Dynamic Structure of Pyrimidine-polyribonucleotides in Solution. ^{1}H NMR Studies[1)]

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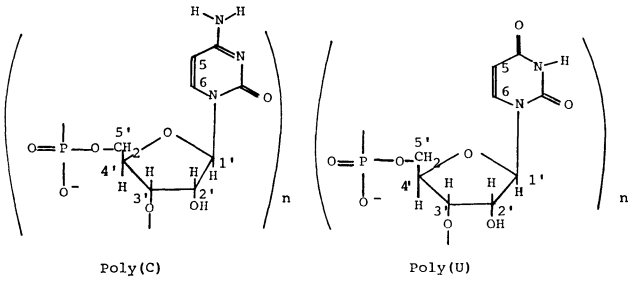
(Received June 25, 1981)

Temperature dependent conformations and molecular motions of pyrimidine polyribonucleotides, poly(C) and poly(U), have been studied in ^2H_2O solutions (pH 5.8±0.3, 5—72 °C) by measuring ^{1}H NMR intensities, chemical shifts, coupling constants, and spin-lattice relaxation times. Below 50 °C, poly(C) exists as a slowly exchanging mixture of double-stranded and single-stranded forms of which only the single-stranded form is observable by ^{1}H NMR. In this temperature range, the single-stranded poly(C) shows a strong base-stacking with dominated C_3'-*endo anti* conformation, whereas above 50 °C it takes partly C_2'-*endo* conformation with destabilization of the helical structure. Poly(U) exists as single strand within the whole temperature range studied, and the bases are nearly unstacked with 58% of C_2'-*endo* conformer. Poly(U) is more flexible than poly(C), which was characterized with rotational correlation times in the order of 10^{-9}—10^{-10} s.

Conformation of synthetic homopolynucleotides has been studied by many investigators as the first step toward the understanding of the structure of nucleic acids. In aqueous solution, each homopolynucleotide shows unique behaviour characteristic of its purine and pyrimidine bases.[2,3)] Nuclear magnetic relaxation measurements of ^{31}P showed different motions for different polynucleotides.[4)] However, their detailed structures in solution are still unknown although some of their structures in fiber states have been analyzed by X-ray diffraction.[5—7)]

At neutral pH, poly(C) takes single-stranded helix stabilized by base stacking, while at acidic pH (pH<5.0) it exists as a double-stranded helix in which the bases are held together by three hydrogen bonds, one of which can be formed only when a proton is added to N-3 for each pair of bases.[8—10)] In general, the ordered double helical form of high polymers does not give a high resolution NMR spectrum.[2)] Our previous ^{31}P NMR study[11)] exhibits that at acidic pH the signal is too broad to be observed presumably due to the double-stranded form, while at an intermediate pH between acidic and neutral the exchange between the double-stranded and single-stranded forms is slow in the NMR time scale, so that the single-stranded form and possibly partially double-stranded form can be observed.

In the present work, temperature dependence of ^{1}H chemical shifts, coupling constants and spin-lattice relaxation times of poly(C) and poly(U) were measured and the conformational features of single-stranded poly(C) are discussed together with those of poly(U) known to exist as a random coiled structure.



Poly(C) Poly(U)

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Experimental

The potassium salts of poly(C) and poly(U) (molecular weight>10^5) were purchased from Sigma Chemical Company. The samples were dialysed against a large volume of 0.1 M (1 M=1 mol dm^{-3}) sodium chloride to remove low molecular species and paramagnetic metal ions. The solution was lyophilized and the residue was dissolved in ^2H_2O (99.7%). The lyophilization and addition of ^2H_2O were repeated three times to reduce ^1H_2O background. About 3 mM of EDTA was added to remove the effect of the remaining paramagnetic metal ions. No buffer was used. The final pH of the solution was 5.8±0.3 (direct pH meter reading). The samples were evacuated on a vacuum line and sealed in an NMR sample tube of 5 mm diameter. The final concentration of poly(U) and poly(C) were 92 mM and 53 mM in monomer unit, respectively, as determined from the optical absorption at 261 nm and 269 nm by using the molar extinction coefficients of 9600 and 6300 for poly(U) and poly(C), respectively.[12)]

Spin-lattice relaxation times (T_1) were measured at 100 MHz on a JEOL PFT Fourier transform NMR spectrometer using the inversion recovery pulse sequence (180°-t-90°). T_1 values were deduced with a non-linear least square fit method. Chemical shifts and coupling constants of poly(C) were measured at 360 MHz on a Bruker HX-360 spectrometer. Relative peak area was determined at 360 MHz by averaging the values obtained by three independent methods, *i.e.* measurement of peak height, integration of the peak area, and weighing the spectral sheet.

