Biophysical Chemistry 11 (1980) 283–294

CONFORMATION STUDIES OF 13 TRINUCLEOSIDE DIPHOSPHATES BY 360 MHz PMR SPECTROSCOPY. A BULGED BASE CONFORMATION. I. BASE PROTONS AND H1′ PROTONS

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Received 29 August 1979
Revised manuscript received 2 January 1980

The 360 MHz NMR spectra of the base protons and the H1′ protons of thirteen trinucleoside diphosphates have been analyzed. The sequences chosen represent all purine-pyrimidine sequences. The chemical shifts of the base protons give evidence for strong next nearest-neighbor effects in some oligonucleotides. Although increasing chain length usually increases nearest-neighbor base-base stacking, it is not always so. Comparing ApCpG, ApUpG and GpUpG to their component dimers, one finds a decrease in stacking of the center pyrimidine with the purine on either side. The coupling constants J1′2′ also show that these three trimers show less stacking for their terminal residues than expected from their component dimers. We conclude that the sequence Pu-Py-Pu favors a conformation in which the pyrimidine is bulged out and the two purines stack on each other.

1. Introduction

Trinucleoside diphosphates (structure shown in fig. 1) are the simplest fragments of nucleic acid which can display next-nearest-neighbor base-base interaction. The conformations of trinucleoside diphosphates are thus important. The conformations of several of the trinucleoside phosphates have been studied by optical methods (UV absorption, ORD and CD) [1–4]. The crystal structure of protonated $ApA^{+}pA^{+}$ was reported by Suck et al. [5]. Ts'o and co-workers published NMR studies of the trimers IpIpI [6], dApTpT and TpTpdA [7]. A stacked right-handed conformation was proposed for these molecules. Longer oligonucleotides ((Ap)nA, $n = 2, 3$ and 4) have also been studied by NMR [8]. Definitive assignment of the NMR signals by specific deuteration of the base protons and 1′ protons of the ribose in ApApA was carried out by Kondo et al. [9]. To study the sequence-dependent conformational properties of trinucleoside diphosphates, we chose thirteen trimers (ApApA, ApApC, UpApA, UpApG, ApCpG, ApUpG, GpUpG, ApCpC, ApUpU, UpApU, CpUpG, UpUpG and UpUpC) which represent all possible purine-pyrimidine sequences. Among these molecules, ApUpG and GpUpG are the initiating codons and UpApA and UpApG are terminating (nonsense) codons for protein synthesis. The trimers together with the component dinucleoside phosphates, nucleoside 3′,5′ diphosphates and mononucleotides were studied by 360 MHz NMR spectroscopy. In this paper, the conformations of the trimers are discussed utilizing the NMR data of the base protons and 1′ protons of the ribose. A second paper of this series will consider the NMR data of the other ribose protons.

2. Experiments

Dinucleoside phosphates and mononucleotides were purchased from Sigma Chemical Company. Nucleoside 3′,5′ diphosphates, pAp and pGp were purchased from P.L. Biochemicals. pUp and pCp were synthesized by

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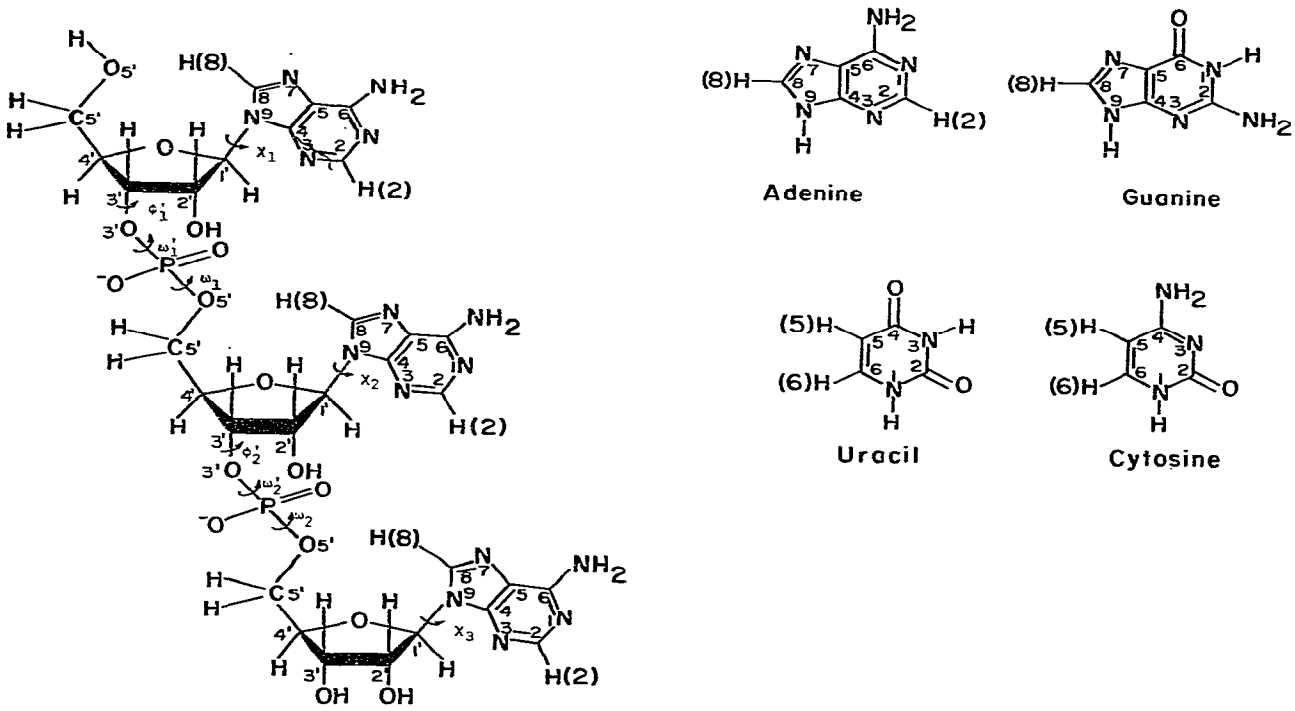

