

Proton Magnetic Resonance Studies on the Conformation of the Hexanucleotide, GmpApApYpAp ψ p, and Related Fragments from the Anticodon Loop of Baker's Yeast Phenylalanine Transfer Ribonucleic Acid[†]

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ABSTRACT: A hexanucleotide, GmpApApYpAp ψ p, and 11 related compounds from the anticodon loop of Baker's yeast tRNA^{Phe} were studied by proton magnetic resonance from 100 to 250 MHz. Totally 19 resonance lines from all the base protons (H₈, H₆, and H₂), H₁, and methyl proton resonances of the hexamer have been assigned by a systematic "incremental procedure" in comparing all the related shorter fragments. Emphasis is given to the Y base and its stacking conformation with respect to its nearest neighboring bases. The results reveal a strong tendency of the purine

bases to have a maximal extent of base-base overlap with their neighbors in the sequence. This tendency is manifested in a zigzag (or balcony-like) mode of base-stacking pattern of the -ApYpA- sequence in the hexamer in which the -pA-residue tends to stack toward the adduct ring (C₁₀, C₁₁, and N₁₂) of Y. This tendency is also shown in the formation of a stack of GmAAA closing the gap left behind by the excision of Y in the hexamer GmpApAp—pAp ψ p. The implication of these findings to the structure and function of tRNA is discussed.

Significant advances in the understanding of yeast tRNA^{Phe} structure in crystals have been made recently by the X-ray diffraction analysis (Kim et al., 1974; Robertus et al., 1974a,b). The structure of tRNA in solution has been investigated by nuclear magnetic resonance (NMR) techniques from various laboratories (Crawford et al., 1971; Danchin and Guéron, 1970; Schweizer et al., 1973; Kearns and Shulman, 1974). Much progress has been obtained, especially about the hydrogen bond formation between the base pairs in tRNA by investigating the NH proton region (Kearns and Shulman, 1974). Much less is known about the single-strand regions and the tertiary structure (such as folding) of tRNA where no intramolecular hydrogen bond of NH takes place.

Recently, in a brief communication we reported a ¹H NMR study on the methyl and methylene resonances of intact tRNA^{Phe} from yeast which revealed tertiary structures of the individual loop sections (Kan et al., 1974). However, interpretation of the ¹H NMR spectra in terms of contributions from tertiary structure demands that contributions from the primary and secondary structure be well characterized. Therefore, the ¹H NMR study on the appropriate fragments of tRNA is a prerequisite to a meaningful ¹H NMR study on whole tRNA. Optical properties of these

fragments have been reported previously by Maelicke et al. (1973, 1975).

In this paper, we report the ¹H NMR studies on a hexanucleotide GmpApApYpAp ψ p¹ and 11 related compounds from the anticodon loop of tRNA^{Phe} from yeast. A preliminary report of these results has been previously presented (Ts'o et al., 1975a).

Materials and Methods

The enzymic digestion, the separation, the purification, and the identification of the 11 compounds listed in Table I have been previously published by Maelicke et al. (1973, 1975). NMR samples were made by dissolving these oligomers (quantities are listed in Table I) into 0.3 ml of D₂O with 0.01 M phosphate buffer (pD 7.4).

The ¹H NMR spectra were recorded on a Varian HR-220 located in the University of Pennsylvania, a HA-100 spectrometer at the Johns Hopkins University, and a 250-MHz spectrometer built by the Mellon Institute, Carnegie-Mellon University, Pittsburgh, Pa. The 250-MHz spectrometer is equipped with a probe operating at ambient temperature of 32°, while the HR-220 and HA-100 are equipped with a V-4257 and a V-6507 variable temperature accessory, respectively. Temperatures were measured by monitoring the separation between a methyl or methylene resonance and the -OH resonance of the methanol or ethylene glycol. All three spectrometers are equipped with Fast Fourier Transform Unit. A trace amount of *tert*-butyl alcohol was added to the sample as our internal standard, but all values of the chemical shifts have been converted to the

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¹ Abbreviations used are: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Cm, 2'-*O*-methylcytidine; m⁵C, 5-methylcytidine; Gm, 2'-*O*-methylguanosine; ψ , pseudouridine; Y, nucleoside Y; N>p, nucleoside 2',3'-cyclic monophosphate; Np, nucleoside 3'-monophosphate; pN, nucleoside 5'-monophosphate; pNp, nucleoside 3',5'-diphosphate; T₁, spin-lattice relaxation time.

Table I: A List for the Anticodon Loop Fragments of Yeast tRNA^{Phe}.^a

Compounds	Quantity (absorbance unit at 260 nm)
Y ₃₇ p	15
Y ₃₇ >p	14.4
Y ₃₇ pA ₃₈ p	15
pA ₃₈ pψ ₃₉ p	15
Gm ₃₄ pA ₃₅ p	15
Gm ₃₄ pA ₃₅ pA ₃₆ pY ₃₇	25
Gm ₃₄ pA ₃₅ pA ₃₆ pY ₃₇ pA ₃₈ p	16
Gm ₃₄ pA ₃₅ pA ₃₆ p— ₃₇ pA ₃₈ pψ ₃₉ p	29
Gm ₃₄ pA ₃₅ pA ₃₆ pY ₃₇ pA ₃₈ pψ ₃₉ p	44
A ₃₁ pCm ₃₂ pU ₃₃ pGm ₃₄ pA ₃₅ pA ₃₆ p— ₃₇ pA ₃₈ pψ ₃₉ p ^m C ₄₀ pU ₄₁ pG ₄₂ p	60
A ₃₁ pCm ₃₂ pU ₃₃ pGm ₃₄ pA ₃₅ pA ₃₆ pY ₃₇ pA ₃₈ pψ ₃₉ p ^m C ₄₀ pU ₄₁ pG ₄₂ p	90

^aThe subscripted numbers (double digits) indicate that the sequential position of individual residue in the yeast tRNA^{Phe}. The numbers (1–3) located at the top of each adenine residue are used for the distinction of the three adenine residue in the ¹H NMR study.

DSS standard (Borer et al., 1975). For the sake of convenience, the negative signs of all chemical shift values are omitted. The chemical shift data of well-separated signals are accurate within 0.01 ppm.

Results and Discussion

Assignment of the Base, H₁', and Methyl Proton Resonances. The easily resolved resonances from the 11 compounds in Table I as well as those from ψp can be divided into three spectral regions: (i) the base proton resonances from the H₈ of A, Gm and Y; H₂ of A, as well as H₆ of ψ at 7.0–8.5 ppm; (ii) the H₁' resonances of A, Gm, and Y at 5.5–6.5 ppm; and (iii) methyl proton resonances from Gm, Y, m⁵C, and Cm in dodecamer and dodecamer–Y at 1.8–4 ppm.

The assignment was determined at relatively high temperature (63°) where base stacking and intermolecular aggregation are minimized. The following sections, (a) to (e), along with Figure 2 trace the identity of the resonances from one base at a time through each fragment in this series of oligomers.

(a) ψ Residue. At the base proton region of the spectrum of ψp, there is only one singlet at 7.7 ppm. This singlet results from the broadening effect on a doublet. The ⁴J_{1'–6} is from 0.6 to 1.3 Hz (M. P. Schweizer, private communication). Obviously, this peak belongs to H₆ in confirmation of the previous assignment of the pseudouridine spectrum published by Cohn (1960). As shown in Figure 1, the H₆ resonates as a singlet in contrast to the H₆ of uridine. The H₁' resonance of the ψ residue is hidden under the HDO peak as reported by Cohn (1960). The H₁' resonance of the residue is located considerably upfield as compared to those of other nucleosides since the C₁' of ψ residue is linked to another carbon atom of the base (C₅) (Figure 1) instead of nitrogen (N₁ or N₉) as in other nucleosides. The assignment of the H₆ resonance of ψ residue in spectra of compound pApψp, hexamer, hexamer–Y, dodecamer, and dodecamer–Y is easy since there is only one singlet at this region around 7.5 ppm (Figures 2–4). In fact, H₆ resonance of ψ residue is the only resonance from the base proton region (Figure 2) that can be unambiguously assigned in the complex spectra of the dodecamer and dodecamer–Y. As shown in Figure 2 and Table II, the chemical shift values of H₆ of ψ residues

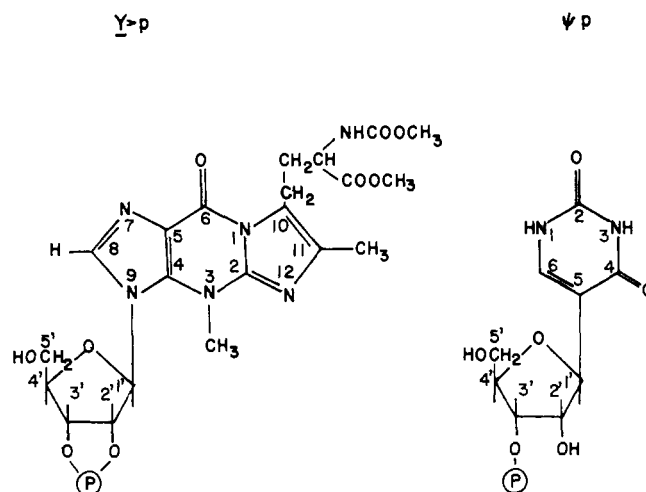


FIGURE 1: Molecular structure of Y>p and ψp.

in the oligomer series became increasingly upfield from dimer to hexamer and finally to dodecamer, and from high temperature to low temperature. These data indicate the expected increase in stacking interaction among the bases under this condition.

It should be noted that a similar singlet was observed in the ¹H NMR spectrum of the hexanucleotide, GmpApA-pYpApψp, isolated from torula yeast tRNA^{Phe} (Kreishman et al., 1974). This singlet was assigned to H₈ of Y base in this report (the Y base in this fragment from torula yeast slightly differs from that shown in Figure 1). The error of this assignment has now been recognized in view of this work (M. P. Schweizer, private communication). One of the most convincing arguments indicating the error is the presence of this singlet in the spectra of hexamer and dodecamer without the Y base (Figures 2 and 4).