Results and Discussion

Poly(C). Figure 1(a) shows the peak area of the three protons (H_5, H_6, and H_1') of poly(C) at pH 5.8 as a function of temperature. It is noted that the peak area of each of the three protons shows an abrupt increase at about 45 °C. The loss of signal intensity below 45 °C seems to be best interpreted as due to the formation of the double-stranded form of poly(C) whose ^{1}H NMR signals are too broad to be observed. Moreover, the exchange between the single-stranded form and the double-stranded form of poly(C) must be slow in the NMR time scale,[11,13)] so that the observed signals correspond to the poly-

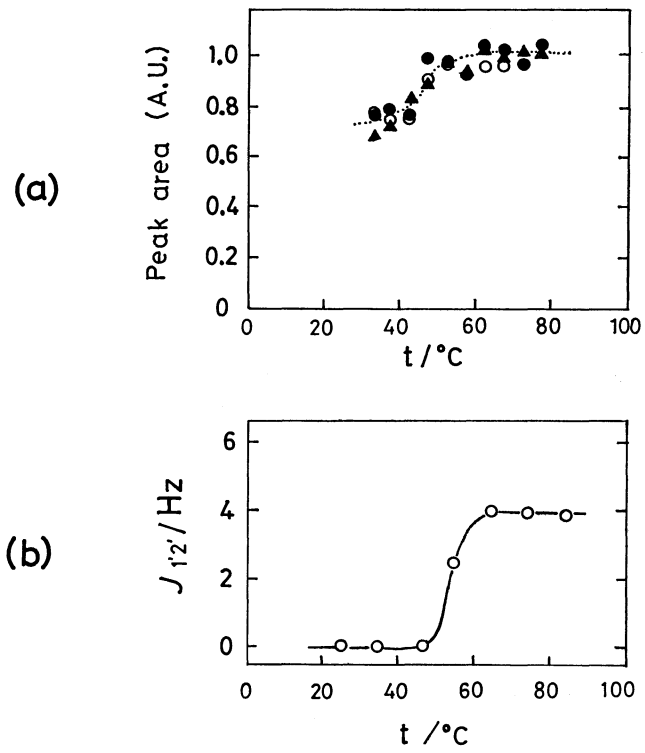

Fig. 1. Temperature dependence of ^{1}H NMR parameters of poly(C) measured at 360 MHz (53 mM in ^2H_2O, pH=5.8±0.3, [Na^+]=0.1).
(a): Peak area of H_5 (●), H_6 (▲), and $H_{1'}$ (○),
(b): coupling constant $J_{1'2'}$.

(C) in the single-stranded form and possibly in the partially double-stranded form. This interpretation is in accordance with the earlier observation that, in the acidic pH range, the signal intensity of ^{31}P resonance decreases upon protonation of poly(C) by the formation of a double strand.[11]

Figure 1(b) shows that the coupling constant between $H_{1'}$ and $H_{2'}$ ($J_{1'2'}$) is almost nil below 50 °C. From the Karplus relation, this result means that the dihedral angle ($\angle H_{2'}C_{2'}C_{1'}H_{1'}$) is close to 90°, *i.e.*, that the ribose ring of the single-stranded poly(C) takes exclusively $C_{3'}$-*endo* (N) conformation below 50 °C. $J_{1'2'}$ shows a sharp increase in the narrow temperature range of 50—65 °C, and above 65 °C becomes constant at 3.9 Hz. The increase in the coupling constant reflects the increase in the population of the $C_{2'}$-*endo* (S) conformer under the assumption of a rapid equilibrium between N and S conformers.[14] The population of the S conformer above 65 °C is then estimated to be 42% by using the equations given by Altona and Sundaralingam.[14]