Fig. 1. The structures of ApApA and the four naturally occurring bases adenine, guanine, uracil and cytosine.

phosphorylation of the corresponding nucleosides by inorganic pyrophosphate [10]. The isolated product, a mixture of 3′, 5′ and 2′,5′ diphosphates, was used without further purification. UpApA, UpApG, GpUpG and CpUpG were purchased from Boehringer Mannheim Biochemicals. ApCpC, ApUpU and UpUpG were purchased from Sigma Chemical Company. Each trimer was analyzed by paper chromatography with 95% ethanol/1 M ammonium acetate (75/25, v/v) as eluting solvent. GpUpG and UpUpG had significant impurities and were further purified by paper chromatography. ApUpG was a gift from Dr. Susan Freier. ApApA was prepared from an alkaline hydrolysate of poly A followed by removal of the terminal phosphate by alkaline phosphatase. ApApC, ApCpG, UpApU and UpUpC were synthesized from the dimer and the nucleoside 5′ diphosphate using polynucleotide phosphorylase [11]. UpApU and UpUpC were isolated directly from the reaction mixture. ApApC and ApCpG were prepared as ApApCp and ApCpGp by treating the reaction mixture with ribonuclease A and T1, respectively. The terminal phosphate was then removed by alkaline phosphatase. The NMR samples containing d_4-trimethylsilylpropionate (TSP) as internal reference and 0.1 mM EDTA to remove contaminating paramagnetic ions, were lyophilized three times with 99.8% D_2O (Bio-Rad) with pH adjusted to the desired value and then dissolved in 100% D_2O (Bio-Rad). The concentration for the trimers was 1–5 mM (10–50 O.D. unit in 0.5 ml), while that for the dimers and monomers was 5 mM. The pH for trimer and dimer samples was 7 while that for the monomer samples was 5, at which the ionization state of the phosphate group is minus 1, comparable to that in the trimers and dimers. The NMR spectra were recorded for all the samples at 4°, 20°, 45° and 75°C, with 16 K data points by the Bruker HSX-360 MHz spectrometer of Stanford Magnetic Resonance Laboratory, Stanford University.

Table 1
The chemical shifts (δ in ppm downfield from TSP) and trimerization shifts (δΔ in ppm upfield from the monomeric components) for H1′ and the base protons for thirteen trinucleoside diphosphates at 20°C.

	Np–		–pNp–		–pN	
	δ	$\Delta\delta^1$	δ	$\Delta\delta^2$	δ	$\Delta\delta^3$
ApApA H1′	5.874	0.26	5.796	0.41	5.916	0.24
H2	7.870	0.43	7.964	0.37	8.075	0.22
H8	8.206	0.20	8.106	0.46	8.154	0.36
ApApC H1′	5.923	0.21	5.822	0.39	5.637	0.33
H2 or H5	7.905	0.39	8.125	0.21	5.557	0.62
H8 or H6	8.288	0.12	8.090	0.48	7.610	0.48
UpApA H1′	5.704	0.25	5.939	0.27	6.006	0.15
H2 or H5	5.633	0.27	8.035	0.30	8.178	0.11
H8 or H6	7.768	0.13	8.320	0.25	8.304	0.21
UpApG H1′	5.710	0.25	5.988	0.22	5.790	0.15
H2 or H5	5.682	0.22	8.136	0.20	–	–
H8 or H6	7.784	0.12	8.335	0.23	7.894	0.22
ApCpG H1′	6.010	0.12	5.708	0.32	5.796	0.14
H2 or H5	8.108	0.19	5.677	0.51	–	–
H8 or H6	8.297	0.11	7.678	0.40	7.940	0.17
ApUpG H1′	6.042	0.09	5.806	0.23	5.808	0.13
H2 or H5	8.146	0.15	5.659	0.30	–	–
H8 or H6	8.280	0.13	7.757	0.22	7.984	0.12
GpUpG H1′	5.860	0.09	5.888	0.15	5.856	0.08
H2 or H5	–	–	5.733	0.22	–	–
H8 or H6	7.953	0.06	7.804	0.18	8.008	0.10
ApCpC H1′	6.044	0.09	5.587	0.44	5.855	0.11
H2 or H5	8.135	0.16	5.536	0.65	5.874	0.31
H8 or H6	8.368	0.04	7.635	0.44	7.856	0.24
ApUpU H1′	6.080	0.05	5.833	0.20	5.877	0.11
H2 or H5	8.207	0.09	5.681	0.28	5.788	0.16
H8 or H6	8.342	0.07	7.773	0.21	7.787	0.20
UpApU H1′	5.726	0.23	6.112	0.10	5.810	0.18
H2 or H5	5.622	0.28	8.222	0.11	5.698	0.21
H8 or H6	7.760	0.14	8.431	0.14	7.828	0.16
CpUpG H1′	5.758	0.18	5.834	0.20	5.891	0.05
H5	5.891	0.28	5.684	0.27	–	–
H8 or H6	7.871	0.18	7.837	0.14	8.086	0.04
UpUpG H1′	5.848	0.11	5.885	0.15	5.901	0.04
H5	5.815	0.09	5.823	0.13	–	–
H8 or H6	7.833	0.07	7.871	0.11	8.056	0.05
UpUpC H1′	5.877	0.06	5.952	0.07	5.955	0.01
H5	5.863	0.05	5.911	0.04	6.147	0.03
H6	7.863	0.00	7.890	0.06	7.975	0.08

$\Delta\delta^1$ = δ(nucleoside-3′-phosphate) − δ(Np–)
$\Delta\delta^2$ = δ(nucleoside-3,5′-diphosphate) − δ(–pNp–)
$\Delta\delta^3$ = δ(nucleoside-5′-phosphate) − δ(–pN)