(b) Gm Residue. The H₈, H₁', and the 2'-O-methyl resonances in Gm residue are readily assigned in GmpAp, since the GpA spectrum has been assigned previously (Ts'o et al., 1969). At 63°, the spectrum of GmpAp is very similar to that of GpA, with an additional methyl resonance located at ~3.4 ppm. On this basis, the most upfield signals are in the base proton region and H₁' region of Gm, respectively (Figure 2). In the tetramer, pentamer, hexamer, and hex-

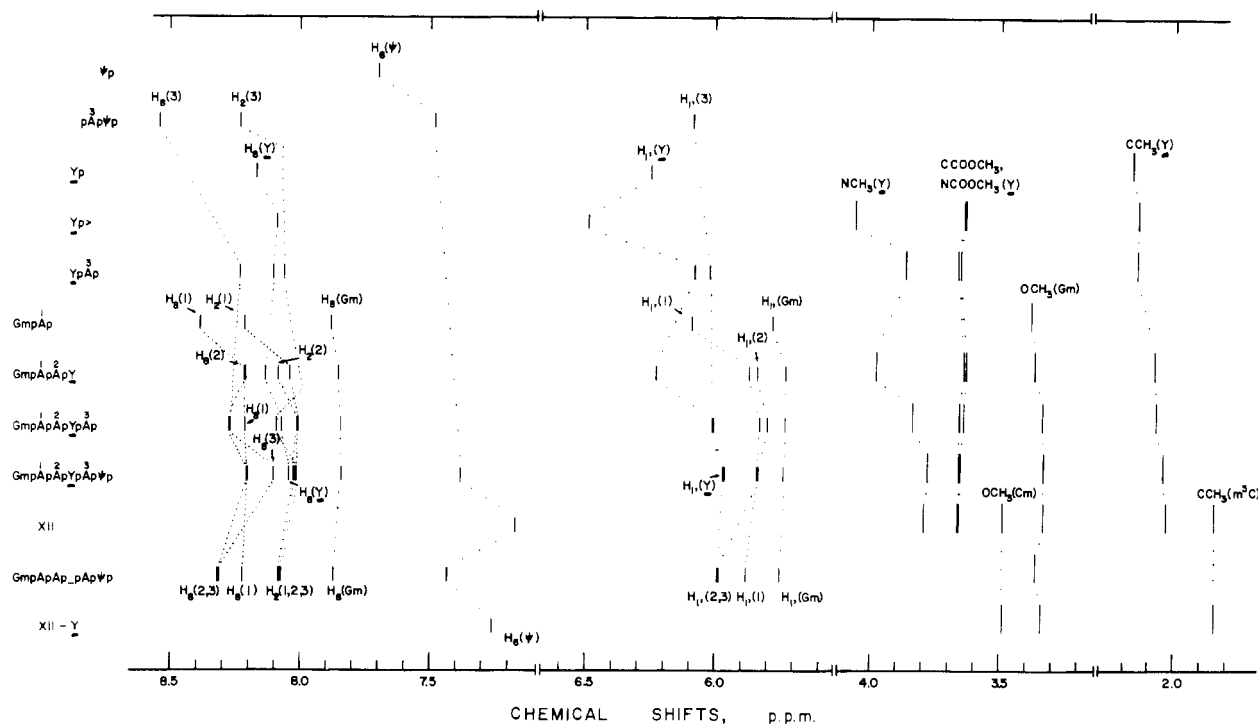


FIGURE 2: Assignments of the proton resonances in 12 compounds by systematic "incremental assignment". The ^1H NMR spectra were recorded at 63° with the quantities of the compounds in Table I dissolved in 0.3 ml of D_2O , containing 0.01 M phosphate buffer (pD 7) and $5 \times 10^{-5} M$ EDTA. XII and XII-Y are dodecamer and dodecamer-Y.

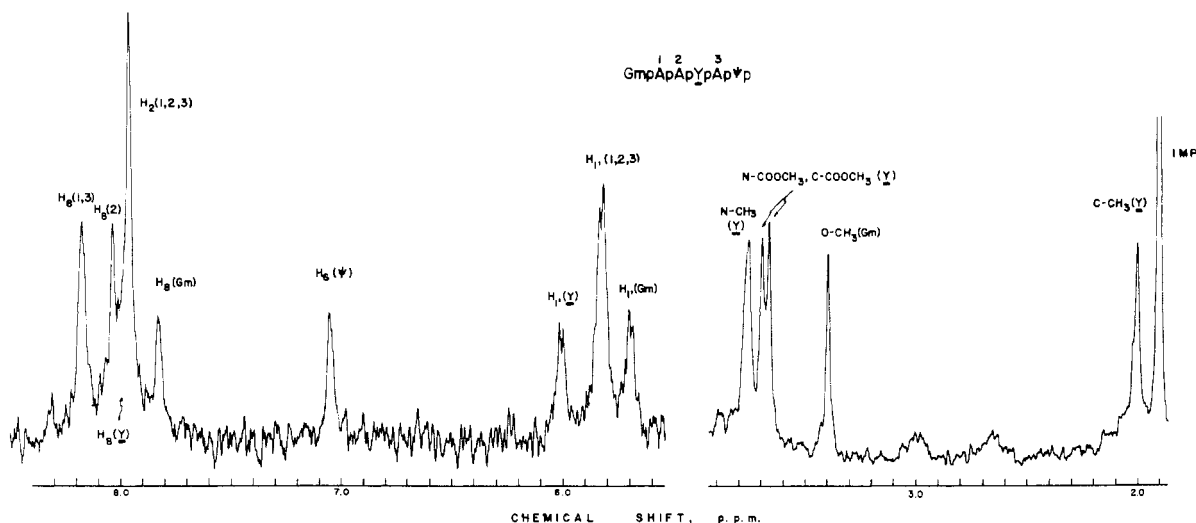


FIGURE 3: A 250-MHz ^1H NMR spectrum of GmpApApYpApΨp recorded by continuous wave mode at 32° together with all the assignments. A signal for impurity was found at ~ 1.9 ppm.

amer-Y, the Gm residue is always located at the 5'-terminus. Thus, the shielding effect received by the Gm residue, which comes chiefly from the nearest adenine residue, is not increased substantially at 63° by the continuous addition of other residues at the 3'-terminus of the oligomer series (Figure 2). At 63° , the H_8 and $\text{H}_{1'}$ resonances from the Gm remain at the most upfield position at the base proton region and the $\text{H}_{1'}$ region, respectively, in the oligomer series up to hexamer. The 2'-O-methyl resonance is also well resolved from others and can be readily assigned by following the spectra of the fragments in this series. The display in Figure 2 demonstrates the utility of the spectra of the constituent fragments for the assignment of the complex ^1H NMR spectrum from oligonucleotides. The approach illustrated in

Figure 2 is similar to the powerful systematic "incremental assignment" procedure employed in the study of the hexamer A_2GCU_2 spectra (Borer et al., 1975). The H_8 and $\text{H}_{1'}$ resonances of Gm cannot be resolved from the complex spectra of the dodecamer and dodecamer-Y.

The chemical shift data of H_8 , $\text{H}_{1'}$, and OCH_3 resonances of the Gm residue in various oligonucleotide fragments at various temperatures are listed in Table III.

(c) *Y Residue*. Two preparations of the mononucleotide of Y base obtained by T_2 RNase digestion of the hexamer were investigated. The ^1H NMR spectrum of one preparation identified as Y>p is shown in Figure 5. From the spectrum of Y>p in dilute solution, only the resonances from the methyl groups, the $\text{H}_{1'}$, and the H_8 can be detected. The

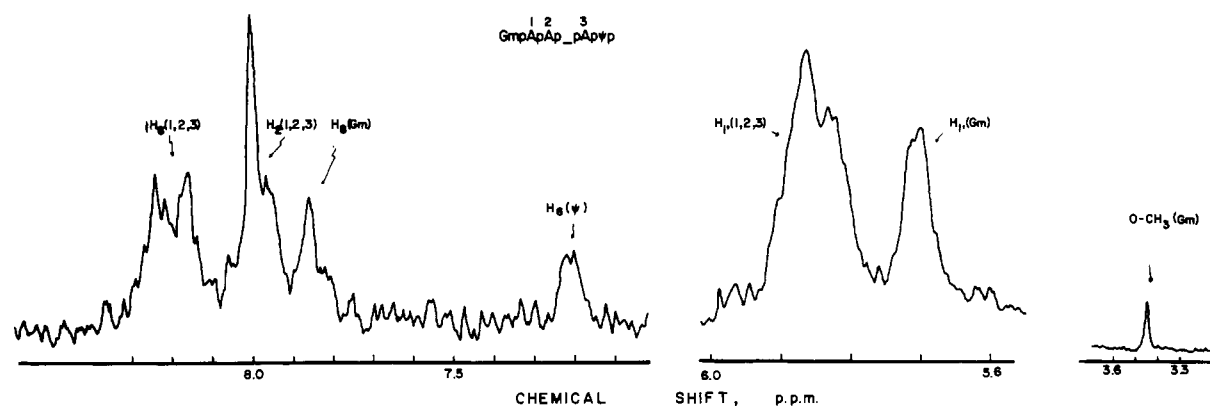


FIGURE 4: A 250-MHz ^1H NMR spectrum of GmpApAp—pApψp recorded by Fourier transform mode (160 pulses) at 32° together with all the assignments.

Table II: The Chemical Shifts of H_8 Resonance of ψ Base in the Following Compounds (in ppm from DSS).

Temp (°C)	pApψp	GmpApAp—pApψp	GmpApApYpApψp	Dodecamer—Y	Dodecamer	ψp
7.5	7.22	7.08	6.92	6.94	6.70	7.70
18	7.26	7.08	6.97	6.96	6.80	
27 ^a				7.00	6.82	7.69
32 ^b		7.21	7.06			
42	7.39	7.30	7.19			7.68
63	7.48	7.43	7.38	7.26	7.17	7.66

^aFrom a 100-MHz NMR spectrometer. ^bFrom a 250-MHz NMR spectrometer.

Table III: The Chemical Shifts of the H_8 , H_1' , and $2'\text{-O-CH}_3$ Resonances of the Gm Nucleoside in the Following Compounds.