In contrast, the chemical shifts of the three protons of poly(C) increase rather monotonically in the temperature range of 28—70 °C as shown in Fig. 2. This observation implies that at low temperature these protons receive strong deshielding effects from the ring currents of neighbouring bases, but that the effects are relieved at higher temperatures by gradual unstacking of bases, in agreement with the conclusion obtained from other methods.[13,15,16]

Lee and co-workers have suggested that the base

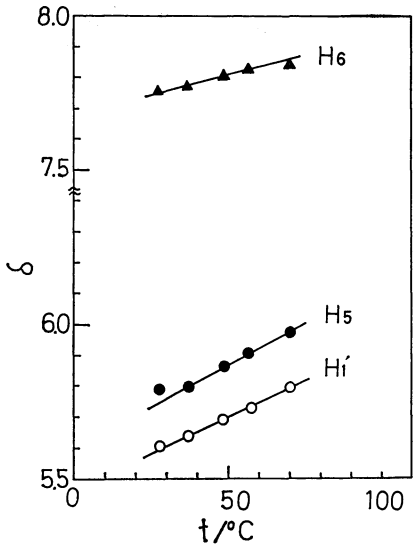

Fig. 2. Temperature dependence of chemical shifts of H_5, H_6, and $H_{1'}$ of poly(C) as measured from an internal standard of DSS (H_6 ▲, H_5 ●, $H_{1'}$, ○).

stacking arrangement prohibits the ribose ring to take the S-conformation, based on the fact that the ribose ring of dinucleotides has a large fraction of N-conformer corresponding to the increase in the fraction of base stacking.[17] The present results have shown that, similarly in poly(C), a single helical or partial double helical form with stacked bases and exclusive N ribose conformation is highly stabilized at low temperature, but that upon destacking of bases, poly(C) is allowed to take the S-conformation.

We may conclude that under the present experimental conditions, the conformational equilibria in poly(C) could be summarized in the following schemes:

$$\left.\begin{array}{ccc} \mathrm{Poly(C)_N{*}} + \mathrm{H^+} & \overset{\text{rapid}}{\rightleftharpoons} & \mathrm{Poly(C)_N{*}H^+} \\ \Big\Vert\!\!\Big\vert \text{rapid} & & \Big\Vert\!\!\Big\vert \text{rapid} \\ \mathrm{Poly(C)_S{*}} + \mathrm{H^+} & \overset{\text{rapid}}{\rightleftharpoons} & \mathrm{Poly(C)_S{*}H^+} \end{array}\right\} \quad (>65\ ^\circ\mathrm{C}), \qquad (1)$$

$$\mathrm{Poly(C)_N{*}} + \mathrm{H^+} \overset{\text{rapid}}{\rightleftharpoons} \mathrm{Poly(C)_N{*}H^+} \quad (<50\ ^\circ\mathrm{C}), \qquad (2)$$

$$\mathrm{Poly(C)_N{*}} + \mathrm{Poly(C)_N{*}H^+} \overset{\text{slow}}{\rightleftharpoons} \mathrm{Poly(C)_N{*}H{+}Poly(C){*}} \quad (<50\ ^\circ\mathrm{C}), \qquad (3)$$

where Poly(C)* and Poly(C)*H+Poly(C)* represent the single- and double-stranded forms of poly(C), respectively, and subscripts N and S represent N and S conformers of the ribose, respectively.

Figure 3 shows the temperature dependence of T_1's of H_5, H_6, and $H_{1'}$ of poly(C). In spite of the remarkable change of the coupling constant in the range of 50—60 °C, T_1 shows no abrupt change in the corresponding temperature range. Akasaka showed that the dynamic structure of a polynucleotide can be studied from the temperature dependence of T_1.[18] Namely, in an isotropic motional approach with a single τ_c, the T_1 minimum occurs at a rotational correlation time τ_c of 9.9×10^{-10} s at 100 MHz, and by using this τ_c and the minimum value of T_1, relevant interproton distances can be obtained. As shown in

