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Poly(inosinic acid) Helices: Essential Chelation of Alkali Metal Ions in the Axial Channel[†]

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ABSTRACT: Poly(I) forms ordered, helical, size-specific complexes with Na⁺, K⁺, Rb⁺, and Cs⁺. Under ordinary conditions, no complex is formed with Li⁺. Formation of helical poly(I) is completely dependent on specific metal-polymer interactions, which are distinct from the usual metal-phosphate counterion association. Na⁺ and K⁺ each form a single complex with poly(I), and the reaction rates are very slow. Rb⁺ and Cs⁺ each give rise to two complexes with poly(I): a metastable form I in a relatively rapid reaction and a stable form II in a slow reaction. Ultraviolet titration curves of poly(I) with K⁺, Rb⁺, and Cs⁺ are presented. Conditions of formation and regions of stability of the different complexes are given. Equilibrium dialysis experiments show specific binding of Rb⁺ to poly(I) in the presence of an excess of Li⁺. The binding ratio is approximately eight inosine residues per Rb⁺. The IR spectra of the form I and form II Rb⁺ (and Cs⁺) complexes are distinct and provide a good tool for observing interconversions of the structures. The CD spectra of the stable (form II) Rb⁺ and Cs⁺ complexes are strikingly different

from those of the metastable forms and from those of Na⁺ and K⁺. The familiar negative first extremum of the Na⁺ and K⁺ helices undergoes a reversal of sign in form II of both the Rb⁺ and Cs⁺ complexes. The CD spectra of the stable (form II) Rb⁺ and Cs⁺ complexes are inconsistent with exciton splitting of a single transition. They thus support assignment of the two long-wavelength extrema to distinct π - π^* transitions in all of the helical poly(I) spectra. The observations are interpreted in terms of a structural model in which the metal ion is specifically bound to inosine carbonyl oxygens at one of two distinct binding sites in the axial channel of a four-stranded helix. Na⁺ is in the center of the planar array of four inosine residues with a M-O distance of ~ 2.3 Å and coordination number of four. K⁺, Rb⁺, and Cs⁺ are on the helix axis midway between the planes of tetramers with a M-O distance of ~ 2.8 - 2.9 Å (for Cs⁺, possibly somewhat greater) and coordination number of eight. Arguments and evidence for the model are presented and predictions of the model discussed.

The ordered form of poly(I) has been studied extensively by both fiber diffraction (Rich, 1959; Arnott et al., 1974; Zimmerman et al., 1975) and solution spectroscopic methods (Sarkar & Yang, 1965; Brahms & Sadron, 1966; Formoso & Tinoco, 1971; Thiele & Guschlbauer, 1973; Cech & Tinoco, 1976). The structure was first considered to be a three-stranded helix (Rich, 1959), but later diffraction studies and model building indicate that it is four stranded (Arnott et al., 1974; Zimmerman et al., 1975). Despite numerous investigations there has not been a satisfactory correlation of physical properties with the accepted structure, and reports of many measured properties have shown rather wide variability. A recent infrared study (Miles & Frazier, 1978) has shown that helix formation is strikingly dependent on the identity of the alkali metal cation present in solution. The results were interpreted in terms of a model based upon size-specific complexing of metal ions to one of two binding sites in the axial

channel of the four-stranded helix (see Discussion).

The present paper has the objectives of examining further the alkali metal ion specificity of complex formation and determining more clearly the range of conditions under which helix formation may be expected. Whereas the infrared measurements were done on relatively concentrated polymer solutions (e.g., 0.02 M), most of the previous UV and CD studies had been done on quite dilute solutions (e.g., 10^{-4} M). In this report, we bring the observations into a common frame of reference and examine the new data for consistency with the model. Since poly(I) is the first and simplest polynucleotide shown to possess a marked alkali metal ion specificity, it is important to characterize the metal-polymer interactions in some detail as a base line for other more complex systems, such as poly(X).