3. Results and discussion

3.1. Assignment of the NMR signals

3.1.1. Base protons of trinucleoside diphosphate

The assignment of the base proton signals (table 1) for the unique bases in trinucleoside diphosphates is straightforward. The pyrimidine base protons show a doublet at 5.7 ± 0.3 ppm (H5) and another doublet at 7.8 ± 0.3 ppm (H6). The coupling constant between H5 and H6 is 7.8 – 8.1 Hz for uracil and 7.4 – 7.7 Hz for cytosine. The H2 signal can be distinguished from the H8 signal in purine residues since H2 is non-exchangeable in D_2O with heating and also has a longer relaxation time, T_1. The H8 signal of adenosine residues appears at lower field than that of the guanosine residues. Thus, the base proton signals of UpApG, ApCpG, ApUpG, CpUpG, pC of ApApC, Up of UpApA, pUp of GpUpG, Ap of ApCpC and ApUpU, pAp of UpApU, pG of UpUpG and pC of UpUpC are assigned. The assignment of the base proton signals for the identical bases in trinucleotide diphosphates is more complicated. The details of each case are discussed below.

The assignments of the base proton signals of ApApA at 20°C follow those of Kondo et al. [9]. When the temperature changes, the NMR signals shift upfield or downfield smoothly. Therefore, the assignments of these protons at the other temperatures are done unambiguously by the chemical shift-temperature curves (fig. 2a). In the case of ApApC, indirect assignment for ApAp-base proton signals was done by comparing their chemical shift-temperature curves (fig. 2b) with those of ApApA. H2 and H8 of Ap– and H8 of –pAp– in ApApC are in similar environment to those protons in ApApA. Therefore, the shapes of their chemical shift-temperature curves are, as expected, alike. H2 of –pAp– in ApApC experiences a weaker ring current shift from the –pC residue than the one in ApApA from the –pA residue so that its chemical shift-temperature curve is shallower. Similar comparison with ApApA was employed to assign the base protons of –pApA of UpApA (fig. 2c) in which H2 of –pAp– and H2 and H8 of –pA behave like those in ApApA.

For the two H8 signals of GpUpG, the one at lower field is assigned to the –pG residue simply because of the presence of the deshielding phosphate group at its 5′ position. In ApCpC and ApUpU the upper field signals of H5 and H6 are assigned to the –pCp– and –pUp– residues because of their nearness to the strong shielding base A. The assignment for UpApU was made by comparison of the spectra of UpApU, ApUpU and UpApUpU (UpApUpU was a by-product of UpApU synthesis). It was found that the addition of a uracil residue to the 5′ OH of ApUpU or to the 3′ OH of UpApU does not change the base proton spectrum pattern.

In the case of UpUpG and UpUpC, tentative assignment was made for the base protons of the two uridine residues. No conformational conclusions were made from such assignment.

3.1.2. 1′ protons of the ribose moieties in trinucleoside diphosphates

The assignment of the NMR signals for the 1′ protons of the riboses in trinucleosides was made by homonuclear decoupling experiments. The details of this method are discussed below for ApApA at 20° (fig. 3).

Spectrum (3a) is a non-decoupled, regular spectrum. The triplets at 1570, 1730 and 1640 Hz become doublets (denoted by asterisks) when the H1′ signals at 2130, 2115 and 2087 Hz are saturated, respectively (spectra 3b, 3c and 3d). Spectrum (3e) is with the decoupling frequency at 1710 Hz (denoted by an arrow). The signals at 1620 and 1640 Hz are partially decoupled compared to those in spectrum (3a). In spectrum (3g), the signals at 1700 and 1500 Hz are partially decoupled when the signals at 1620 Hz are partially saturated. Similarly, when the decoupling frequency is at 1570 Hz, the signals at 1500 and 1620 Hz are partially decoupled. In ApApA, H3′, H4′, H5′ and H5″ are coupled to ^{31}P in –pAp–. From spectra (3d), (3e), (3g) and (3h) and the characteristic line shape of H3′ and H5″ which are coupled to ^{31}P, one sees that the NMR signals for the ribose protons H1′, H2′, H3′, H4′, H5′ and H5″ of the –pAp– residue are located at 2087, 1640, 1710, 1620, 1570 and 1500 Hz, respectively. Because of coupling to H2′ and H4′, the signal of H3′ is a triplet (H3′ of pA, fig. 3a). This triplet becomes a multiplet (i.e. quartet, quintet or hextet) when the coupling constant J3′p is introduced (H3′ of Ap and pAp, fig. 3a). The line shape of H5″ is of the ABM type since it couples to H5′ and H4′ (H5″ of Ap, fig. 3a). It becomes an ABMX system when it additionally couples

