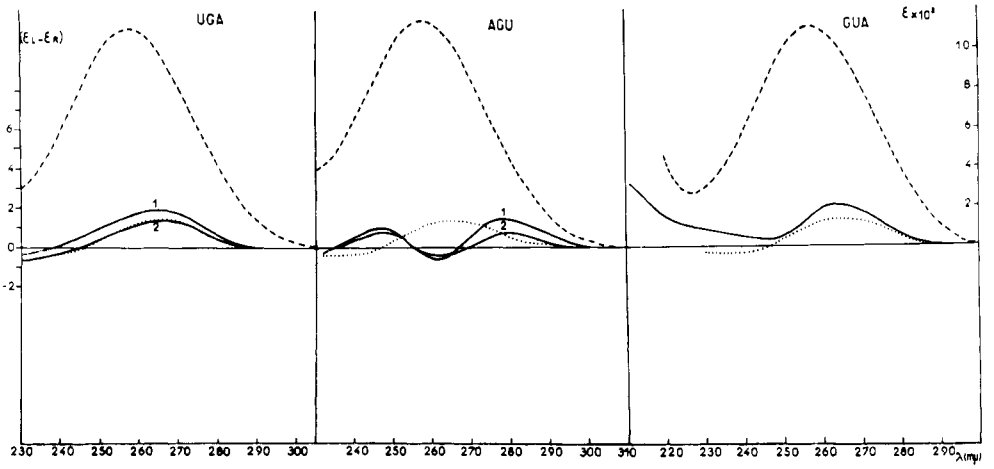

FIGURE 10: Circular dichroic spectra of (——) guanine containing trinucleotides isomers. Left: UpGpA (1) at 2° and (2) at 30°, concentration 8.6 $\times$ 10^{-4} M per residue. Center: ApGpU, (1) at 2° and (2) at 25°, concentration 7.7 $\times$ 10^{-5} M per residue. Right: GpUpA, at 0° in 0.01 M Tris (pH 7.4), concentration 8.7 $\times$ 10^{-5} M. (. . . . .) Circular dichroism of the corresponding monomers constituents; (- - - - -) absorption spectra of trinucleotides.

band structure in GpUpGp. Furthermore, for these two trinucleotides the predicted spectra, which were calculated on the basis of the nearest-neighbor semiempirical method (Cantor and Tinoco, 1965), disagree with the observed results. Thus, these two guanine-containing trinucleotides cannot be considered in the stacked conformation.

It is to be noted that in some cases the circular dichroic spectrum, despite its very low intensity, is different in shape from the sum of contributions of the corresponding monomers (*e.g.*, ApGpU). We feel that the optical activity of a disordered oligonucleotide may not always be equal to the sum of contribution of the monomers. The absence of a temperature-dependent circular dichroism and also the absence of circular dichroic bands (other than that similar to mononucleotides) is indicative of, but is not considered as absolute evidence for, the existence of random conformation. At present we are extending our studies of polynucleotides using other highly sensitive spectroscopic methods including hypochromicity in order to gain more information about the conformational characteristics of these oligonucleotides.

Discussion

The present investigation of trinucleotides has revealed some new aspects of polynucleotide conformational characteristics. One of the most important results is the indication that the presence of the guanine residue seems to weaken the stacking interaction in a single-stranded structure. This is first indicated by the relatively low value of the free-energy change at 0° for GpApUp as compared with other trinucleotides containing similar purine and pyrimidine sequences, such as ApApU and ApApCp (Table I). A second indication is obtained from a comparison of the thermal dependence of circular dichroism of ApGpUp and UpGpA which differs from that of ApApU. Finally, the unusually low intensity of the circular dichroism, or even the absence of the bands (*e.g.*, in GpUpG spectrum), at the lowest temperature, of guanine-containing trinucleotides, seems to bring further support concerning the disordered features of their conformation. The interpretation of these results is consistent with the particularity of the mononucleotide conformation defined in terms of the rotation about the glycosidic band. The torsion angle (ϕCN) between the purine and pyrimidine plane with the sugar C_1–O_1 will lead to two ranges of values corresponding to the *syn* and *anti* conformation (Donohue and Trueblood, 1960). The majority of the crystal structures of mononucleotides was found to be in the *anti* conformation (Haschemeyer and Rich, 1967) which is also suggested by the nuclear magnetic resonance studies in solution (Schweizer *et al.*, 1968). However, the calculations of Haschemeyer and Rich (1967) have shown that guanosine (deoxyguanosine) may also adopt the *syn* conformation. These authors calculated that there is only a small energy difference between the *syn* and *anti* conformation, which may render possible the existence of two stable isomers and their interconversion (see Jordan and