Materials and Methods

Poly(I) purchased from P-L Biochemicals, Inc. (lot no. 200-15) was converted to the Li⁺ salt and characterized as described previously (Howard & Miles, 1982).

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UV spectra were measured with a Cary Model 118 spectrophotometer. A preliminary account of the design of the laboratory data acquisition system (LDACS) used to control the spectrophotometer and process the data has been reported (Powell et al., 1980).

CD spectra were measured with a Cary Model 60 spectropolarimeter equipped with a Model 6003 CD accessory or with a Jasco J-500A spectropolarimeter, both using the above LDACS system.

Equilibrium dialysis was carried out at $5 \pm 1^\circ\text{C}$ with 1-mL cells (New York Laboratory Supply, Inc.) separated by semipermeable membranes (Spectrum Medical Industries, Inc., Los Angeles, CA) designed to prevent diffusion of compounds with molecular weights greater than 12 000–14 000. One side contained 1 mL of 0.025 M poly(I) [or 0.025 M poly(A) in control experiments] and the other side 1 mL of a solution containing 0.1 M LiCl and RbCl of an appropriate concentration. Cells were shaken for at least 24 h before analysis. In control experiments Rb^+ in the absence of poly(I) was shown to reach the same concentration on either side of the membrane within 16 h. Aliquots were delivered from a calibrated microburet (Micro Metric Instrument Co., Cleveland, OH) to glass scintillation vials. Ten milliliters of Aqueous Counting Scintillant (Amersham Corp.) were added and ^{87}Rb counted with a Nuclear-Chicago liquid scintillation counter at 84% counting efficiency. A standard curve was established relating cpm to micromoles of Rb. The linear least-squares regression line $\text{cpm} = 3.125(\text{micromoles of Rb}) + 2.768$ was found to describe the relation with a correlation coefficient of 1.000.

Results

We shall summarize here several of our findings in order to facilitate following the rather complicated details which follow. Under ordinary conditions (e.g., 0.1 M Li^+) helix formation is not observed in Li^+ solution. This fact has enabled us to use Li^+ as a supporting electrolyte, capable of screening mutual repulsion of phosphates, while examining specific helix-forming interactions of the other cations. In Na^+ solutions, a helix of rather low stability ($T_m = 29^\circ\text{C}$ in 0.1 M Na^+) is formed. In K^+ solutions, a markedly more stable helix ($T_m = 56^\circ\text{C}$) is formed. We have now found that NH_4^+ ion also permits formation of a helix only slightly less stable than that in K^+ (Howard & Miles, 1982). Both Rb^+ and Cs^+ form stable complexes with poly(I) but form as well metastable helices of low stability. The stable complex with Rb^+ has the highest T_m (62°C , 0.1 M Rb^+) of all complexes formed with alkali metal cations. It is convenient to divide the cations studied here into two classes with respect to their speed and extent of interaction with poly(I): (1) Na^+ , K^+ , and NH_4^+ , which react very slowly with poly(I) under most conditions to form a single, stable complex, and (2) Rb^+ and Cs^+ , which interact much more rapidly with poly(I) and form two distinct complexes.

With cations of the first class (Na^+ , K^+ , and NH_4^+) helix formation is strongly influenced by (1) cation concentration, (2) poly(I) concentration, (3) length of reaction period, and (4) freezing the solution containing both poly(I) and cation. The consequences of changes in these experimental variables on the interaction between poly(I) and each of the alkali metal cations are considered below.

Interaction of Poly(I) with K^+ . The UV spectrum of 0.01 M poly(I) shows little or no change on addition of K^+ , provided that the cation concentration is below about 3×10^{-2} M. With an increase in $[\text{K}^+]$, however, the spectrum undergoes a progressive decrease in molar extinction [from 10 000 to 6 800