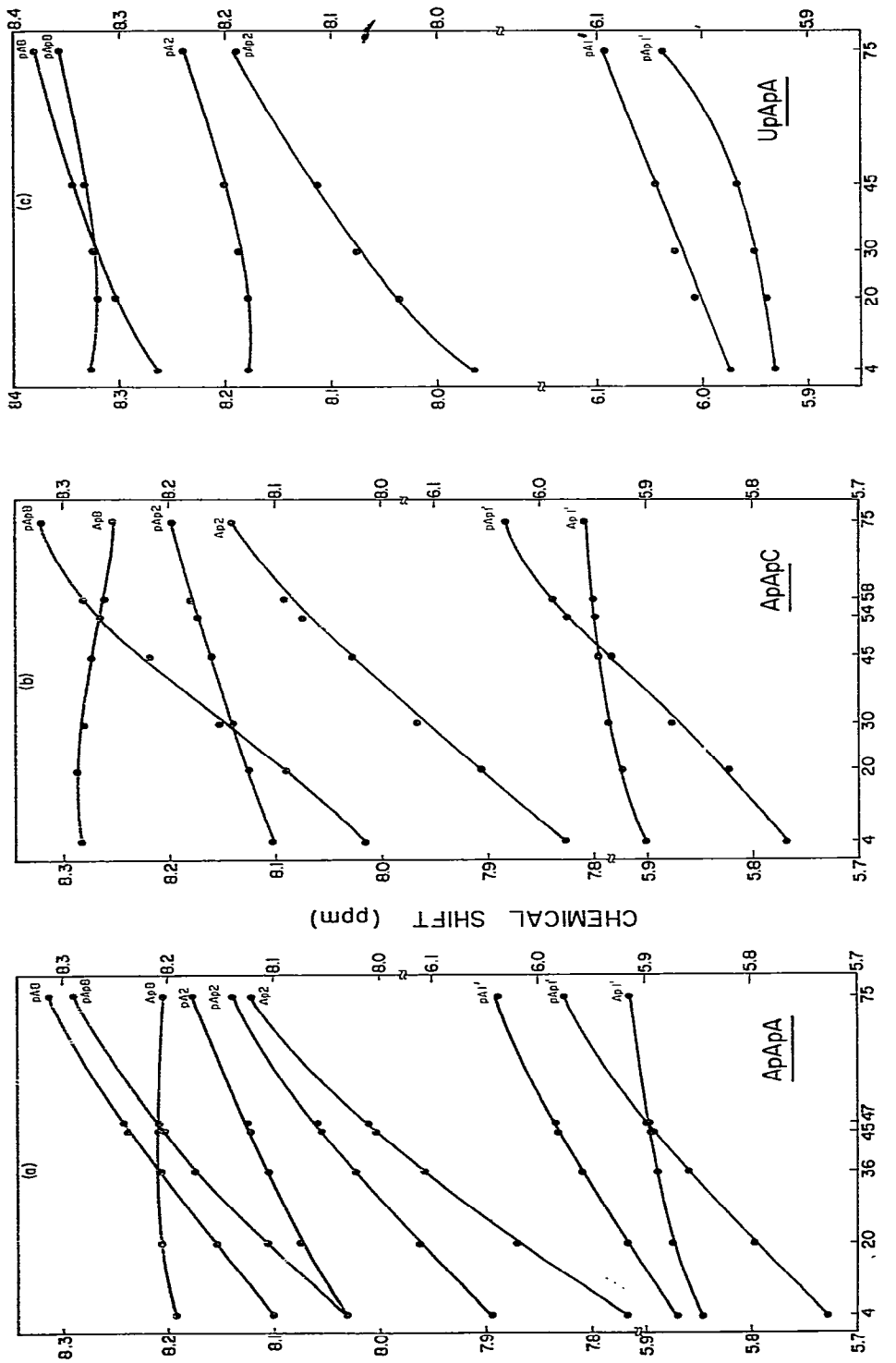

Fig. 2. The temperature dependence of the chemical shift of H1′ and the base protons for the adenosine residues of the trimers ApApA(a), ApApC(b) and UpApA(c). The chemical shift is downfield from the internal reference trimethylsilylpropionate (TSP).

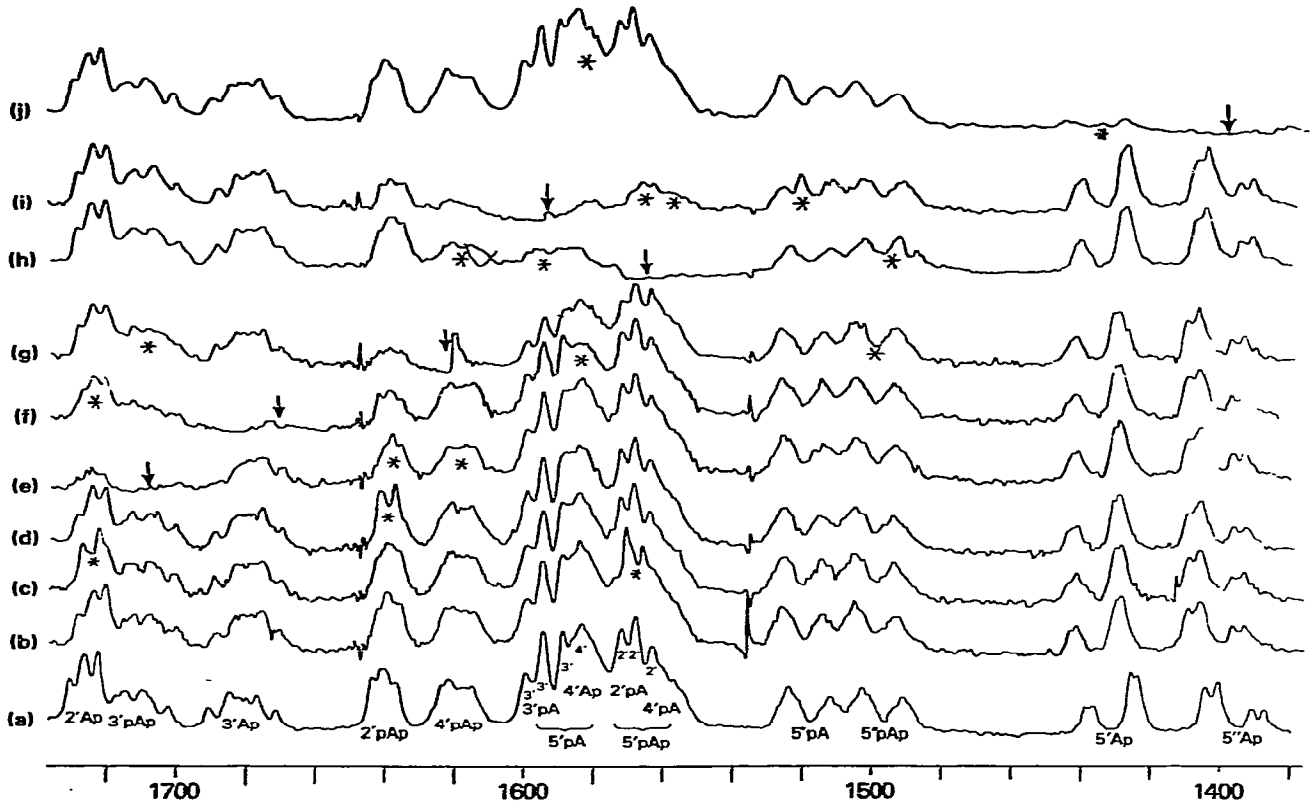

Fig. 3. Homonuclear decoupling spectra of the ribose protons of ApApA, 20°C. The signals for the three H1′ at 2130, 2115 and 2087 Hz (or 5.916, 5.874 and 5.796 ppm) are not shown. (a) Nondecoupled spectrum; (b)–(j) decoupled spectra with arrow indicating the decoupling frequency and asterisk denoting the decoupled signals.

to a ^{31}P (H5″ of pAp and pA, fig. 3a).