	Temp (°C)	(ppm from DSS)		
		H_8	H_1'	OCH_3
GmpAp	7.5	7.90	5.73	3.50
	18	7.89	5.73	3.47
	42	7.89	5.76	3.43
	63	7.88	5.78	3.39
		7.84	5.73	3.56
GmpApApY	7.5	7.84	5.73	3.56
	18	7.84	5.74	3.52
	32 ^a	7.84	5.74	3.47
	42	7.85	5.76	3.43
	63	7.85	5.73	3.37
GmApApYpAp	7.5	7.83	5.70	3.44
	18	7.83	5.69	3.42
	32 ^a	7.82	5.69	3.39
	42	7.83	5.71	3.37
	63	7.84	5.73	3.34
GmpApAp—pApψp	7.5	7.88	5.74	3.51
	18	7.88	5.75	3.50
	32 ^a	7.86	5.71	3.45
	42	7.87	5.75	3.42
	63	7.87	5.75	3.36
GmpApApYpApψp	7.5	7.83	5.73	3.47
	18	7.84	5.72	3.43
	32 ^a	7.83	5.71	3.39
	42	7.84	5.71	3.36
	63	7.84	5.74	3.33
Dodecamer—Y	7.5			3.61
	18			3.55
	27 ^b			3.43
	40			3.39
	62			3.34
Dodecamer	7.5			3.48
	18			3.45
	27 ^b			3.41
	63			3.33

^aFrom a 250-MHz NMR spectrometer. ^bFrom a 100-MHz NMR spectrometer.

other methylene or methine proton resonances (multiple) from the side chain and the other ribose proton resonances could not be detected from this spectrum presumably due to either low intensity of the signal or to the masking by the HDO peak. In Figure 5, the singlet at 8.12 ppm and the doublet at 6.55 ppm can be readily assigned to the H_8 base proton and the H_1' , respectively. The four methyl singlets (3 protons), one near 4.1 ppm, two at 3.7 ppm, and one at 2.2 ppm, are assigned to the $\text{N}_3\text{-CH}_3$, two COOCH_3 , and the $\text{C}_{11}\text{-CH}_3$, respectively, according to the work of Nakanishi et al. (1970). The two methyl resonances from the methyl esters of the Y residue are very close to each other (less than 0.03 ppm apart) in the spectra from all the fragments; therefore, it is unnecessary to assign them separately. In the other preparation, designated as Yp, certain impurities were present which masked the spectral region from 3.6 to 4.2 ppm. However, the H_8 resonance, the H_1' resonance, and the C-CH_3 resonance could be readily recorded (Table IV and Figure 2). The chemical shift of the $\text{C}_{11}\text{-CH}_3$ resonance of the Yp sample is exactly the same as that from the Y>p sample. The H_1' resonance (6.25 ppm) in the Yp spectrum has a coupling constant of 5.5 Hz and is 0.26 ppm more upfield than the H_1' resonance (6.51 ppm) in the Y>p spectrum, which, in addition, has a coupling constant of 4.0. It is known that the H_1' resonance of a nucleoside 2',3'-cyclic phosphate tends to be more deshielded and has a lower coupling constant as compared to the H_1' of a nucleoside 3'-phosphate (Lapper and Smith, 1973). For example, the chemical shift of H_1' resonance in 3'-AMP and 2',3'-cAMP is 6.13 and 6.54 ppm, respectively (L. S. Kan, unpublished data). In fact, these ^1H NMR data and this reasoning provide the elucidation of the chemical nature of these two mononucleotides of Y base. The H_8 resonance (8.21 ppm) of the Yp is also downfield from the H_8 resonance (8.12 ppm) of the Y>p, reflecting a small change of glycosyl bond rotation influenced by the phosphate attachment to the ribose.

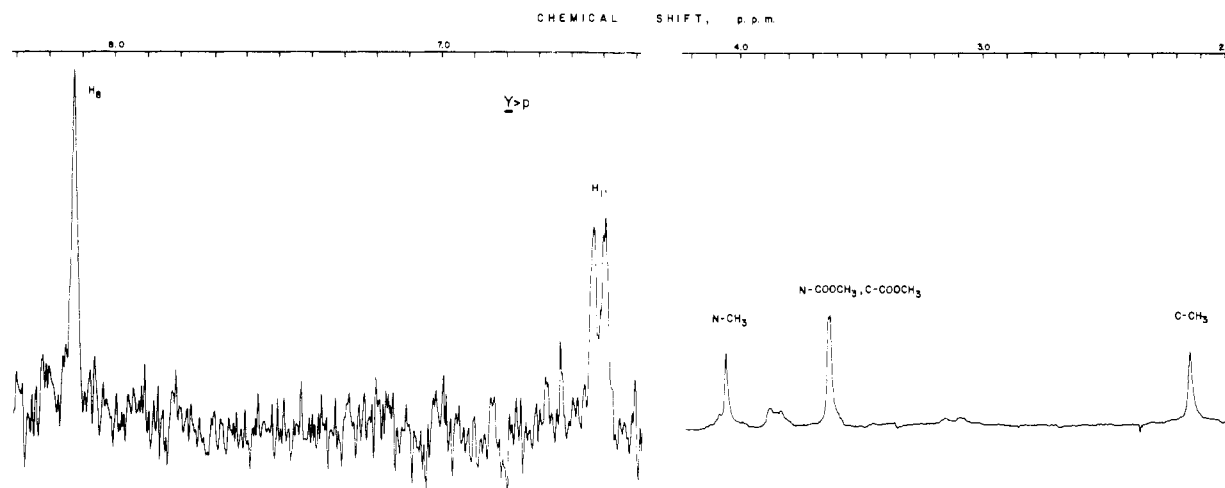


FIGURE 5: A 100-MHz ^1H NMR spectrum of Y>p recorded by Fourier transform mode (500 pulses) at 27°.

Table IV: The Chemical Shifts of H_8 , $\text{H}_{1'}$, and Methyl Resonances of the Y Nucleoside in the Following Compounds (in ppm from DSS).

	Temp (°C)	H_8	$\text{H}_{1'}$	$\text{N}-\text{CH}_3$	$\text{N}-\text{COOCH}_3$ and $\text{C}-\text{COOCH}_3$	$\text{C}-\text{CH}_3$
Y>p	6	8.12	6.52	4.06	3.64, 3.64	2.13
	18	8.12	6.52	4.07	3.64, 3.64	2.14
	27 ^a	8.12	6.51	4.08	3.65, 3.64	2.16
	36	8.12	6.51	4.08	3.66, 3.65	2.16
	60	8.10	6.49	4.07	3.66, 3.64	2.17
	66 ^a	8.09	6.49	4.07	3.67, 3.64	2.17
Yp	7.5	8.21	6.25			2.13
	19	8.21	6.26			2.16
	20.5 ^a	8.20	6.25			2.14
	28	8.20	6.25			2.16
	42	8.19	6.26			2.17
	63	8.17	6.25			2.19
YpAp	7.5	8.23	6.02	3.77	3.67, 3.63	2.10
	18	8.21	6.06	3.79	3.66, 3.64	2.12
	42	8.15	6.07	3.88	3.66, 3.66	2.14
	63	8.10	6.08	3.87	3.66, 3.65	2.16
GmpApApY	7.5	8.1	6.16	3.95	3.65, 3.63	2.12
	18	8.12	6.16	3.95	3.64, 3.63	2.11
	32 ^b	8.14	6.17	3.97	3.64, 3.64	2.10
	42	8.14	6.18	3.97	3.64, 3.64	2.10
GmpApApYpAp	63	8.13	6.23	3.98	3.64, 3.63	2.09
	7.5	8.02	5.94	3.75	3.68, 3.66	2.06
	18	8.03	5.95	3.77	3.68, 3.67	2.07
	32 ^b	8.07	5.97	3.78	3.66, 3.66	2.06
GmpApApYpApψp	42	8.08	5.99	3.80	3.66, 3.66	2.08
	63	8.07	6.01	3.84	3.66, 3.64	2.08
	7.5	7.96	6.04	3.75	3.71, 3.66	1.98
	18	7.97	6.03	3.76	3.70, 3.66	1.99
Dodecamer	32 ^b	8.00	6.02	3.75	3.69, 3.66	2.00
	42	8.02	6.01	3.76	3.66, 3.66	2.03
	63	8.02	5.97	3.78	3.66, 3.66	2.05
	7.5			3.75	3.72, 3.72	1.96
	18			3.76	3.70, 3.70	1.97
	27			3.78	3.69, 3.69	1.99
	63			3.79	3.66, 3.66	2.03

^aData obtained from a 100-MHz NMR spectrometer. ^bData obtained from a 250-MHz NMR spectrometer.

The assignment of the resonances of the Y residue in the spectra of the fragments is straightforward in the methyl resonance region from 2 to 4 ppm. As shown in Figure 2, the four methyl resonances from the Y base are well resolved from the 2'-O-methyl resonances of the Gm residue in the hexamer and from the 2'-O-methyl resonance of Cm residue and the 5-methyl resonance of m⁵C residue in the dodecamer. The methyl resonances from Cm and m⁵C residues can be assigned in reference to the chemical shifts of

the monomers. The assignment of the methyl resonances from the Y base in the spectra of the hexamer and dodecamer can be further confirmed by comparison with the spectra from hexamer-Y and dodecamer-Y. As indicated in Figure 2, the spectra from these two fragments without Y base do not contain the methyl resonances assigned to the Y base. It is interesting to point out that the assignment of the N₃-Me, C₁₁-Me of Y; and C_{2'}-O-Me of Gm in the hexamer agreed with that of the hexamer with the same se-

quence from torula yeast tRNA^{Phe} (Kreishman et al., 1974).

The major problem in the assignment is the H₈ and the H_{1'} resonances. The resonance of H₈ from the Y base can be distinguished from the resonance of H₂ of adenine because of the larger *T*₁ value of the latter (Ts'o et al., 1973). With increasing 90° pulse rate, the intensity of the H₂ resonance tends to be lower than that of the H₈ because of its slow recovery. Another important fact is that the H₈ resonances of adenine (~8.6 ppm), of the Yp (~8.2 ppm), and of the guanine in GmpAp (~7.9 ppm) are well separated. In addition, the resonances of the Y residue in the fragments should be more shielded than those from the mononucleotide Yp; therefore, the H₈ resonance of Y residue in the oligonucleotide should be located at a higher field than that of Yp. From all these considerations, the H₈ resonance of Y base can be assigned in the spectra of YpAp, tetramer, pentamer, and hexamer at 63° (Figure 2). The H₈ resonance of Y is the only H₈ resonance with a short *T*₁, which is at a lower field position than the already assigned H₈ of Gm residue. The H₈ of Y base is located at a higher field position than the H₈ resonances of all the A residues in these fragments. This assignment is confirmed by the spectrum of hexamer-Y which does not contain this signal.