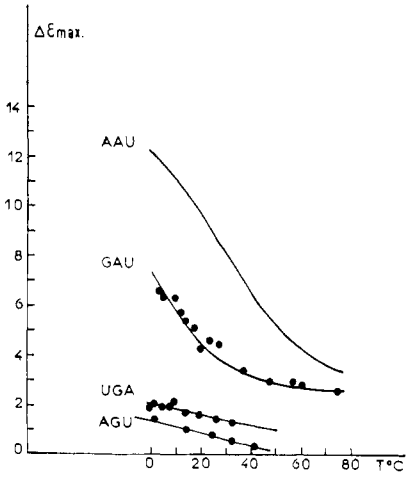

FIGURE 11: Circular dichroism temperature dependence for three-sequence isomers ApGpU, UpGpA, and GpApU, measured at the longer wavelength positive band ($\Delta\epsilon_{max}$). The ApApU thermal denaturation profile is shown for comparison.

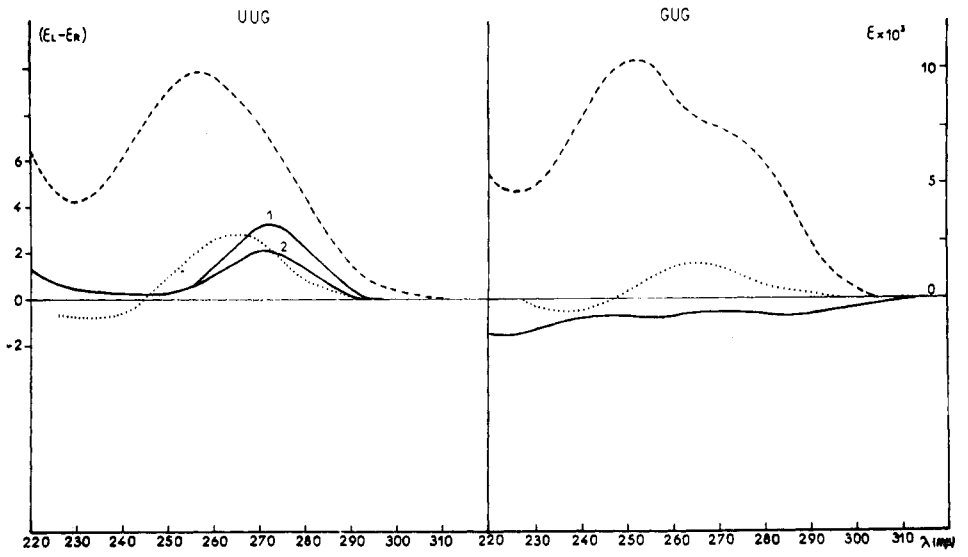

FIGURE 12: Circular dichroism spectra (——) of GpUpG in 0.01 M Tris (pH 7.4) at 0°, $5 \times 5 \times 10^{-5}$ M and UpUpG (1) at 0° and (2) at 25°, concentration 1.34×10^{-4} and 1×10^{-5} M per residue. (- - - -) Absorption spectra; (.....) represents the circular dichroism of the corresponding monomers constituents.

Pullman, 1968). This possibility of existence of *syn* and *anti* conformers of G in solution may well explain the exceedingly feeble rotatory power and very weak stacking interaction of trinucleotides like UpUpG, UpGpA, GpUpG, and GpUpA, where the guanosine residue is next to uridine. This guanosine tendency to form two conformers which differ by the rotation about the glycosidic C–N bond will be particularly facilitated by the presence, as nearest neighbor, of the uridine residue which has a tendency to unstack, particularly at low ionic-strength (Simpkins and Richards, 1967a,b). In contrast, one can expect the formation of the stacked conformation in the sequences when the guanine residue is next to other bases, *e.g.*, cytosine and adenosine. This can be illustrated by the properties of GpApU which displays the spectrum characteristic of a stacked base dissymmetric structure (Figure 8), and the thermal denaturation curve of a structural transition (Figure 11). Another example of the same hypothetical rule must be displayed by the two isomers GpApC and ApGpC. Both these compounds exhibit relatively intense circular dichroic bands (Figure 13) and melting behavior (in aqueous solutions without added salt) characteristic of single-stranded helical structures. However, one cannot definitely exclude the possibility of formation of intermolecular associations.