In spectrum (3f), when the signals at 1680 Hz are partially saturated, the signals at 1585 and 1730 Hz show partial decoupling. Partial decoupling arises at 1430 and 1585 Hz when the decoupling frequency is set at 1400 Hz (spectrum 3j). From spectra (3c), (3f) and (3j), the characteristic line shape of H3′ which is coupled to ^{31}P and the typical ABX signals for H5′ and H5″, the signals of the ribose protons H1′, H2′, H3′, H4′, H5′ and H5″ of the Ap– residue are assigned at 2115, 1730, 1680, 1585, 1430 and 1400 Hz, respectively. The absolute assignment of H5′ and H5″ follows that of dinucleoside phosphates by Lee and Tinoco [19].

The triplet at 1595 Hz collapses when the decoupling frequency is set at 1570 Hz (spectrum 3h). If the decoupling frequency is applied at 1590 Hz, the signals at 1520, 1560 and 1570 Hz are partially decoupled. From spectra (3b), (3h) and (3i), the signals at 2130, 1570, 1595, 1560, 1590 and 1520 Hz are assigned to H1′, H2′, H3′, H4′, H5′ and H5″ of the –pA residue, respectively. Thus, the unambiguous assignment of the NMR signals for protons 1′ and the other ribose protons is obtained. Our assignment of proton 1′ signals is consistent with that by Kondo et al. [9] using specific deuteration. However, we found that the chemical shift of H1′ of Ap– crosses that of –pAp– at 45°C (fig. 2a). Thus our assignment of these two protons above 45°C is in disagreement with the earlier report of Evans and Sarma [12].

Similar procedures were carried out for the spectra at the other temperatures and for the spectra of the

other trinucleosides. Smooth chemical shift-temperature curves were observed for these protons. The computer line-shape similation using the above assignments generates calculated spectra in excellent agreement with the experimental spectra.

3.1.3. Base protons and 1′ protons of riboses in monomers and dimers

The assignment of H1′ and base protons for the monomers (except 3′, 5′ nucleoside diphosphate) and dimers followed the work of Ts'o et al. [13], Lee et al. [14] and Ezra et al. [15]. The assignment of the signals of 3′, 5′ pAp and 3′, 5′ pGp was made by comparison with those of the corresponding 3′ and 5′ mononucleotides, while that for 3′, 5′ pUp and 3′, 5′ pCp (present as a mixture with their 2′, 5′ diphosphate isomers) was made by comparison with the spectra of 3′ and 5′ mononucleotides and a mixture of 3′ and 2′ mononucleotides.

3.1.4. Measurement of the coupling constant J1′2′

In general, the coupling constant J1′2′ was measured directly from the splitting of the H1′ signals. The error is estimated to be ±0.2 Hz from the resolution of the spectrum when there is little virtual coupling or line broadening involved. When virtual coupling (e.g. Ap— of ApApA, 75°C; —pA of UpApA, 20°C; Gp— of GpUpG, 75°C; —pU of ApUpU, 45° and 75°C; Up— of UpApU, 4°C; —pAp— and —pU of UpApU, 45° and 75°C, etc. Fig. 4) or line broadening (e.g. both ApApC and ApCpC at 4° and 20°C) appeared, accurate measurement of J1′2′ could only be achieved by line shape simulation of the ribose proton signals and the temperature-dependent tendency of this value. However, the accuracy of the coupling constants in such cases is not less than ±0.5 Hz. The values of J1′2′ were used to estimate the amount of 3′ endo conformation from the equation % 3′ endo = 10(10—J1′2′) [14,15]; the data are given in table 2.

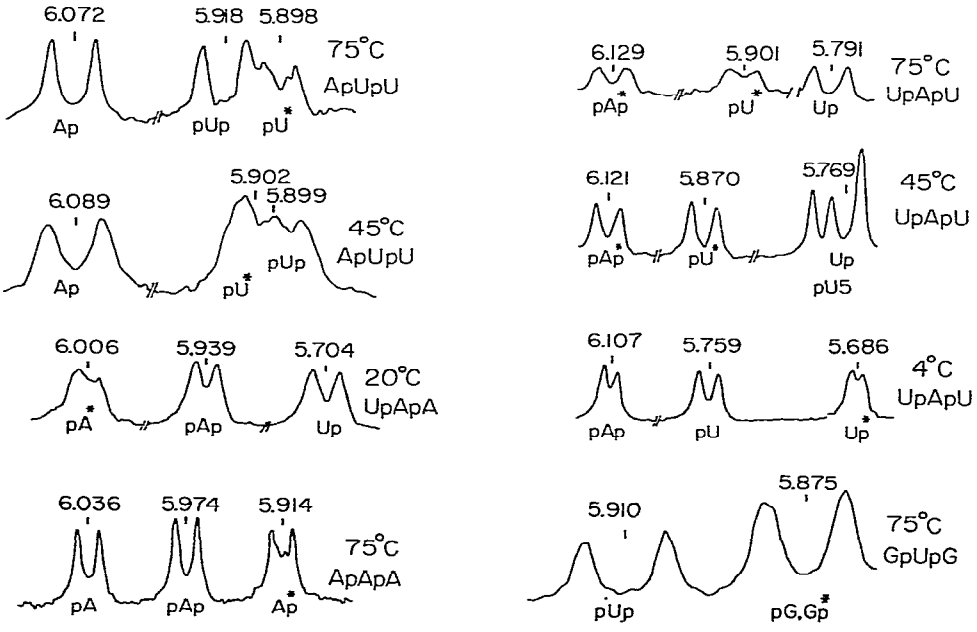

Fig. 4. Spectra of the H1's of the trimers showing virtual coupling line shape (see ref. [22] for a good discussion of virtual coupling). The signals with virtual coupling are labelled with an asterisk. In the case of GpUpG (75°C), ApUpU (45°C), UpApU (4°C, 45°C) the virtual coupling is not clearly shown in the line shape.