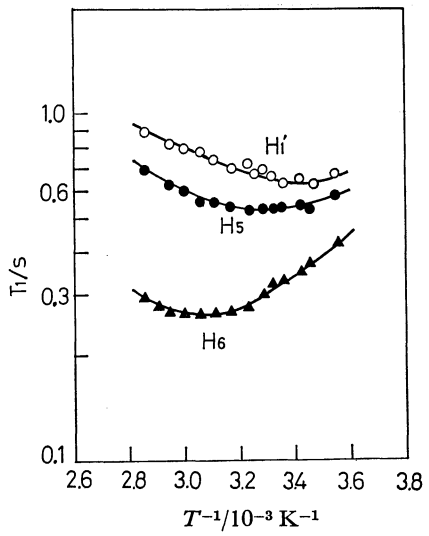

Fig. 3. Semilogarithmic plots of spin-lattice relaxation times (T_1) of poly(C) against reciplocal absolute temperature (H_6 ▲, H_5 ●, $H_{1'}$ ○).

Fig. 3, T_1's of all the three protons of poly(C) go through minima. However, they occur at different temperatures.

In principle, the occurence of different $T_{1\,min}$ temperatures indicates the possibility of differential motions of the three protons. The effect does not seem to arise from the cross-relaxation effect among protons,[19] since around $T_{1\,min}$, where $\omega\tau_c \approx 1$ holds, the cross-relaxation term is close to nil. By taking a simple approach of isotropic motion, the value of r_{iobsd} was obtained for each proton from the minimum values of T_1 (see Appendix), and the results are shown in Table 1.

The values of r_{iobsd} may be compared with r_{icalcd} calculated with atomic coordinates given for 5'-CMP,[20] by taking into account of dipolar interactions within the same nucleotide unit only. The r_{icalcd} values were computed for different torsional angles about the glycosidic bond (χ) varied at an interval of 10° and the dihedral angle ($\angle C_{3'}C_{4'}C_{5'}O_{5'}$) ($\psi$) at interval of 30°, respectively. The ribose conformation was fixed to $C_{3'}$-*endo*. The values of r_{icalcd} for H_6 was not calculated since T_1 for H_6 goes through minimum above 50 °C where the fraction of $C_{2'}$-*endo* conformer cannot be neglected.

On computation r_{icalcd} showed little dependence on ψ. Therefore, the curves in Fig. 4 show r_{icalcd} as a function of χ for a fixed ψ (=60°).[21] For H_5, r_{icalcd} and r_{iobsd} coincide in the whole region of χ within the experimental errors of T_1 (±5%), as is expected from the chemical structure of cytidine base

Table 1. r_{iobsd}'s deduced from minimum values of T_1 by using Eq. 3 in Appendix

	r_{iobsd}/Å		
	H_6	H_5	$H_{1'}$
Poly(C)	2.16±0.12	2.43±0.14	2.50±0.14
Poly(U)	2.15±0.12	2.48±0.15	2.48±0.15

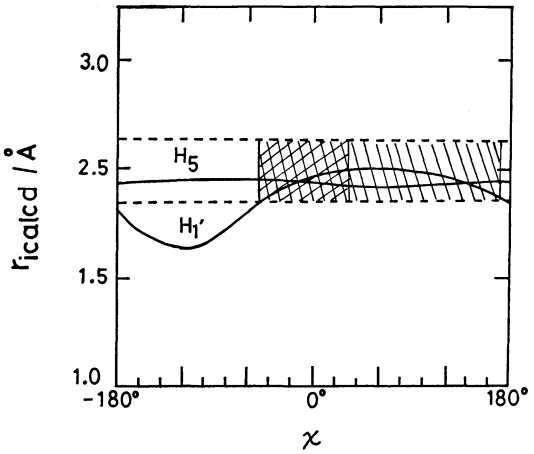

Fig. 4. r_{icalcd}'s $(\sum_j r_{ij}^{-6})^{-1/6}$ are plotted against torsional angle about glycosidic bond (χ).
Ribose ring and ψ are fixed to $C_{3'}$-*endo* and 60°, respectively. The region where r_{icalcd} and r_{iobsd} coincide within experimental errors is shown by hatched line for $H_{1'}$. The cross-hatched area shows the allowed and plausible region obtained by taking account of the van der Waals exclusion.

where T_1 of H_5 is mainly determined by the dipolar interaction with H_6. The hatched region between the dotted lines in Fig. 4 shows where r_{icalcd} and r_{iobsd} of $H_{1'}$ coincide within experimental errors of T_1. The allowed region of χ for van der Waals contact was estimated to be −130°—40°, where the exclusive atomic radii for H and O were taken to be 0.95 and 1.1 Å, respectively. Therefore, the allowed and plausible region of χ for $H_{1'}$ is in the range of −50°—40° as is represented by the cross-hatched area in Fig. 4, showing that the orientation of the base is restricted to *anti*.