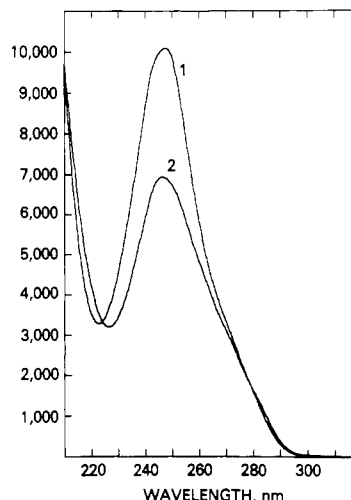


FIGURE 1: Ultraviolet spectra of single-stranded poly(I) (curve 1) and of the K^+ -poly(I) complex (curve 2). Conditions: 0.01 M polymer; 0.01 M K^+ (curve 1) or 0.19 M K^+ (curve 2); 0.02 M lithium cacodylate, pH 6.8. Curve 2 was measured 18 days after the components were mixed.

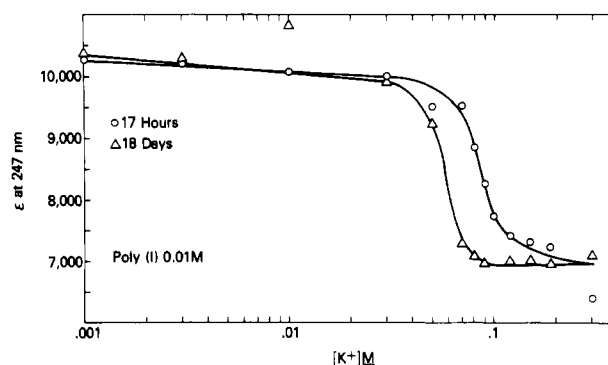


FIGURE 2: Spectrophotometric titration of poly(I) with K^+ . Measurements were made 17 h (O) and 18 days (Δ) after preparation of a separate solution for each point. Conditions: 0.01 M poly(I); 0.025 M lithium cacodylate, pH 6.8; 20.0°C .

at 247 nm for fully interacted poly(I)] in a manner characteristic of a cooperative structural transition (Figures 1 and 2). We have used this decrease in extinction coefficient as a measure of the extent of complex formation. Complex formation is very slow when $[\text{K}^+] \leq 0.1$ M, and spectral changes remain incomplete even when measurements are made after reaction periods of many days, resulting in nonequilibrium titration curves (Figure 2). Poly(I) is apparently half-titrated at $[\text{K}^+] = 0.09$ M if measurements are made after 17 h, but the value shifts to 0.06 M after 18 days, though even this does not represent equilibrium.

The rate of helix formation is also strongly dependent on poly(I) concentration. Thus, for example, when $[\text{poly(I)}] = 10^{-2}$ M (at 0.2 M K^+), complex formation is 90% complete in 0.7 h. When the polymer concentration is reduced 10-fold to 10^{-3} M, however, 17.5 h are required to achieve the same extent of reaction. Helix formation between poly(I) and K^+ thus is favored by high concentrations of both reactants and by extended reaction times. With poly(I) at 6×10^{-4} M and K^+ at 0.01 M, no change in molar extinction and, consequently, no appreciable helix formation occurs within 24 h. Failure to observe complex formation has a kinetic rather than a thermodynamic origin, however. Preformed K^+ -poly(I) complex, prepared at higher concentrations of both reactants, is quite stable when diluted to 6×10^{-4} M poly(I) and 0.01 M K^+ ($T_m = 38^\circ\text{C}$). Once formed, the complex is stable over