Table 2
The percent 3′ endo population of the ribose moieties in trinucleoside diphosphates and in the component dinucleoside phosphates. The equation, percent 3′ endo = 10(10-J1′2′), was used.

Temperature	Np–				–pNp–				–pN			
	4°C	20	45	75	4°C	20	45	75	4°C	20	45	75
ApApA	75	67	58	(48)	82	74	62	52	68	65	58	52
ApApC	84	83	63	55	87	86	69	58	83	79	69	58
UpApA	72	63	56	52	78	70	67	61	72	(63)	55	52
UpApG	68	63	58	51	74	66	58	48	52	51	49	46
ApCpG	73	60	53	46	78	67	58	52	52	46	45	43
ApUpG	57	53	49	46	62	56	49	44	52	50	47	44
GpUpG	(46)	44	42	40	55	51	47	45	49	47	45	43
ApCpC	82	77	64	52	83	80	73	59	75	72	67	60
ApUpU	62	56	50	46	61	54	50	46	60	58	(55)	(50)
UpApU	70	65	55	52	74	68	(60)	(50)	66	62	(60)	(55)
UpUpG	57	54	51	49	55	51	48	45	46	45	43	41
CpUpG	80	74	66	58	72	62	52	48	40	40	40	40
UpUpC		60	55	49		55	50	44		65	62	57
ApA	70	65	60	56					69	65	58	53
ApC	74	68	58	48					80	74	68	62
ApG	63	57	53	50					61	56	51	47
ApU	69	61	53	46					74	66	61	56
CpC	79	72	66	60					78	72	67	61
CpG	74	68	62	57					60	56	52	48
CpU	75	71	66	60					72	66	60	54
GpU	57	53	49	43					64	62	58	54
UpA	62	58	53	51					58	54	51	48
UpC	67	61	56	50					74	68	62	58
UpG	62	58	51	48					52	50	48	46
UpU	60	56	52	48					(62)	(56)	(52)	(48)

Figures in parentheses are estimated values, due to virtual coupling.

3.2. Temperature dependence of chemical shifts and base stacking

There are three major factors which determine the chemical shifts of the protons in nucleotides: the ring current effect of the base, the diamagnetic anisotropy of the electron-rich groups on the molecules and electric field effects. In stacked oligonucleotides the ring current of the neighboring bases shifts the base and H1′ resonances upfield (i.e. shielding occurs). As stacking decreases with increasing temperature we expect these resonances to move downfield (δ increases) with increasing temperature. This was always true for H2 of adenine and H5 of the pyrimidines. The largest downfield shifts seen with increasing temperature between 4°C and 75°C were for H5 of pCp in ApCpC ($\Delta\delta$ = 0.43 ppm) and H2 of Ap in ApApA ($\Delta\delta$ = 0.35 ppm). All but two nucleotides showed downfield shifts with increasing temperature for H1′. The Ap1′ in ApUpU and the pG1′ in UpUpG showed slight (<0.01 ppm) upfield shifts with increasing temperature. For H6 of the pyrimidines and H8 of the purines, however, the diamagnetic anisotropy of the ribose ring oxygen and the 5′ phosphate group exert a significant effect which depends on the glycosidic torsion angle χ. These protons are sometimes found to shift upfield and sometimes downfield with increasing temperature. The range of effects for the temperature interval from 4°C to