This result is consistent with the theoretical calculation in mononucleotides which predicts that the most probable value of χ for the pyrimidine base lies in the range of 0°—30° for the $C_{3'}$-*endo* sugars.[21] In solution, mononucleotides are shown to exist as equilibrium mixtures of *syn* and *anti* conformers with preference of *anti*. However, in CpC the angle decreases with increasing stacking of base.[17] Our conclusion that single-stranded poly(C) takes predominantly $C_{3'}$-*endo anti* orientation of the base at low temperatures is in accordance with these results. A consistent result has also been presented for single stranded poly(C) in the fiber state by X-ray analysis ($C_{3'}$-*endo anti*, χ=80°).[7] The fraction of *syn* conformers, however, might not be neglected above 50 °C, in view of the coexistence of a substantial fraction of $C_{2'}$-*endo* ribose conformers (42%).

Poly(U). In contrast with poly(C), no temperature dependence of peak area of protons was observed for poly(U). As shown in Fig. 5, neither the chemical shifts of H_5 nor H_6 of poly(U) show significant temperature dependence, indicating that the base-stacking is negligible in this polymer, in accordance with the reported random coil property of poly(U).[2,3]

Temperature dependence of coupling constant $J_{1'2'}$

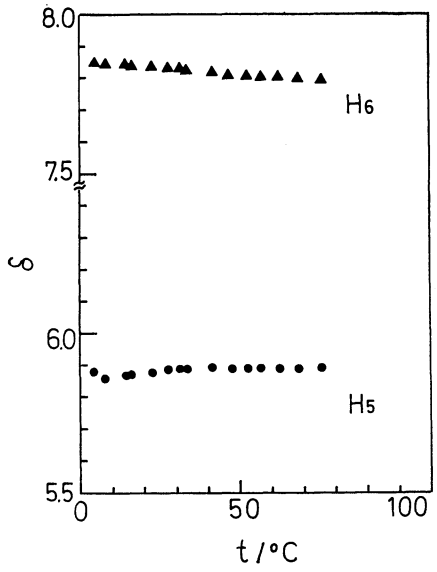

Fig. 5. Temperature dependence of chemical shifts of H_5 and H_6 of poly(U) as measured from an internal standard of DSS (H_5 ●, H_6 ▲).

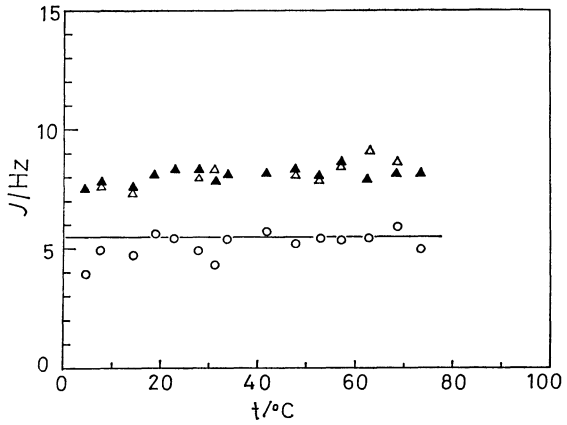

Fig. 6. Temperature dependence of coupling constants $J_{1'2'}$ and J_{56} of poly(U).
$J_{1'2'}$ ○, J_{56} (measured on H_5 signal) △ J_{56} (measured on H_6 signal) ▲.

of poly(U) is shown in Fig. 6 together with that of J_{56}. $J_{1'2'}$ is measured on the $H_{1'}$ signal under homo-decoupling H_6 proton, since the H_5 and $H_{1'}$ signals partially overlap in poly(U). Since J_{56} shows little temperature dependence, it appears safe to conclude that $J_{1'2'}$ is also invariant with temperature within the experimental accuracy of ±1.5 Hz. Based on the detailed analysis of coupling constants of ribose protons at 36 °C at 220 MHz by Kreishman and Chan,[22] Davies and Danyluk have estimated ring-conformer population of poly(U) to be 42% and 58% for N and S conformers, respectively.[23] The result of Fig. 6 shows that such conformation of the ribofranosyl ring of poly(U) does not change within the temperature range studied. Richards and co-workers[24] reported that poly(U) could undergo a co-operative transition to an order form below 15 °C. However, since their optical experiments were performed under high salt concentration, it could not be compared directly with the present results.