The assignment of the H_{1'} resonance of the Y residue is more complex. In the spectrum of YpAp, the H_{1'} resonance of Y residue is the doublet at a lower field position, since the H_{1'} signal at a higher field position must belong to the A residue. The assignment of this H_{1'} resonance to the A residue in YpAp is based on the fact that the already assigned H_{1'} resonance (Figure 2) of A residue from pAp ψ p is located at about the same field position as the designated H_{1'} resonance of A in YpAp, but significantly higher than the remaining doublet in the spectrum. Also, the assignment is based on the reasoning that the H_{1'} resonance of A residue in YpAp should not be more deshielded than the same H_{1'} resonances in pAp ψ p. The H_{1'} resonances of Y in GmpApApY can be assigned also from the following deductions. The H_{1'} resonance of Gm residue has been previously assigned to be the one in the highest field at this region. The molecular model of the GmpApApY clearly indicates the two H_{1'} from the two A residues (1 and 2) are sandwiched in the stack with the H_{1'} from the Y residue located outside of the stack. In addition, the H_{1'} resonance occupying the lowest field position (6.23 ppm) is more deshielded than the H_{1'} resonances from A residue in GmpAp and in pAp ψ p (Figure 2). Therefore, this low-field H_{1'} resonance at 6.23 ppm must belong to the H_{1'} of Y residue. The addition of a -pAp residue to GmpApApY to form GmpApApYpAp should not affect the resonance positions of the H_{1'} of the Gm, the A(1), or of the A(2) residue as indicated in Figure 2. The resonance position of H_{1'} of the A(3) residue should be similar to that found for H_{1'} of the A(3) residue in YpAp; in addition, the H_{1'} of the Y residue in the pentamer should be much more shielded than that in GmpApApY and should be slightly more shielded than that in YpAp. From the above reasoning, the signal at 6.01 ppm (Figure 2, Table IV) which contains two resonances can be unambiguously assigned to both H_{1'} of the A(3) residue and H_{1'} of the Y residue. The hexamer is formed by the addition of ψ p residue to the pentamer. As described above, the H_{1'} resonance of ψ residue is shifted to the HDO region and is absent from the H_{1'} region of the common nucleosides. In comparison to the spectrum of pentamer, the five H_{1'} resonances from the hexamer are not greatly affected by addi-

tion of the ψ p residue which is not expected to have a large ring-current shielding effect. The signal containing both H_{1'} resonances from the Y residue and the A(3) residues is moved slightly upfield as anticipated and there is a very minor downfield shift of both H_{1'} resonances of the two A residues (1 and 2) which now merge together (Figure 2). The H_{1'} resonances in the spectrum from the hexamer-Y can also be assigned readily with the understanding that the H_{1'} resonance from the ribose moiety previously attached to the Y base now becomes part of an aldehyde (-CH₁O). This transformation took place during the excision of the Y base in mild acid (Thiebe and Zachau, 1968) with the concomitant opening of the ribose ring. The proton of the aldehyde group usually resonates at about 10 ppm, totally outside of the present spectral range. The H_{1'} resonance at 5.75 ppm has been previously assigned to the Gm residue and the H_{1'} resonance at 5.88 can be assigned to A(1) residue with certainty since the chemical shifts of this residue should be least affected by the excision of the Y (Figure 2). The remaining signal at 5.99 ppm which contains two resonances can now be assigned unambiguously to both H_{1'} protons in the A(2) and A(3) residues.

(d) *A Residue*. Since there are three A residues in the hexamer, these A residues are numbered as 1, 2, and 3, starting from the 5'-terminus (Table I). It should also be noted that the H₂ resonance can be separated from the H₈ resonance based on the difference in their *T*₁ values as described above. The assignments for the resonances from A residues in the spectra of the dimers pAp ψ p, YpAp, and GmpAp are straightforward and in fact have been described in the above sections concerning the assignments for resonances from the ψ , Y, and Gm residues.

For the tetramer, the resonances of the Gm residue and Y residue have been assigned in the preceding sections. The main problem is to distinguish the resonances from one A residue from those of the other. At present, such a distinction cannot be based entirely on chemical data and has to rely, at least in part, on considerations from the molecular model. The two H₈ resonances from the two A residues merge together and thus can be assigned with one δ value. This observation is consistent with the prediction from the model which has all nucleotides in anti conformation and from the knowledge that the ring-current shielding effects of the five-membered imidazole ring from Gm, A, and Y are about the same. On the other hand, in this model, the H₂ of A(1) is mostly overlapped by the pyrimidine ring of A(2), while the H₂ of A(2) is mostly overlapped by the pyrimidine ring of Y. As described below, the ring-current shielding effect of the pyrimidine ring of Y (~0.83 ppm) is less than the same ring of A (~1.28 ppm, Giessner-Prettre and Pullman, 1970). From this consideration, therefore, the more shielded H₂ resonance at 8.04 ppm (63°) is assigned to the A(1) residues and the less shielded H₂ resonance at 8.08 ppm assigned to A(2) residue (Table V, Figure 2). The situation is different for the H_{1'}, which is more overlapped by the imidazole ring of the 3' residue and less by the pyrimidine ring of the 5' residue (see Table VI, B). The H_{1'} of A(1) is sandwiched between the pyrimidine ring of the 3'-Gm residue and the imidazole ring of the 5'-A(2) residue, while the H_{1'} of A(2) is sandwiched between the pyrimidine ring of the 5'-A(1) residue and the imidazole ring of the 5'-Y residue. Based on this model and the consideration of the magnitudes of the ring-current shielding effect of different rings in various bases, the H_{1'} resonance at a higher field (5.84 ppm, 63°) is assigned to the A(2) residue

Table V: The Chemical Shifts of H₂, H₈, and H₁' Resonances of the Adenosine in the Following Compounds (in ppm from DSS).

	Temp (°C)	H ₈			H ₂			H ₁ '		
		1	2	3	1	2	3	1	2	3
1 GmpAp	7.5	8.38			8.18			6.09		
	18	8.38			8.18			6.08		
	42	8.39			8.20			6.09		
	63	8.38			8.21			6.09		
3 pApψp	7.5			8.63			8.16			6.08
	18			8.61			8.18			6.09
	42			8.57			8.21			6.09
	63			8.54			8.23			6.09
3 YpAp	7.5			8.23			7.92			6.10
	18			8.26			7.95			6.07
	42			8.30			8.00			6.04
	63			8.33			8.06			6.02
1 2 GmpApApY	7.5	8.08	8.08		7.90	8.00		5.78	5.78	
	18	8.11	8.11		7.93	8.01		5.80	5.80	
	32 ^a	8.14	8.14		7.96	8.03		5.81	5.79	
	42	8.17	8.17		8.00	8.05		5.85	5.82	
1 2 3 GmpApApYpAp	63	8.21	8.21		8.04	8.08		5.87	5.84	
	7.5	8.09	8.10	8.10	7.92	7.92	7.95	5.80		5.99
	18	8.13	8.15	8.15	7.93	7.93	7.95	5.80	5.78	5.99
	32 ^a	8.15	8.19	8.19	7.95	7.95	7.95	5.80	5.78	5.99
1 2 3 GmpApAp—pApψp	42	8.18	8.23	8.23	8.00	8.00	8.08	5.81	5.78	5.99
	63	8.21	8.27	8.27	8.01	8.01	8.09	5.83	5.80	6.01
	7.5	8.14	8.18	8.18	7.92	7.96	7.96	5.82	5.82	5.82
	18	8.16	8.23	8.23	7.94	7.97	7.97	5.86	5.86	5.86
1 2 3 GmpApApYpApψp	32 ^a	8.17	8.24	8.24	7.96	8.01	8.01	5.83	5.86	5.86
	42	8.19	8.27	8.27	8.02	8.04	8.04	5.87	5.94	5.94
	63	8.22	8.31	8.31	8.08	8.08	8.08	5.88	5.99	5.99
	7.5	8.14	8.14	8.06	7.92	7.92	7.96	5.81	5.81	5.86
	18	8.17	8.17	8.05	7.94	7.94	7.97	5.84	5.84	5.86
	32 ^a	8.17	8.17	8.04	7.96	7.96	7.96	5.84	5.84	5.87
	42	8.20	8.20	8.07	7.99	7.99	8.02	5.84	5.84	5.91
	63	8.20	8.20	8.11	8.02	8.02	8.04	5.84	5.84	5.97

^aFrom a 250-MHz NMR spectrometer.

Table VI: The Dimerization Shift of (A) YpAp and (B) ApA (0.001 M) at Various Temperatures (in ppm).

A. Temp (°C)	Yp					-pAp		
	H ₈	H ₁	N-CH ₃	C-COOCH ₃ N-COOCH ₃	C-CH ₃	H ₈	H ₂	H ₁ '
7.5	-0.02	0.23	0.28	-0.03, 0.01	0.03	0.35	0.34	0.09
18.0	0.0	0.20	0.28	-0.02, 0	0.03	0.31	0.31	0.11
42	0.04	0.20	0.20	0, -0.01	0.02	0.26	0.27	0.14
63	0.07	0.17	0.20	0, -0.01	0.02	0.19	0.22	0.15
B. Temp (°C)	H ₈		H ₂		H ₁ '			
	Ap-	-pA	Ap-	-pA	Ap-	-pA		
5.5	0.125	0.32	0.31	0.125	0.28	0.195		
20	0.12	0.27	0.26	0.11	0.26	0.17		
26	0.12	0.25	0.24	0.11	0.24	0.16		
46	0.13	0.21	0.18	0.085	0.21	0.125		
65.5	0.12	0.16	0.14	0.08	0.18	0.09		

and the H₁' resonance at a slightly lower field (5.87 ppm) is assigned to the A(1) residue.

As for the pentamer GmpApApYpAp, the resonances of the Gm and Y residues have been assigned already (Tables III and IV). Therefore, only the resonances from the three A residues have to be designated. The chemical shifts of the resonances in the A(3) residue of the pentamer at 63° should be very similar to those of the A(3) residue in dimer YpAp (Table V). Thus, one of the two H₈ resonances at 8.27 ppm, the H₂ resonance at 8.09 ppm, and the H₁' resonances at 6.01 ppm belong to the A(3) residue (Figure 2,

Table V). Similarly, the resonances from the A(1) and A(2) residues in the pentamer should be very similar to those in the tetramer. Therefore, the H₂ and H₁' resonances of these two A residues can be readily identified (Figure 2, Table V). The two H₂ resonances actually merge into one signal, thereby eliminating the necessity of a separate identification. An interesting situation develops for the two H₈ resonances from A(1) and A(2), which merge as one signal in the spectrum of the tetramer. In the pentamer spectrum, one of the H₈ resonances remains at the same field position, while the other one is shifted downfield by 0.06 ppm (Fig-

ure 2, Table V). At present, there is insufficient data to distinguish these two H₈ resonances between A(1) and A(2) residues with certainty. We tentatively assign the resonance at 8.21 to H₈ of A(1) residue and the resonance at 8.27 to H₈ of A(2) residue at 63°; the reason for this tentative assignment is that the effect of adding a residue to the 3'-terminus of tetramer normally should have a greater effect on A(2) than A(1) residue. In this tentative assignment the δ value for H₈ of A(1) residue in the pentamer is unchanged as compared to that in tetramer, while the δ value for H₈ of A(2) residue in the pentamer is shifted downfield slightly (~ 0.06 ppm) in comparison to that in tetramer. As discussed in later sections, this assignment is supported by the molecular model. It should be noted that at low temperatures (7.5–18°), the three H₈ resonances of the three A residues all merge together within 0.01 ppm; the three H₂ resonances of these three A residues also merge within 0.02–0.03 ppm (Table V).