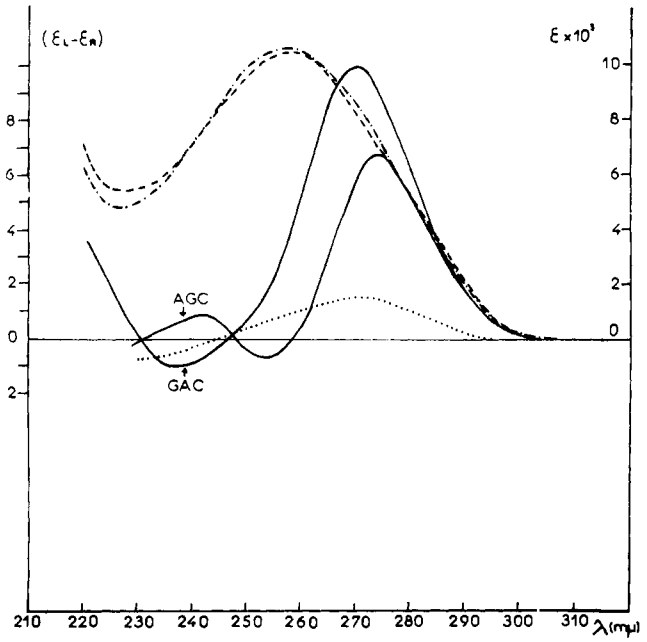

FIGURE 13: Circular dichroism spectra of ApGpC, 7.1×10^{-5} M per residue, and GpApC in 0.01 M Tris (pH 7.4) at 2°, concentration 7.1×10^{-5} and 8.3×10^{-5} M per residue, respectively.

The resulting biological implication seems particularly important for the three-dimensional structure of tRNA. The primary structure determinations have shown that the guanine residue is neighboring to uracil in almost all known tRNAs: alanine (Holley *et al.*, 1965), serine from yeast (Zachau *et al.*, 1966), valine (Baev *et al.*, 1967), serine from rat liver (Staehelin *et al.*, 1968), phenylalanine (RajBandhary *et al.*, 1967), and tyrosine (Madison *et al.*, 1966). Furthermore, these GpU-like sequences have been suggested to be located in the loops and particularly in the anticodon loop, which is in good agreement with the clover leaf arrangement proposed for various yeast tRNAs (Madison, 1968). This particular flexibility of the guanine and uracil sequences may first facilitate the formation of loops. Then the guanine-containing flexible loops may easily form base pairs with another arm or with the terminal end of the same tRNA molecule, and adopt a compact tertiary structure at appropriate temperatures (Cramer *et al.*, 1969; J. Bakes, G. Dirheimer, J. Brahms, in preparation; Henley *et al.*, 1966; Brahms, 1968).

The comparison of circular dichroic spectra of trinucleotides measured at various conditions of ionic strength, nucleotide concentration, and temperature, allows one to detect the presence of two other groups of compounds differing in their ordered conformation.

The first group is represented by compounds able to form,

at low temperatures, single-stranded structures, like ApApC, ApApU, and GpApU. This conclusion is supported by the similarity of spectroscopic and thermodynamic characteristics exhibited by these compounds under various conditions (Figures 6–8 and Table I). Also, the form of the spectra of these trinucleotides can be quite correctly predicted from the corresponding dinucleotides on the basis of semiempirical calculations (Cantor and Tinoco, 1965; Aubertin, 1968).

Our conclusion for the formation of the stacked-bases single-stranded structure by these trinucleotides is in agreement with previous studies of Cantor and Tinoco (1965, 1967), Zavil'gel'skii *et al.* (1966), and Inoue *et al.* (1967).

In the second group of compounds we found trinucleotides containing two guanine residues, like GpGpUp and GpGpCp, in which both spectral and thermodynamic properties are markedly different from previously described single-stranded structures. Their tendency to form double- or multiple-stranded associative forms appears clearly from the studies of the influence of ionic strength or nucleotide concentration on the spectral characteristics. Thus, the complex spectrum of GpGpUp at high ionic strength, composed of at least three positive and negative bands (Figure 1), disappears and approaches the one main band spectrum of monomers.