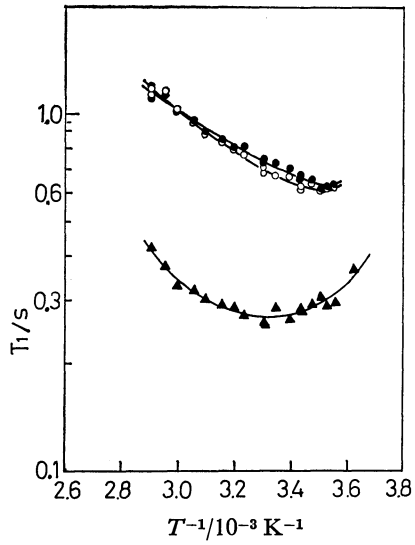

Fig. 7. Semilogarithmic plots of spin-lattice relaxation times (T_1) of poly(U) against reciprocal absolute temperature (H_5 ●, H_6 ▲, $H_{1'}$ ○).

Figure 7 shows the temperature dependence of T_1 for H_5, and H_6, and $H_{1'}$. T_1 of the three protons go through minima, but at different temperatures. Interproton distances, r_{lobsd} were obtained similarly as in poly(C) (Table 1). These distances must reflect local conformation of poly(U). The r_{lcalcd} values were computed for many conformers and compared with r_{lobsd} similarly as for poly(C). As the result, the base orientation of poly(U) is restricted to *anti* and *syn*.

The population of S conformer above 65 °C is 16% higher in poly(U) than in poly(C). This would arise from the fact that poly(U) is free from base stacking at all the temperatures studied, whereas poly(C) takes partial base stacking even above 65 °C as judged from Fig. 2, where N conformer is preferable to S conformer discussed for poly(C).

From the T_1 values, rotational correlation times τ_c were estimated to be in the order of 10^{-10}—10^{-9} s, corresponding to the local motions of polynucleotides.[4,18,25,26] The higher flexibility of poly(U) is implied from the fact that $T_{1\,\text{min}}$ of poly(U) protons appear at lower temperature than those of poly(C). The above observation is in accordance with the earlier conclusion from ^{31}P T_1 measurements.[4]

Appendix

When T_1 of a proton i is determined by dipolar interaction which is modulated by an isotropic rotation of the molecule with a characteristic time constant τ_c, the following equation is given for the interacting protons separated by a fixed distance r,[27]

$$\frac{1}{T_1} = \sum_j \frac{\gamma^4\hbar^2}{r_{ij}^6}\left(\frac{3}{10}\frac{1}{1+\omega^2\tau_c^2}+\frac{12}{10}\frac{1}{1+4\omega^2\tau_c^2}\right)\tau_c, \qquad (1)$$

where γ and ω are the gyromagnetic ratio and Larmor angular frequency of a proton, respectively, and suffix i and j represent the observed proton and the other proton interacting with proton i, respectively. Equation 1 predicts that T_1 goes through a minimum when $\omega\tau_c \simeq 0.62$ or $\tau_c =$

9.9×10^{-10} s. Therefore, for T_1 at the minimum, Eq. 1 could be rewritten as follows,

$$\frac{1}{T_{1\,\mathrm{min}}} = \sum_{j}\frac{\gamma^4\hbar^2}{r_{ij}^6}\times 6.81\times10^{-10}. \quad (2)$$

Accordingly, with the minimum value of T_1 we can evaluate interacting proton-proton distance in a form of

$$r_{\mathrm{lobsd}} = (T_{1\,\mathrm{min}}\gamma^4\hbar^2\times 6.81\times10^{-10})^{1/6} \quad (\text{in Å}). \quad (3)$$

Chemical shifts and coupling constants of poly(C) were measured at 360 MHz at the Max-Planck Institut für Medizinische Forschung, Heidelberg, FRG. The authors are grateful to Professor K. H. Hausser for this opportunity.

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