As for the assignment of the resonances for the three A residues in GmpApApYpAp ψ p, one should consider the effect of the addition of a ψ p residue to the 3'-terminus of the pentamer. While the direct ring-current effect of the ψ base may not be large, the added residue will restrict the movement of A(3) residue and increase the stacking of the A(3) residue with the Y base. Again the effect of the added ψ residue to the resonances from A(1) and A(2) residues should be minimal, while the resonances of the A(3) residue should be more affected toward a greater degree of shielding. The molecular model also suggests that the H₂ and H_{1'} of A(3) residue should be less shielded than the H₂ and H_{1'} of the A(1) and A(2) residues. Based on these considerations, the assignments for the resonances from these three A residues were made in Table V and Figures 2 and 3. As for the H₈ resonances, at 63° the resonance at 8.11 ppm which has the largest increase in shielding over that of the pentamer is assigned to the H₈ of A(3) residue; the two remaining resonances which have the same δ value (8.20) are assigned to the H₈ of the A(1) and A(2) residues. In comparison to the H₈ resonances of A(1) and A(2) in the pentamer, the H₈ of A(1) in the hexamer still has the same δ value while the H₈ of A(2) has a small increase in shielding.

Concerning the resonances for the three A residues in hexamer-Y, the assignments are made on the basis that the resonances from the A(1) residue should be the least affected by the excision of the Y base and should be located at the most upfield positions as compared to those from A(2) and A(3) residues. The data in Table V and Figure 2 show that at 63° all three H₂ resonances have the same δ values, and two of three H_{1'} resonances have the same δ values. At 7.5°, two of the H₈ resonances have the same δ value, and a similar situation was found for two H₂ resonances and two H_{1'} resonances. A 250-MHz spectrum of hexamer-Y is shown in Figure 4.

It should be remembered that the assignments for the often closely clustered resonances from the three A residues in pentamer, hexamer, and hexamer-Y are aided by model building and other logical deductions, and are not determined entirely on the basis of chemical data. While there is much credibility in these assignments, indicated especially in a later discussion on the molecular model which summarizes all the NMR data, the certainty of these assignments remains to be established unambiguously in the future.

(e) *Assignments for the Resonances in Dodecamer and Dodecamer-Y.* Except for the H₆ resonance from the ψ residue, the base proton and the H_{1'} resonances are not suffi-

ciently resolved in spectra of dodecamer and dodecamer-Y. The methyl resonances, on the other hand, can be readily identified. The methyl resonances from the Y base have been assigned already and their assignments are confirmed by the comparison of the spectra from dodecamer and from dodecamer-Y. Similarly, the 2'-O-methyl resonance from Gm residue has also been assigned for compounds GmpAp, to hexamer in the above section; this resonance is well separated from others and can be clearly identified in the spectra of dodecamer and dodecamer-Y as shown in Figure 2. The two additional methyl resonances derived from Cm residue and m⁵C residue in the dodecamers (Figure 2) have been assigned in our previous publication (Kan et al., 1974) by comparison with the chemical shift data on the Cm and m⁵C mononucleotides.

The Stacking Model of Y Residue in the Di- and Oligonucleotides. At the onset of the discussion on the conformation of the oligonucleotides, it should be remembered that these molecules are constantly in dynamic motion in solution. The model in discussion represents either the population-averaged conformation at a given time or the time-averaged conformation for a given molecule. The mode of the dynamic motion of a dimer has been previously investigated by NMR technique (Kondo et al., 1972; Kan et al., 1973). These studies suggested that the mode of motion of the two bases relative to each other in the dimer is oscillation-rotation. At low temperature, the amplitude of the oscillation is small and the two bases stay with each other for a large fraction of time; this population assumes a stacked conformation. At moderate temperature, the two bases begin to oscillate with a larger amplitude and the bases spend a larger fraction of time away from each other. Finally, when the temperature becomes sufficiently high, a complete rotation of the two bases relative to each other takes place. In such a rotation movement, the time fraction spent by the bases with each other represents the moment when the two bases pass over each other, perhaps with a pause, in the rotating process. The dynamic motion of trimers of inosine has also been compared with the inosinic dimer by NMR technique (Tazawa et al., 1972). The comparison between the IpI and IpIpI reveals the following information: (1) a possible existence of the distant-neighbor field effect; (2) the absence of right-handed to left-handed interconversion in the trimer; (3) a reduction of the rotation freedom of the nucleosidyl unit in the interior residue in the trimer (more rigidly fixed in an anti conformation); (4) a possible asymmetry in the geometrical relationship among the residues in the trimer. A general discussion on the conformation dynamics on di- and oligonucleotides has been presented (Ts'o, 1974). The above consideration and understanding provide the basis of the discussion on the conformation models of various di- and oligonucleotides at different temperatures in this paper.

The following discussion is also benefited by the ring-current magnetic anisotropy isoshielding curve of Y base at 3.4 Å distance sent to us by Professor B. Pullman before publication. In the first shielding contour, the center of the shielding zone is in between the five-membered "adduct ring" (the imidazole ring with the side chains, Figure 1) and the six-membered pyrimidine ring with a value of 0.83 ppm (less than the value of 1.28 ppm in the center of the first contour zone of adenine or the value of 1.0 ppm in the second zone of adenine) covering an ellipsoidal area of both rings. The second zone of shielding with a value of 0.62 ppm (about the same as that of the five-membered imidazole ring of the guanine) covers mainly the five-membered imid-

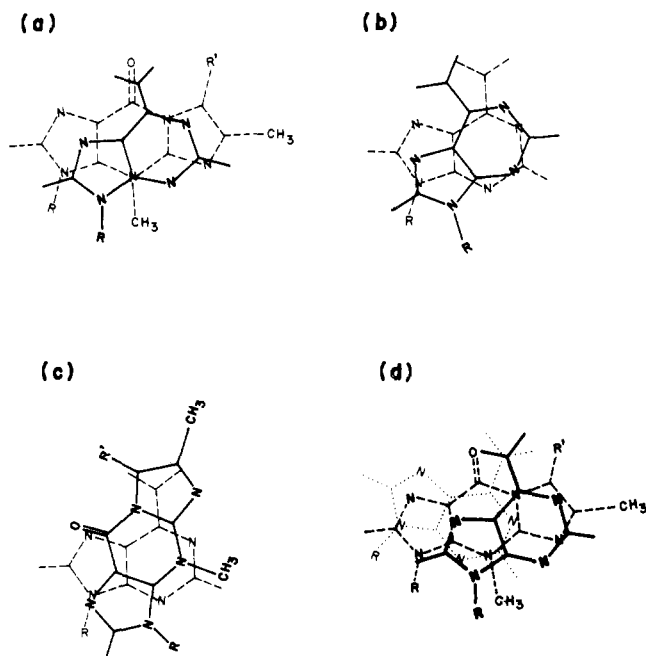


FIGURE 6: Schematic presentation of the average base stacking patterns of two dimers and two sequences in the oligomers at low temperature as viewed from the 3' to 5' direction. The bases are parallel to the plane of the paper with the $H_{1'}$ out of the plane. The distance between the adjacent bases is 3.4 Å. Bases with solid lines are above bases with broken lines which in turn are above the bases with dotted lines. The nucleotidyl units have an anti conformation and the screw axis is right-handed. The construction of these models is guided by the isoshielding zone values of the bases calculated by Professor B. Pullman's laboratory and the geometry of the Kendrew models. (a) YpAp; (b) ApA; (c) -pApY sequence in GmpApApY; and (d) -pApYpAp- sequence in GmpApApYpApψp.

azole ring and the periphery of the adduct ring and the pyrimidine ring.

(a) *YpAp*. Table VIA lists the dimerization shifts ($\Delta\delta$) of the base protons, $H_{1'}$ protons, and the methyl protons of YpAp in the temperature range of 7.5–63°, where $\Delta\delta$ is defined as $\delta_{\text{dimer}} - \delta_{\text{monomer}}$. In addition, Table VIB lists the $\Delta\delta$ of resonances from ApA for comparison; these $\Delta\delta$ values are slightly different from those published from our laboratory earlier. The previously published values were based on an extrapolation to infinite dilution with the lowest concentration at 4 mM; while the present values are based on measurement at 1 mM concentration. The $\Delta\delta$ values of pAp are used for the calculation of $\Delta\delta$ for YpAp, since these values are slightly different from those of Ap or pA used in the calculation of $\Delta\delta$ for ApA.

In comparing the $\Delta\delta$ values of -pAp residue in YpAp with those of -pA in ApA, there are major differences. It is not surprising that the δ for H_2 in -pAp of YpAp is substantially larger than that in -Ap of ApA, since the Y has an additional ring (adduct ring) attached to the pyrimidine moiety which should be located adjacent to the H_2 of -pAp if the -pAp residue is in anti conformation. It is important to note that H_8 in -pAp residue of YpAp is also more shielded than the H_8 in -pA of ApA. This fact indicates that the H_8 of -pAp residue has to be much closer to the pyrimidine ring of adenine in Ap- since the imidazole ring of Y base has somewhat less ring-current shielding effect than the imidazole ring of adenine. This mode of stacking in YpAp is strongly supported by the $\Delta\delta$ value for H_8 of the Y. The $\Delta\delta$ value from H_8 of Ap- residue in ApA is around 0.12 ppm

(Table VIB), while the $\Delta\delta$ value from H_8 of Yp- is nearly zero at 7.5–18°. These data unequivocally indicate that the distance between the two imidazole rings of Y and A in YpAp must be considerably greater than that between the two imidazole rings of two adenine in ApA. Interestingly, only at high temperature (63°), the $\Delta\delta$ value of H_8 of Y becomes higher, in contrast to the common trend for base protons which are usually deshielded at high temperature. This observation indicates that only upon destacking of the Y and A bases by oscillations of large amplitude or by a full rotating process can the H_8 of Y experience the shielding effect of the neighboring A. This relatively uncommon phenomenon, i.e., increase in shielding during a destacking process, serves to confirm the interpretation that when the Y and A bases are firmly stacked at low temperature, the imidazole ring of Y is relatively far away from the imidazole ring of A, at least in comparison to the reference model ApA.