It is to be emphasized that from all the trinucleotides studied, only GpGpUp and GpGpCp, that is two guanine-containing trinucleotides, form double- or multiple-stranded complexes. This is also different from previous investigations of oligoadenylate (Brahms *et al.*, 1966), where it was found that the double-strand formation occurs only at chain length greater than the hexamer. The explanation of these differences probably resides in the tendency of guanine to form aggregates which is known from the studies of monomer GMP gels (Gellert *et al.*, 1962), and of polymers, poly G (Pochon and Michelson, 1965). Thus, the nature of these complexes should involve G–C base pairing, but also G–G and G–U hydrogen bonding. This particularity of guanine to form hydrogen-bonded complexes allows one to explain the absence of formation of double-stranded structure with any other trinucleotide. The possible formation of hydrogen-bonded base pairs between U and G, but not A and G (Crick, 1966), is confirmed by the studies of circular dichroism temperature dependence of corresponding polynucleotides: poly (U,G) and poly (A,G) (J. Brahms and M. Grunberg-Manago, in preparation). Unfortunately, it was not possible to obtain direct evidence about guanine–guanine hydrogen bonding because of experimental difficulties in the investigating of GpG. The feasibility of formation of double-stranded structures by trinucleotides was theoretically predicted by Eigen (1968), and is in agreement with a recent paper of Jaskunas *et al.* (1968), which appeared when this work was completed.

In summary, the presence of guanine in an oligonucleotide chain has a dual role. First, in conditions allowing only the formation of single-stranded structures, guanine appears to have a tendency for unstacking which is particularly pronounced when it is neighboring with uridine. This weakening of stacking interaction can be explained by the rotation of guanine around the glycosidic C–N bond which will lead to the formation of *syn* and *anti* conformers. Secondly, the presence of guanine at appropriate conditions of ionic strength and nucleotide concentrations will facilitate complementary G–C base pairing or G–G, G–U hydrogen bonding and the formation of double- or multiple-stranded helical structures. It will also be of importance for the tertiary structure of RNAs.

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Aminoacyl Nucleosides. VI. Isolation and Preliminary Characterization of Threonyladenine Derivatives from Transfer Ribonucleic Acid*

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ABSTRACT: Transfer ribonucleic acid was hydrolyzed by acid under conditions that released the purine residues. Examination of the hydrolysate by means of ion-exchange chromatography on a sulfonic acid resin revealed the presence of a "new" component. This component contains 1 mole of adenine and 1 mole of threonine. It has been detected in the unfractionated transfer ribonucleic acid of yeast (0.28 mole %), *Escherichia coli* (0.07 mole %), and calf liver (0.19 mole %).

A number of articles reports the presence of amino acids or small polypeptides, apart from the amino acids attached to the acceptor end of tRNA molecules, bound to nucleic acids. Ingram and Sullivan (1962) and Akashi *et al.* (1965), for example, have reported the presence of amino acids bound to RNA which cannot be removed through the use of extensive deproteinizing procedures. Balis *et al.* (1964) and Olenick and Hahn (1964) have reported the presence of amino acids in highly purified preparations of DNA isolated from a variety of sources. The nature of the amino acid–nucleic acid linkage, however, has not been elucidated. Bogdanov *et al.* (1962) have reported that tRNA contains amino acids attached to the phosphate residues. Harris and Wiseman (1962) have also reported the presence of small polypeptides attached to the phosphate residue of yeast nucleic acid; the exact nature of such complexes has not been described.

Hall (1964) and Hall and Chheda (1965) reported that amino acid nucleoside derivatives had been isolated from yeast tRNA in which the aminoacyl group was attached to the N^6 position of adenosine. It appeared that a series of these compounds existed and that they all possessed the same basic structure. We have continued our investigations of these amino acid adenosine derivatives of tRNA in order to clarify their exact nature and to establish their significance to tRNA structure and function.

The isolation procedure used in the original work (Hall, 1964, 1965) was lengthy and the yield of the desired amino acid adenosine derivatives was very low. Therefore, more convenient and efficient methods for isolation were sought. Purines are readily cleaved from RNA by mild acid hydrolysis and this procedure seemed to offer a relatively fast and effective means of obtaining the free base of the amino acid nucleosides. Yeast tRNA was subjected to such a procedure and the released purine derivatives were separated by means of ion-exchange chromatography. The acid hydrolysate of yeast tRNA yielded three amino acid adenine derivatives, each of which contained threonine. This paper describes the method of isolation, the preliminary characterization of these compounds, and the detection of threonine–adenine residues in tRNA of *E. coli* and mammalian tissue. The accompanying

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