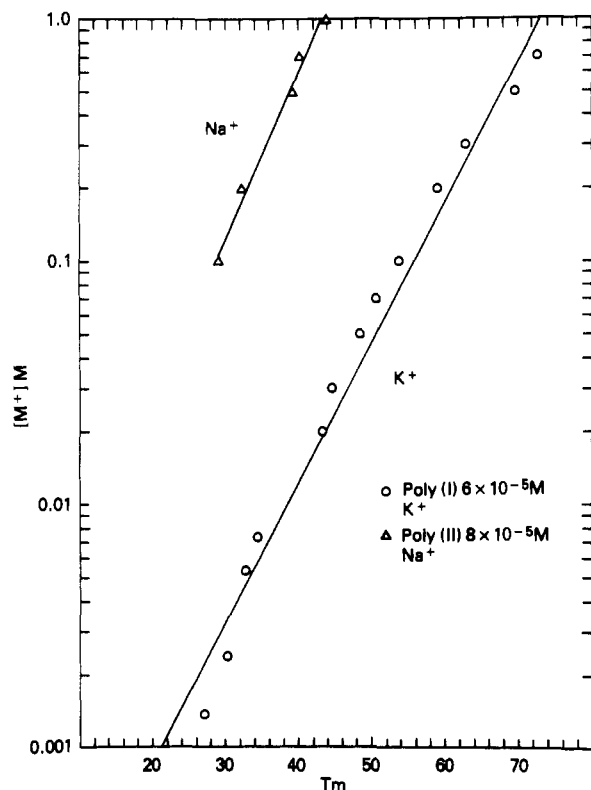


FIGURE 3: Dependence of T_m of metal ion complexes of poly(I) on $[Na^+]$ (Δ) or $[K^+]$ (O). Concentrated metal ion complexes were diluted for measurement of melting curves, as described below. Poly(I) (0.01 M) in 0.01 M lithium cacodylate, pH 6.8, was made 1.15 M in K^+ (total volume 0.06 mL), resulting in precipitation of the K^+ -poly(I) complex. The precipitate was redissolved in 1 mL of water and further diluted to 6×10^{-5} M poly(I) in a solution containing K^+ and buffer at the indicated concentrations. The Na^+ -poly(I) complex was prepared by holding 5×10^{-4} M poly(I) in 1 M NaCl at room temperature for 27 days prior to dilution to 8×10^{-5} M poly(I). Least-squares lines were fitted by computer to the data. $dT_m/d \log [Na^+] = 14.7 \pm 1.0$ °C. $dT_m/d \log [K^+] = 17.3 \pm 0.7$ °C. The breadth of melting curves is 10–12 °C over the range 0.001–1.0 M K^+ .

a wide range of concentrations (Figure 3). The salt-dependence curve is shown in Figure 3.

An alternative method of promoting helix formation, even when poly(I) and K^+ are so dilute that they otherwise do not interact, is freezing the solution. We assume that the factor responsible for enhancing the conversion is the concentrating effect of freezing on solutes excluded from the ice phase [cf. Zimmerman & Coleman (1972), Zimmerman (1975), Howard et al. (1977)]. Complex formation at 0.01 M poly(I) and 0.03 M K^+ , for example, was only 5% complete after 18 days, but freezing and thawing resulted in essentially complete conversion to the complex. At 6×10^{-5} M, poly(I) and 0.01 M K^+ conversion is only 18%. Elevation of $[K^+]$ above 0.1 M with 0.01 M poly(I) results in formation after freezing and thawing of a precipitate which can only be redissolved either by extensive dilution or by raising the temperature above T_m of the K^+ -poly(I) complex.

The extent and rate of helix formation can thus be seen to depend on the previous history of a poly(I) solution. The common practice, for example, of storing poly(I) solutions in a freezer may result in an unintended but extensive and unknown degree of complex formation. This source of uncontrolled variability may be responsible for many of the discrepancies in experimental data collected on poly(I) by different groups. Conversion of poly(I) to the helical state may be avoided by use of Li^+ as the counterion, since the complex