This conformational model for YpAp is also supported by the $\Delta\delta$ values of N-CH₃ from the Y (Table VIA). The substantial shielding value of NCH₃ indicates that this group is fairly close to the shielding center of the adenine ring of the 5'-residue, in fact closer than the H_2 of the 3'-residue in ApA (Table VIB). The $\Delta\delta$ value for the C-CH₃ group of the Y is small but measurable, indicating that even a group so far away from the pyrimidine ring of the Y has received a small degree of shielding from the adenine of the 5'-residue in YpAp. Interestingly, the shielding of this relatively distant group becomes much larger in pentamer and hexamer as described in a later section. The $\Delta\delta$ values for the two methyl groups from the two esters (N-COOCH₃ and C-COOCH₃) of the side chain in Y base are nearly zero or slightly negative (Table VIA), indicating that these groups are far away from the neighboring A residue as might be expected from the molecular structure of Y.

The $\Delta\delta$ values for the two $H_{1'}$ resonances also contribute useful information to the conformational model (Table VIA and B). The $H_{1'}$ of Y residue in YpAp is slightly less shielded than the corresponding $H_{1'}$ of Ap in ApA. This information again indicates that the imidazole ring (which is attached to the $C_{1'}$) of the Y residue is farther away from the shielding center of the adjacent A residue as compared to the corresponding imidazole ring of A in ApA. The $H_{1'}$ of the pAp in YpAp is also less shielded than the corresponding $H_{1'}$ of -pA in ApA. The $H_{1'}$ of the 5'-A residue in YpAp becomes more shielded at high temperature; in fact it possesses a higher $\Delta\delta$ value than the corresponding $H_{1'}$ in ApA at 60°. These data suggest the $H_{1'}$ of the 5'-A residue in YpAp becomes closer to the shielding center of the Y. Again, this is done presumably by the oscillation of the bases with a large amplitude or even by a full rotating process.

The conformational models for YpAp and for ApA as reference are presented in Figure 6a and b, respectively. These base-stacking patterns are constructed with the aid of the Kendrew model and represent the *average* modes of stacking for the two dimers at about 7.5°. In comparison to ApA, the overlap of 5'-adenine in the YpAp is shifted much closer to the adduct ring of the Y and farther away from the imidazole ring of the Y base. In addition, the A and the Y bases are stacked in a more parallel fashion while two A bases in ApA are stacked in a more oblique manner. The relatively large difference between these two stacking models can be explained on the following basis. Within the constraints of the 3'-5' linkage and the steric hindrance of the

Table VII: The Oligonucleotide Shifts ($\Delta\delta$) of the H₂, H₈, H_{1'}, and Methyl Resonances of A and Y Residues in the Compounds GmpApApY (IV), GmpApApYpAp (V), GmpApAp—pApΨp (VI—Y), and GmpApApYpApΨp (VI) (in ppm).^a

Temp (°C)	Compds	-pY or -pYp ^b				
		H ₈	H _{1'}	N-CH ₃	N-COOCH ₃ , C-COOCH ₃	C-CH ₃
7.5	IV	0.11	0.09	0.11	-0.01, 0.01	0.01
	V	0.19	0.32	0.31	-0.04, -0.02	0.07
	VI	0.25	0.21	0.31	-0.07, -0.02	0.15
18	IV	0.09	0.10	0.11	0.00, 0.01	0.03
	V	0.18	0.31	0.30	-0.04, -0.02	0.08
	VI	0.24	0.21	0.31	-0.06, -0.02	0.15
32	IV	0.06	0.09	0.11	0.01, 0.00	0.05
	V	0.13	0.29	0.29	0.00, -0.02	0.10
	VI	0.20	0.24	0.33	-0.04, -0.02	0.16
42	IV	0.05	0.08	0.10	0.02, 0.01	0.06
	V	0.11	0.28	0.27	0.01, -0.02	0.10
	VI	0.17	0.25	0.32	0.01, -0.01	0.15
63	IV	0.04	0.02	0.09	0.02, 0.01	0.08
	V	0.10	0.27	0.24	0.01, -0.01	0.09
	VI	0.11	0.28	0.29	0.01, -0.02	0.12

Temp (°C)	Compds	-pAp-'s								
		H ₈			H ₂			H _{1'}		
		1	2	3	1	2	3	1	2	3
7.5	IV	0.50	0.50		0.35	0.26		0.41	0.41	
	V	0.49	0.48	0.48	0.34	0.34	0.31	0.38		0.19
	VI-Y	0.44	0.40	0.40	0.34	0.30	0.30	0.36	0.36	0.36
	VI	0.44	0.44	0.52	0.34	0.34	0.30	0.37	0.37	0.34
18	IV	0.46	0.46		0.33	0.25		0.38	0.38	
	V	0.43	0.42	0.42	0.32	0.32	0.31	0.38	0.40	0.19
	VI-Y	0.40	0.34	0.34	0.31	0.31	0.29	0.32	0.32	0.32
	VI	0.40	0.40	0.51	0.32	0.32	0.29	0.34	0.34	0.32
32	IV	0.42	0.42		0.31	0.23		0.36	0.39	
	V	0.37	0.31	0.41	0.31	0.31	0.31	0.38	0.40	0.19
	VI-Y	0.39	0.32	0.32	0.30	0.25	0.25	0.35	0.32	0.32
	VI	0.39	0.39	0.51	0.30	0.30	0.30	0.34	0.34	0.31
42	IV	0.38	0.38		0.28	0.23		0.34	0.36	
	V	0.38	0.33	0.33	0.28	0.28	0.20	0.37	0.40	0.17
	VI-Y	0.37	0.29	0.29	0.26	0.24	0.24	0.31	0.24	0.24
	VI	0.36	0.36	0.49	0.29	0.29	0.26	0.34	0.34	0.29
63	IV	0.31	0.31		0.24	0.20		0.30	0.33	
	V	0.31	0.25	0.25	0.26	0.26	0.20	0.33	0.37	0.15
	VI-Y	0.30	0.21	0.21	0.19	0.21	0.21	0.27	0.18	0.18
	VI	0.32	0.32	0.41	0.27	0.27	0.25	0.34	0.34	0.19

^a $\Delta\delta = \delta_{\text{oligomer}} - \delta_{\text{monomer}} (-pN \text{ or } -pNp-)$. ^b The chemical shifts of Yp and Y>p were used as reference for the calculation of $\Delta\delta$.

2'-OH group, the ApA tends to establish a *maximal* degree of base-base overlap by adopting the pattern shown in Figure 6b (Kondo et al., 1972). As for YpAp, the presence of the hydrophobic adduct ring with side chain in the Y base provides a larger degree of contact with the 5'-adenine by shifting the overlap toward the adduct ring with a more parallel mode. In addition, the presence of the N₃-methyl in Y base also tends to keep the 5'-adenine away from the imidazole ring of the Y base. This steric obstruction can be overcome at higher temperature. The comparison between the two stacking patterns between YpAp and ApA provides the explanation for $\Delta\delta$ values observed in Table VI. A later discussion emphasizes the importance of this difference in the stacking patterns of YpAp and ApA which may provide some understanding about the biological role of Y base.

(b) *GmpApApY*. The $\Delta\delta$ values of -pY residue in this tetramer provide certain important information about the influence of the 3'-adenine residue on the Y residue. Unfortunately, due to the unavailability of the 5'-monophosphate of Y nucleoside (pY), the $\Delta\delta$ values for the Y residue in Table VII are computed with the δ values from 3'-monophosphate of Y (Yp) instead. A previous study on the deshielding ef-

fect of the 5'-phosphate on the H₈ of purine nucleoside in anti conformation revealed that δ values of H₈ in the 5'-nucleotide are about 0.13–0.15 ppm downfield from those in the 3'-nucleotides or nucleosides. The nucleosidyl conformation of the 5'-Y nucleotide most likely is in anti conformation because of the steric hindrance of the bulky N₃-methyl group. Therefore, the $\Delta\delta$ values for the H₈ of Y residue are probably underestimated by at least 0.1 ppm. With this correction in mind, the values of both H₈ and H_{1'} of the Y residues are still smaller and more temperature dependent than the $\Delta\delta$ values of the corresponding H₈ and H_{1'} of the 3'-A residue in the ApA. There is a significant shielding of the N₃-methyl group ($\Delta\delta$ value = 0.11 ppm) which is not greatly affected by temperature variation. On the other hand, the $\Delta\delta$ value for C₁₁-methyl is small at low temperature but becomes rather substantial at high temperature (Table VII).

The $\Delta\delta$ values in Table VI indicate that the two A residues in the tetramer are well shielded. In fact, the $\Delta\delta$ values for the two H₈ and the two H_{1'} from these two A residues are nearly the same from 7.5 to 63°. The $\Delta\delta$ value of H₂ of A(2) residue is about 0.1 ppm less than the $\Delta\delta$ value of H₂

of A(1) residue. This is expected since the H_2 of A(1) is shielded by pyrimidine ring of A(1), while the H_2 of A(2) is shielded by the pyrimidine ring of Y which has a smaller ring-current magnetic anisotropy as compared to the pyrimidine ring of A. An approximate estimation of the shielding effects received by A(2) residue from both A(1) and Y residues was made by adding the $\Delta\delta$ values of -pA and Ap-residues for each proton in ApA (Table VI). These values for H_2 and $H_{1'}$ are close to those for the A(2) residue listed in Table VII but not to that for H_8 . The higher $\Delta\delta$ values (by 0.07 ppm) for the H_8 of A(2) could come from the distant shielding effect of the imidazole ring of Gm residue as the next neighbor in the tetramer.

A tentative model for the base-stacking pattern of the residue -ApY in tetramer is proposed in Figure 6c. This model is to account for the significant shielding to the N_3 -methyl of Y base received from the neighboring A residue, and for the slight reduction of the shielding received by H_8 in the Y base as compared to the H_8 of 5'-A in ApA. In this conformation, the two carboxyl groups of the side chain of the Y base (Figure 1) could be in position to form hydrogen bonds with the 6-amino group of the 3'-adenine residue. There are no NMR data, however, to support directly such an interaction.