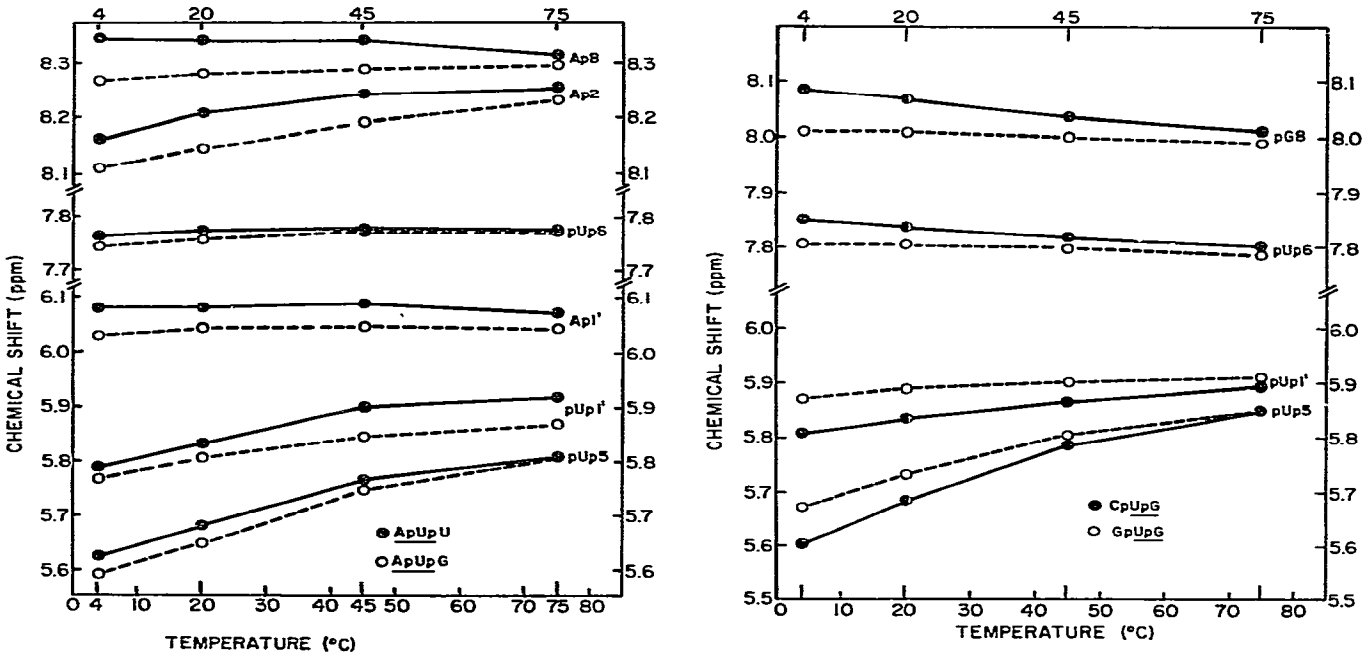

Fig. 5. Comparison of the temperature dependence of the chemical shifts of (a) the ApU part of ApUpU and ApUpG and (b) the UpG part of CpUpG and GpUpG. The chemical shift is downfield from TSP.

75°C was a 0.15 ppm upfield shift for H6 of pU in UpApU and a 0.31 ppm downfield shift for H8 of pAp in ApApC. Fig. 2 shows the temperature dependence of protons in ApApA, ApApC, UpApA; fig. 5 shows ApUpU, ApUpG, CpUpG and GpUpG. Complete data are available on request. We conclude that the best measures of base-base stacking from chemical shifts are obtained from H2 of adenine and H5 of the pyrimidines.

3.3. Ribofuranose conformation and base-base stacking

The conformation of the ribose can be described as an equilibrium between 2′ endo and 3′ endo conformations [16,17]. Table 2 shows the % 3′ endo conformation of trimers and the component dimers, estimated from 10 · (10–J1′2′) [14,15]. The component monomers exhibit 36–45% of 3′ endo conformation for purine nucleotides and 46–61% 3′ endo for pyrimidine nucleotides at 20°C. Thus the trimers as well as the dimers generally have more 3′ endo conformation than the monomers. In double-stranded RNA the ribose groups are 3′ endo [18] and it has been assumed [14,15,19] that completely stacked oligonucleotides also have 3′ endo ribose groups. Thus the % 3′ endo has been taken to be a measure of the amount of stacking. The 3′ endo population decreases when the temperature increases (table 2), consistent with the destacking caused by increasing temperature. Furthermore, one would expect that increasing chain length of an oligonucleotide would favor stacking and thus produce an increase in % 3′ endo. Comparison of the amount of 3′ endo of the two terminal residues in a trimer with their component dimers (table 2) shows the following. ApApA, ApApC, ApCpC, UpApA and UpUpC do have larger, or equal, 3′ endo conformation than their component dimers. ApUpU, UpApU, UpApG and CpUpG have less 3′ endo for one terminal residue, but more for the other. However, ApCpG, ApUpG, GpUpG and UpUpG have less, or equal, 3′ endo conformation than their component dimers at all temperatures studied. This is contrary to expectations; it indicates a special conformation for these trimers.

3.4. Bulged bases and next-nearest neighbor interaction

Optical studies of dinucleoside phosphates [20] and trinucleoside diphosphates [1—4] showed that a nearest neighbor model was adequate to explain most of the data. However, for some trimers, notably ApUpG, there was evidence [4] for strong interaction between the Ap— and —pG. It was suggested that the —pUp— looped (or bulged) out and that the Ap— and —pG stacked.

The NMR data can provide new information, because the change in chemical shifts for the base and H1′ protons in going from monomer to dimer to trimer depends on the amount of base-base stacking. As discussed in the section on temperature dependence of chemical shifts and base stacking, the H2 of adenine and H5 of pyrimidines are the least affected by glycosidic torsion angle, and are the best measures of stacking. Table 1 gives the trimerization shifts, δ(nucleotide) − δ(residue in trinucleoside diphosphate), for the molecules at 20°C. All the trimerization shifts are positive (or zero) thus indicating an upfield shift mainly due to the neighboring stacked bases. However, to address the question of next-nearest neighbor interaction we must compare trimerization shifts with dimerization shifts. If there were only nearest-neighbor effects, we would approximately expect the following. The terminal nucleotides would have a trimerization shift equal to the dimerization shifts of the component dimers, and the internal nucleotide would have a trimerization shift equal to the sum of the dimerization shifts of the component dimers. Positive deviations from the nearest-neighbor prediction would indicate the shielding effect of the ring current of the next nearest base and/or an increase in stacking. Negative deviations from the nearest-neighbor prediction would most probably indicate a decrease in nearest-neighbor stacking of the trimer relative to the dimer. Table 3 presents the difference of the trimerization shifts and appropriate nearest-neighbor dimerization shifts. A conservative estimate for the accuracy of each dimerization and trimerization shift is ±0.02 ppm. Therefore, even for the internal nucleotide (—pNp—) the differences are correct to ±0.06 ppm. However, only one significant figure is given so as to emphasize the most significant effects.

All trimers starting with Ap— give a 0.1 ppm increase in the measured trimerization shift of the —pN nucleotide relative to the nearest-neighbor calculated trimerization shift. Adenine has the largest ring current and an increase of this magnitude is reasonable for a combination of direct shielding in a right-handed stack and/or a slight increase in amount of stacking. Three trimers show a marked decrease in trimerization shift (compared to that expected from nearest-neighbor effects) for the internal nucleotide: ApCpG, ApUpG and GpUpG. These are all the type Pu—Py—Pu and we think that they are characteristic of this class. The decrease in chemical shifts for *all* three protons of the center pyrimidine nucleotide compared to the expected magnitude is a strong indication of a decrease in stacking with the purine on each side of it. Although H1′ and H6 can be affected by a change in glycosidic torsion angle, the H5 chemical shift depends mainly on base stacking. For example, the —Cp— in ApCpG is stacked less with the A and the G than it is in ApC and CpG. The obvious explanation for this result is that the strongly-stacking purines will tend to stack with each other and the intervening pyrimidine will be bulged out.