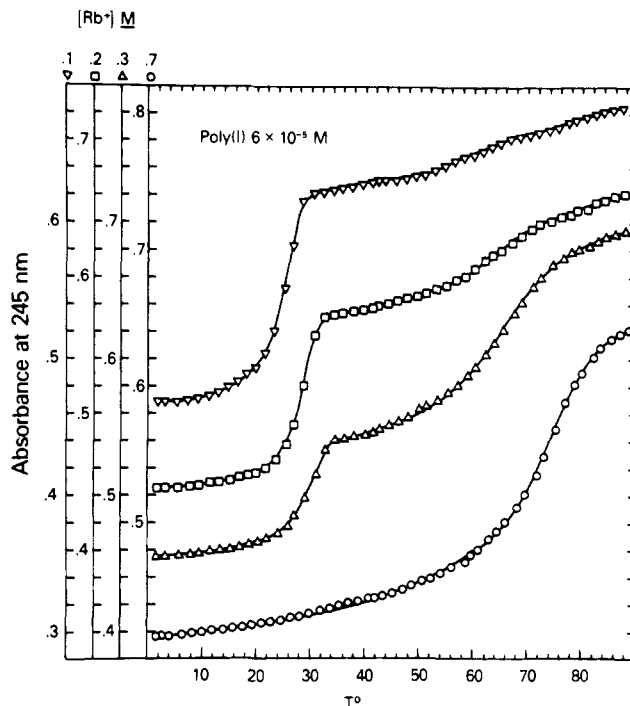


FIGURE 4: Melting curves of 6×10^{-5} M poly(I) in 0.1 (∇), 0.2 (\square), 0.3 (Δ), and 0.7 M (O) RbCl and 0.002 M lithium cacodylate, pH 6.8. The lower transition (visible in 0.1, 0.2, and 0.3 M Rb^+) is that of form I and the higher transition (visible in 0.7, 0.3, and, to some extent, in 0.2 M Rb^+) is that of form II. Before the experiment, the solutions were held at 40 °C for 66 h to remove any preexisting form I, then cooled to 2 °C, and melted. Form I is quickly re-formed at the lower Rb^+ concentrations, and repeated meltings of these solutions follow essentially the same temperature profiles. With more concentrated poly(I) solutions (e.g., 0.01–0.04 M), the polymer can be converted entirely to form II, which then gives a single, sharp transition observable in either IR (cf. Figure 5) or UV (Figure 2, supplementary material).

formed with this cation is unstable except at low temperatures and high poly(I) concentrations.

Interaction of Poly(I) with Na^+ . Complex formation between poly(I) and Na^+ also exhibits marked hypochromicity, but the rate of reaction is slower than that between poly(I) and K^+ . Increasing the concentrations of both reactants also favors helix formation, but higher concentrations are required to achieve the same rate. When $[Na^+] = 0.4$ M, the half-times of complex formation at 20 °C with $[poly(I)] = 10^{-4}$, 10^{-3} , and 10^{-2} M are 115, 45, and 9 h, respectively. Total conversion of poly(I) to the helical state, however, is slow, and with $[Na^+] = 0.4$ M and $[poly(I)] = 10^{-2}$ M, the process remains incomplete even after 6 days. Increasing $[Na^+]$ to 1 M results in a large increase in the rate of reaction: $t_{1/2}$ is less than 2 min with $[poly(I)] = 10^{-4}$ M. At higher concentration of both reactants [1 M Na^+ , 0.2 M poly(I)], the Na^+ -poly(I) complex is insoluble.

Interaction of Poly(I) with Rb^+ . Poly(I) reacts with Rb^+ to produce two complexes, which we designate as forms I and II. These may be distinguished by the two resolved thermal transitions (Figure 4), by infrared spectra in the carbonyl region, and as we see in a later section by CD spectra.

The relative amounts of the two forms produced depends on the reaction conditions, with form I being favored by relatively short reaction periods, low concentrations of reactants, and low temperatures. At 8×10^{-5} M poly(I) and $[Rb^+] \leq 0.2$ M, the rate of formation of the less stable complex (form I) is much more rapid than that of the more stable form II. Thus, at 7.76×10^{-5} M poly(I) and 2.93×10^{-2} M Rb^+ , production of form I was half complete in 0.76 h [0.03 h in