(c) *GmpApApYpAp* and *GmpApApYpAp ψ p*. A comparison between the $\Delta\delta$ values of Y residue in the pentamer and in the tetramer in Table VII reveals the interesting influence of the added A(3) residue in the 3'-terminus on the mode of stacking of the Y base. If the geometrical relationship of the Y residue to its 3' and 5' nearest neighboring residues in *GmpApApYpAp* is the same as that to the 3'-neighboring residue in *GmpApApY* and as that to the 5'-neighboring residue in *YpAp*, then the $\Delta\delta$ values of Y in pentamer would be equal to the sum of the $\Delta\delta$ values of Y in tetramer and the $\Delta\delta$ values of Y in *YpAp*. The data in Table VII clearly indicate that this is not the case. Taking the $\Delta\delta$ values at 7.5° for instance (Table VII), the observed $\Delta\delta$ for H_8 (0.19 ppm) is higher than the sum (0.07 ppm); the observed $\Delta\delta$ for $H_{1'}$ (0.32 ppm) is identical with the sum (0.32 ppm); and the observed $\Delta\delta$ for N_3 -methyl (0.31 ppm) is lower than the sum (0.39 ppm). The same conclusion also holds for the comparison of the $\Delta\delta$ values at 18°. This comparison immediately suggests that the stacking pattern of the Y base in the pentamer may be different from that expected from the tetramer. Comparison of the $\Delta\delta$ of the A(3) residue in the *YpAp* (Table VI) and those in pentamer (Table VII) confirms this suggestion. The H_2 of the A(3) residue is shielded to the same extent in both the dimer and the pentamer, while the H_8 of A(3) residue is much more shielded in the pentamer ($\Delta\delta = 0.48$ ppm at 7.5°) than in the dimer ($\Delta\delta = 0.35$ ppm). Part of this increase in the shielding of H_8 could come from the distant shielding effect of the imidazole ring of A(2) residue in the pentamer, but some of the increase must be due to the shortening in distance of the H_8 of A(3) to the shielding zone of the Y base. This deduction is supported by the increase in shielding of $H_{1'}$ of A(3) in the pentamer (0.19 ppm) vs. that (0.09 ppm) in *YpAp*. The data again indicate a closer proximity of the imidazole ring of A(3) to the Y base.

As for the A(1) and A(2) residues in the pentamer, the $\Delta\delta$ values (Table VII) indicate the extent of shielding for H_8 is slightly reduced especially for H_8 of A(2) at high temperature as compared with the corresponding H_8 in the tetramer. Similarly, the shielding of $H_{1'}$ from A(1) and A(2),

particularly at high temperature, is slightly less in the pentamer than in the tetramer. These observations are interpreted to mean that the oscillation-rotation of the Y base (and possibly the A(2) base as well) is less restricted in the *GmpApApY* and more restricted in *GmpApApYpAp*. The $\Delta\delta$ values for H_2 from A(1) residue are the same for both the tetramer and pentamer, while the $\Delta\delta$ values from A(2) residue are larger in the pentamer as compared to those in the tetramer. These observations are expected since the addition of the A(3) residue to the 3'-terminus of the tetramer will tend to increase the stacking of the Y base on A(2) as well as to increase the shielding of the H_2 from A(2) residue directly by the distant shielding effect.

Addition of ψ p residue to the pentamer in the formation of the hexamer has only small effects on the $\Delta\delta$ values of Gm, A(1), and A(2) residues in the pentamer as anticipated (Figure 2 and Table VII), since these nucleotides are situated 3 to 5 residues away from the added nucleotide, ψ p. The $\Delta\delta$ values of H_8 and $H_{1'}$ of Gm, of two H_2 's of A(1) and A(2) are nearly identical for both pentamer and hexamer, while the small decrease in $\Delta\delta$ values of H_8 in A(1) and A(2) residues from the hexamer as compared to those from the pentamer is within the experimental uncertainty of measurement on clustered signals in this region. On the other hand, the $\Delta\delta$ values from the A(3) residue are influenced notably by the addition of the ψ p residue to the pentamer in a manner indicating an increase in the restriction of the motion of A(3) residue in the hexamer. At low temperature, the $\Delta\delta$ values of H_2 and H_8 of A(3) are about the same for the pentamer and hexamer, while at high temperature, the $\Delta\delta$ values are lower for the pentamer as compared to those for the hexamer. This result suggests that with considerable stacking of the bases at lower temperature, addition of ψ p to the pentamer does not increase the shielding of A(3) located at the end of the pentamer. At high temperatures which favor the destacking process of bases, however, the added ψ p prevents the oscillation-rotation of the A(3) residue, thereby maintaining a high degree of shielding for this penultimate A(3) residue. The increase of shielding of $H_{1'}$ of A(3) in the hexamer over that in the pentamer (Table VII) is also expected, since the diamagnetic double bond in the ψ base is in close proximity to the $H_{1'}$ of A(3) and the restricted rotation of A(3) residue will increase the shielding of its own $H_{1'}$ by the neighboring Y base.

The $\Delta\delta$ values of the Y residue in the hexamer provide additional information about the stacking mode between the Y and the A(3) residue (Table VII). There is a noticeable increase in $\Delta\delta$ value (0.08 ppm at 7.5°) of the C_{11} -methyl from pentamer to hexamer, while the $\Delta\delta$ value of N_3 -methyl remains the same for both pentamer and hexamer at 7.5° but becomes higher for the hexamer at temperatures above 32°. Since these two methyl groups can only be shielded by the overlap from the A(3) residue or the 5'-neighbor, the 1H NMR results on the C_{11} - and N_3 -methyl groups suggest a further shift of the A(3) base toward the adduct ring of Y in the hexamer at low temperature as compared to the pentamer, as well as a restriction of movement of A(3) vs. Y base at higher temperature. This suggestion is supported by the $\Delta\delta$ values of $H_{1'}$ (Table VII). At low temperature, the $\Delta\delta$ of $H_{1'}$ in the hexamer is smaller than that of the pentamer, but at 63° the $\Delta\delta$ values of $H_{1'}$ become the same for both pentamer and hexamer. In addition, the $\Delta\delta$ of $H_{1'}$ increases with increasing temperature. The interpretation of these data is that at low temperature, the stacking of

the A(3) base in the hexamer tends to be shifted further toward the adduct ring of the Y base, therefore tends to be away from H_{1'}, as compared to the situation in the pentamer. Part of the decrease in shielding of H_{1'} of the Y residue can also come from the reduction of the conversion from the right-handed stack to the left-handed stack when A(3) residue is transformed from a terminal residue in the pentamer to become a penultimate residue in the hexamer. A reduction of the population in left-handed stack tends to reduce the shielding of the H_{1'} of the 3'-residue and such a reduction has been reported in comparative study on IpIpI vs. IpI (Tazawa et al., 1972) as discussed above. A small increase in the $\Delta\delta$ value of H₈ of the Y base in the hexamer is noted at low temperature in comparison to that in the pentamer; such an increase vanishes at 63°. It is possible that the increase in stacking interaction between Y and A(3) may also increase the shielding of the H₈ of Y by the pyrimidine ring of A(2); however, this possible enhancement of interaction between A(2) and Y is not reflected in the $\Delta\delta$ value of H₂ of A(2). The 250-MHz spectrum of the hexamer at 32° with the assignment for all the resonances is presented in Figure 3.

In summarizing all the ¹H NMR data, a stacking pattern of the three bases in -ApYpA- sequence of GmpApApYpAp ψ p at low temperatures can be constructed with some confidence as shown in Figure 6d. In this scheme, all bases are in anti conformation. The mode of overlap between A(2) and Y is similar to that of ApA (Figure 6b), i.e., the imidazole ring of Y is over the imidazole ring of A(2) and the pyrimidine ring of Y is over the pyrimidine ring of A(2). This mode of A(2)-Y stacking in -ApYpA- sequence is different from that in the tetramer (Figure 6c). The mode of overlap of the Y and A(3) in -ApYpA- sequence is close to that established for YpAp (Figure 6a). In this mode, the imidazole ring of A(3) overlaps a large portion of the pyrimidine ring of Y and the pyrimidine ring of A(3) overlaps both the pyrimidine and adduct rings of Y. Therefore, there is a definite shift of base-stacking pattern in the sequence -ApYpA-, and the significance of this shift to the structure and function of tRNA will be discussed later. Addition of the ψ p residue to the -ApYpA- sequence restricts the overall motion of the sequence especially the relative motion of A(3) vs. Y.

The Effect of the Excision of Y Base on the Conformation of the Hexamer and the Dodecamer. A 250-MHz spectrum of the hexamer-Y, GmpApAp—pAp ψ p, at 32° is shown in Figure 4. The chemical shifts of the Gm residue which is located at the 5'-terminus three residues removed from the Y residue in the hexamer are not much affected (less than 0.05 ppm) by the excision of the Y base (Table III). The most striking ¹H NMR properties of this hexamer-Y are the $\Delta\delta$ values of the three A residues (Table VII). At 7.5 and 18°, the $\Delta\delta$ values for H₂ and H_{1'} of all three A residues and the $\Delta\delta$ value for H₈ of A(1) and A(2) residues in hexamer-Y are practically the same as those in the hexamer; only the $\Delta\delta$ value for H₈ of A(3) is slightly reduced by about 0.1 ppm. In fact, the $\Delta\delta$ values for the three A's in hexamer-Y are close to those of ApApA (L. S. Kan, unpublished data). Clearly, the three A bases in hexamer-Y stack on each other, closing the gap created by the excision of the Y. As discussed earlier, the excision of the Y base leads to the concomitant opening of the ribose ring. Thus, there is little steric hindrance in the backbone to prevent the A(3) base to overlap the A(2) base. It can be safely concluded that at low temperature, the three A residues

stack with each other to close out the gap in the conformation similar to ApApA and not as two segments of GmpApAp and pAp ψ p loosely linked to each other. In fact the four purine bases are likely to form a cohesive stack of GmAAA. Only at higher temperatures, the $\Delta\delta$ values for H₈, H₂, and H_{1'} of the A(2) residue begin to decrease notably, thereby indicating a greater separation between the segment GmpApAp and the segment pAp ψ p in the GmpApAp—pAp ψ p.