Further evidence is given in fig. 5a,b where chemical shifts are compared for the ApU part of ApUpG and ApUpU and the UpG part of GpUpG and CpUpG. Guanine has a larger ring current than uracil has, so we expect the ApU protons in ApUpG to be more shielded than those in ApUpU. This is found. However, we also expect the difference in shielding to be greater for pUp protons than for Ap. This is not found. In fig. 5a it is clear that the increase in deshielding for ApUpG versus ApUpU upon increasing temperature is greater for the Ap— protons than it is for the pUp protons. The guanine is apparently nearer to adenine than it is to uracil. Comparing the UpG in CpUpG with GpUpG (fig. 5b) we see a similar phenomenon for the H8 of pG and the H6 of pUp. The difference in shielding (GpUpG versus CpUpG) is greater for H8 of pG than for H6 of pUp. Even more striking is the fact that contrary to expectations based on a right-handed-stacked conformation, both the H1′ and H5 of pUp are shielded less in GpUpG than in CpUpG. All these data strongly suggest a significant proportion of a conformation in which the two purines are stacked and uracil is bulged out of the stack.

Table 3
The difference between dimerization shifts and trimerization shifts for H1′ and the base protons for thirteen trinucleoside diphosphates at 20°C.

Trimer	Np– Δ^1	–pNp– Δ^2	–pN Δ^3	Trimer	Np– Δ^1	–pNp– Δ^2	–pN Δ^3
ApApA H1′	0.0	0.0	0.1	ApCpC H1′	0.0	0.0	0.0
H2	0.1	0.0	0.1	H2 or H5	0.0	0.0	0.1
H8	0.0	0.1	0.1	H8 or H6	0.0	0.0	0.1
ApApC H1′	0.0	0.2	0.1	ApUpU H1′	0.0	–0.1	0.1
H2 or H5	0.1	–0.1	0.1	H2 or H5	0.0	–0.1	0.1
H8 or H6	0.0	0.2	0.1	H8 or H6	0.0	0.0	0.1
UpApA H1′	0.0	0.0	0.0	UpApU H1′	0.0	0.0	0.0
H2 or H5	0.1	0.0	0.0	H2 or H5	0.2	0.0	–0.1
H8 or H6	0.0	0.0	0.0	H8 or H6	0.0	0.0	0.0
UpApG H1′	0.0	0.0	0.0	CpUpG H1′	0.0	0.0	0.0
H2 or H5	0.1	0.0	–	H2 or H5	0.0	0.0	–
H8 or H6	0.0	0.0	0.0	H8 or H6	0.0	–0.1	0.0
ApCpG H1′	0.0	–0.1	0.1	UpUpG H1′	0.0	0.0	0.0
H2 or H5	0.0	–0.3	–	H2 or H5	0.0	0.0	–
H8 or H6	0.1	–0.3	0.1	H8 or H6	0.1	–0.1	0.0
ApUpG H1′	0.0	–0.1	0.1	UpUpC H1′	0.0	0.0	0.0
H2 or H5	0.1	–0.2	–	H5	0.0	0.0	–0.1
H8 or H6	0.1	–0.1	0.1	H6	0.0	0.0	–0.1
GpUpG H1′	0.0	–0.1	0.1				
H5	–	–0.1	–				
H8 or H6	0.0	–0.1	0.0				

Δ^1 = δ(Np– in trimer) – δ(Np– in dimer)
Δ^2 = δ(–pNp–) – δ(Np– in dimer) – δ(–pN in dimer) – [δ(nucleoside-3,5-diphosphate) – δ(nucleoside-3′-phosphate) – δ(nucleoside-5′-phosphate)]
Δ^3 = δ(–pN in trimer) – δ(–pN in dimer)

4. Conclusion

We have studied the effect of chain length and composition on nucleotide conformation by analyzing the NMR data of the base protons and the H1′ proton of the ribose of 13 different trinucleoside diphosphates. Comparing the results with that of the component dimers and monomers, we find marked sequence-dependent effects. ApApA, ApApC, ApCpC, UpApA and UpUpC are more stacked than their component dimers, but ApCpG, ApUpG, GpUpG and UpUpG are less stacked. Evidence from next nearest-neighbor effects was obtained for a conformation of trinucleoside diphosphates in which the interior base is bulged out and the two terminal bases stack on each other. This conformation is most pronounced for the sequence Pu–Py–Pu and is most important when the middle base is uracil – the weakest stacking base. Earlier optical studies had already suggested this conformation [4]. We do not know if this bulge will also occur in longer oligonucleotides, but we think it likely. In the presence of ethidium a bulge of this type is stabilized enough so that a 1:1:1 complex of GpUpG:CpC:ethidium forms a minihelix with two G·C base pairs and a bulged base [21]. It may be that bulges in both single and double strands have significant biological functions such as enzyme recognition signals and sites of mutagenesis.

Acknowledgment

The authors thank Ms. Barbara Dengler, Mr. David Koh and Ms. Leni Grey for helping synthesize trimers and pUp and pCp, and Dr. Susan Freier for providing ApUpG. Mr. Arthur Pardi and Mr. Stephen Winkle made helpful suggestions. We also thank the Stanford Magnetic Resonance Laboratory (supported by NSF grant GR 23633 and NIH grant RR 00711) for use of the LHS-360 MHz facilities. This work was supported by NIH grant GM 10840 and by the Division of Biomedical and Environmental Research of the Department of Energy.

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