Excision of the Y base has other interesting effects, particularly on the shielding of the ψ base which is one residue away from the Y in the hexamer-Y. At 7.5°, the shielding of the H₆ of ψ is reduced by 0.16 ppm in hexamer-Y as compared to the hexamer; the reduction is about 0.11 ppm at 42° and is about 0.05 ppm at 63°. The molecular model of the hexamer clearly indicates that the shielding of H₆ in the ψ base is directly proportional to the distance between the A(3) and ψ . As described above, at 7.5° the $\Delta\delta$ values of the two nearest neighboring A's are hardly reduced by the excision of the Y base; a more significant reduction was noticed only at high temperature. Thus, the temperature influence on the deshielding effects of the excision of the Y base is positive for the two neighboring A residues but negative for the ψ residue. At low temperatures, the deshielding effects of Y excision are small for the two neighboring A residues but large for the more distant ψ residues, while at high temperature, the deshielding effects of Y excision became large for the two A's but small for the ψ .

Combining the information in the two preceding paragraphs, a more complete picture about the conformation of GmpApAp—pAp ψ p begins to emerge. At low temperature, the three A residues all stack together in closing the gap left by the excision of Y. However, the close stacking of three A's causes a separation between the A(3) and the ψ p residue in the hexamer. Thus, at low temperature, the conformation of GmpApAp—pAp ψ p is represented by a cohesive stack of four purines, GmAAA, with a small separation from the ψ residue. At high temperature, the stack of the four purines in hexamer-Y begin to break into the two segments of GmpApAp and pAp ψ p.

The chemical shift data of H₆ of ψ in the dodecamer and the dodecamer-Y (Table II) suggests that the separation of A(3) and ψ also takes in the dodecamer-Y. The excision of Y base apparently decreases the shielding of H₆ in the dodecamer by 0.24 ppm at 7.5°.

The Coupling Constants of H_{1'} And H_{2'} (J_{1'-2'}). As mentioned earlier, the J_{1'-2'} values of Yp were around 5.5 Hz from 18 to 63°. In YpAp, the J values for the A and Y residues are both less than 4 Hz at 18°, and are 3.9 and 5.6 Hz, respectively, at 63°. At 32°, the J values in the tetramer are 4.5 Hz (Gm), 3.6 Hz (A(1)), 4.7 Hz (A(2)), and 4.2 Hz (Y); in the pentamer are 5.0 Hz (Gm), ~4 Hz (A(1)), 4.8 Hz (A(2)), ~4 Hz (Y), and 3.6 Hz (A(3)); in the hexamer are 4.5 Hz (Gm), ~4 Hz (A(1)), ~4 Hz (A(2)), ~4.5 Hz (Y), and ~4 Hz (A(3)). The ¹H NMR spectrum in the H_{1'} region for the J values measurements of GmpApApY is shown in Figure 7. The ¹H NMR spectra of the H_{1'} region of the hexamer and hexamer-Y are shown in Figures 3 and 4. At 63°, all the J values became larger, approaching 5–6 Hz.

It has been well recognized that at low temperatures the J_{1'-2'} of purine ribosyl nucleotidyl residues in dimer and trimer become significantly smaller (2–4 Hz) than those of the mononucleotides (5–6 Hz). This observation is attributable to the steric hindrance of the 2'-OH group in the stack-

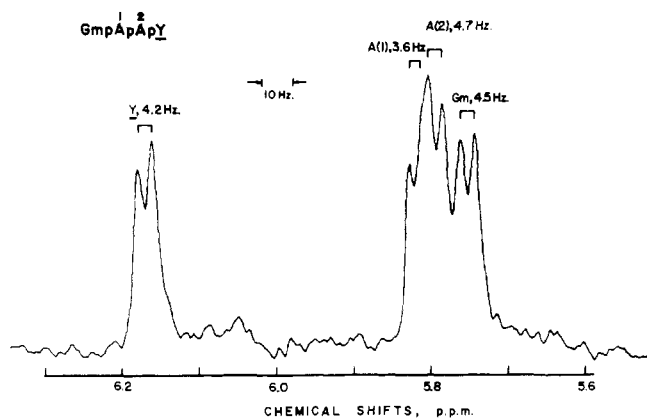


FIGURE 7: A 250-MHz spectrum at the $H_{1'}$ region (5.6–6.3 ppm) of the tetramer GmpApApY with all the assignments and the $J_{1'-2'}$ values at 32°.

ing of the bases (Kondo et al., 1972). This steric hindrance of the 2'-OH group causes a change of the ribose conformation in a situation of a rapid equilibrium between 2'- and 3'-endo forms. A general review on this subject has been recently presented (Ts'o, 1974). Obviously, the ribose conformation of this hexamer from the tRNA follows this general rule just described.

Concluding Remarks

The significance of this research can be viewed from the standpoint of physical chemistry of oligonucleotides and from the standpoint of the study on the function and structure of tRNA.

(a) *Physical Chemistry of Oligonucleotides.* The 1H NMR spectrum of the hexamer GmpApApYpAp ψ p consists of 19 proton/methyl resonances (Figure 3) in the region from 5.5 to 8.5 ppm (base protons and $H_{1'}$'s) as well as from 2 to 4 ppm (methyl resonances). In this comparative study with 12 compounds (ψ p and 11 compounds listed in Table I) together with the information obtained in our previous studies on mononucleotide dimers (ApA, GpA, etc.) and trimers (ApApA), 15 resonances have been assigned with certainty and four with a high degree of confidence. Other than the self-complementary hexamer ApApGpCpUpU studied by us (Ts'o et al., 1975b), so far this hexamer is the largest oligonucleotide which has ever been characterized by 1H NMR to this extent of completeness. This hexamer contains a continuous sequence of five purine nucleotides (including a Y base) and one pyrimidine (ψ base) nucleotide at the 3'-terminus. This continuous sequence of five purine residues (including three adenine residues), from the standpoint of secondary structure, endows this oligonucleotide with a very high degree of base stacking. From the standpoint of 1H NMR properties the oligonucleotides impose a formidable problem in spectral assignment on one hand but provide a wealth of information about the conformation on the other hand. It is because the purine bases (especially the A and Y) exert a large shielding effect on nearby neighboring residue protons. These rather unique properties of this hexamer together with its related series of compounds enable this 1H NMR study to reveal a strong tendency of the purine bases to have a maximal extent of base-base overlap with their neighbors in the sequence.

The conformation of YpAp (Figure 6a) is a good example. The base-stacking pattern of this dimer indicates a shift of the adenine base toward the pyrimidine ring and the ad-

duct ring of Y as compared to the base stacking pattern of ApA (Figure 6b). Such a shift presumably is to provide a maximal overlap between A and Y and would not allow the adduct ring of Y to be uncovered and exposed. The stacking pattern of the -ApY sequence in GmpApApY all illustrates this concept (Figure 6c). The stacking pattern of A and Y in this -ApY sequence is more oblique as compared to ApA or YpAp, presumably again to provide a maximal degree of overlap. The zigzag stacking pattern of the sequence -ApYpA- in the hexamer of the pentamer demonstrate another interesting situation (Figure 6d). When the Y base is now sandwiched between two adenines, the base-stacking pattern of the trimer sequence is so adjusted as to provide a maximal overlap with all three bases. The Y base is now more in parallel with the two A residues with the adduct ring partially covered from the 3' side but exposed from the 5' side. The two adenines are totally overlapped by the Y base in the stack (Figure 6d). The carboxyl groups in the side chain of Y base could be in a position to form hydrogen bonds with the 6-amino group of the two neighboring adenines. Unfortunately, the present 1H NMR data do not support or deny the existence of such an interesting interaction. Addition of the ψ residue to a stack of five purine nucleotides clearly restricts the motion of the penultimate A residue relative to the neighboring Y base and possibly increases the rigidity of the entire hexamer stack.

The most dramatic demonstration comes from the hexamer without Y, GmpApAp—pAp ψ p. At temperatures below 20°, the penultimate adenine forms a stack with the remaining three purines closing the gap left behind by the excision of Y. Such a formation of a four-purine stack (GmAAA) takes place even in causing a slight separation between the penultimate adenine and the terminal ψ . Only at high temperature, the influence of the gap in the sequence begins to emerge in breaking up the stack of four purines.

The present NMR study confirms the conclusion from the optical studies on this hexamer previously reported by Maelicke et al. (1973). From the data on hypochromicity from the melting curve, the fluorescence of Y base in the hexamer, as well as optical properties of the hexamer-Y, it was concluded that the hexanucleotide exists in a rather rigid conformation maintained by stacking interactions.

(b) *Yeast tRNA^{Phe} Structure and Function.* There are two most important contributions from the present 1H NMR investigation to the studies on the structure of the yeast tRNA^{Phe}. First of all, the assignment of all the methyl resonances in the hexamer and in the dodecamer (Figure 2) provides the basis for the assignments of these methyl resonances in the spectrum from the intact tRNA recently reported by us (Kan et al., 1974). Second, a comparison between the 1H NMR properties of these methyl resonances from the oligonucleotides (both hexamer and dodecamer) and those from the tRNA allows us to reach the conclusion that the anticodon loop in the intact tRNA does not associate with other components of the molecule and the side chain of the Y base protrudes out into the solvent (Kan et al., 1974).

The zigzag (or balcony-like) shift of the base overlapping pattern (Figure 6d) in the stack of -ApYpA- sequence could be of structural importance in the intact tRNA. This phenomenon of the zigzag shift should be considered in the structure determination of tRNA^{Phe} in crystals by X-ray diffraction analysis (Kim et al., 1974; Robertus et al., 1974a,b). Currently, differences do exist between the two

proposed models of tRNA^{Phe} in the geometry of the base-stacking pattern of this sequence -A₃₆Y₃₇A₃₈- in the anticodon loop. The shift of the overlap between the Y and its nearest 3'-neighboring base may help to stabilize the anticodon sequence in an exposed position. If this proposal about the structure is correct, then such a shift may have a major contribution to the function of tRNA. This shift of stacking pattern with the 3'-neighboring base should not restrict to only the Y base, but can take place with any purine base substituted with hydrophobic groups at the pyrimidine ring of the purine. It is tempting to speculate that this could be the structure-function consideration why the residue located at the 3'-end of the anticodon in many tRNAs is a purine (particularly A) heavily substituted with hydrophobic side chain at the pyrimidine moiety (Dirheimer et al., 1972; Barrell and Clark, 1974). Shifting of stacking pattern with the 3'-neighboring base can take place with these substituted purines.

In case of tRNA^{Phe}, the excision of the Y base by mild acid does not completely remove the biological activity of the tRNA^{Phe} (Thiebe and Zachau, 1969). This partial retention in function may be due to the stacking of the A₃₈ residue on the A₃₆ residue in closing the gap left behind in the whole tRNA as in the case of the hexamer-Y.

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