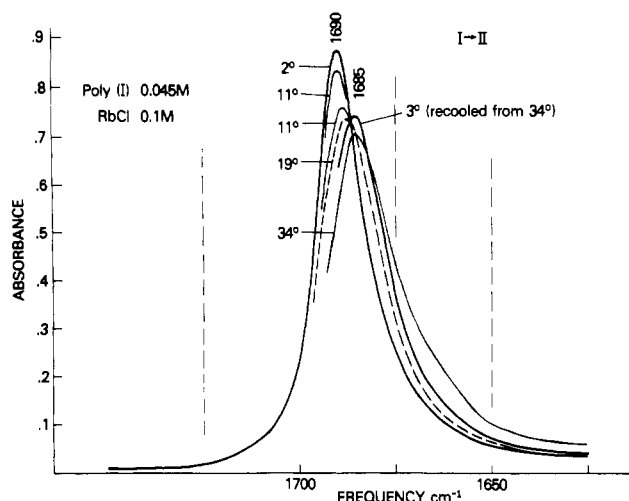


FIGURE 5: Infrared spectra of 0.045 M poly(I) Li^+ salt as a function of temperature in the presence of 0.1 M RbCl. The D_2O solution was heated to 70 °C and cooled quickly to 2 °C, producing form I (1690 cm^{-1}). On gradual warming to 34 °C poly(I) is converted to form II (1685 cm^{-1}). Cooling again to 3 °C increases the intensity of the form II carbonyl band and removes the slight absorbance of the random coil on the low-frequency side of the band, $\nu_{\text{max}} = 1685 \text{ cm}^{-1}$. Time intervals allowed between measurement of the 2 °C spectrum and spectra at high temperature were as follows: 11 °C (higher absorbance), 1 h; 11 °C (lower absorbance), 16.5 h; 19 °C, 19 h; 34 °C, 21 h. When the solution which has been warmed to 34 °C and recooled is heated from 3 °C, there is a single, sharp transition of $T_m = 57 \text{ °C}$, indicating absence of form I and complete conversion to form II. For these conditions, this procedure is the most convenient way to prepare pure form II. The path length of the cell was $2.36 \times 10^{-3} \text{ cm}$. The ordinate was expanded 5.8-fold.

$5.47 \times 10^{-2} \text{ M Rb}^+$; supplementary material (see paragraph at end of paper regarding supplementary material), Figure 1] whereas no form II was observed in 16 h at 23 °C or 3 days at 5 °C.

Form I may be converted completely to the more stable form II. This conversion [at $6 \times 10^{-5} \text{ M poly(I)}$] is favored by increasing the Rb^+ concentration and holding the solution for extended periods (66 h) above the melting range (40 °C) of form I (Figure 4). The fraction of poly(I) in form II also progressively increases with $[\text{Rb}^+]$, and at $[\text{Rb}^+] \geq 0.7 \text{ M}$ conversion to form II is essentially complete (Figure 4). A long reaction time (66 h) is required at 40 °C owing to the slow rate of formation of form II at this temperature and low polymer concentration. The $\text{I} \rightarrow \text{II}$ conversion is greatly accelerated at high $[\text{poly(I)}]$. IR spectroscopy may be used to monitor the process by observation of changes in bands characteristic of form I (1690 cm^{-1}) and form II (1685 cm^{-1}) (Figure 5). By this technique we have determined that at 0.03 M poly(I) and 0.1 M Rb^+ the change from form I to form II is complete in 1 h at 26 °C.

Once it has been produced, form II is stable and shows no reversion to form I. We explored some of the limits of its stability in the following experiments. A 0.025 M solution of poly(I) was made 1 M in Rb^+ and became opalescent when the salt was added. Conversion to form II was achieved by heating at 48 °C for 20 h. A white precipitate appeared during the heating. The polymer was then dialyzed for 2 days at 5 °C against five changes of distilled water (4 L each). During the dialysis, the white precipitate went into solution and was replaced by a thick, translucent gel. After standing 6 days at 5 °C, the gel was diluted to $8 \times 10^{-5} \text{ M poly(I)}$ in 0.01 M Rb^+ and held for a day at 5 °C. During melting the material exhibited a monophasic transition with $T_m = 53 \text{ °C}$ (supplementary material, Figure 2, 0.01 M Rb^+), the value observed

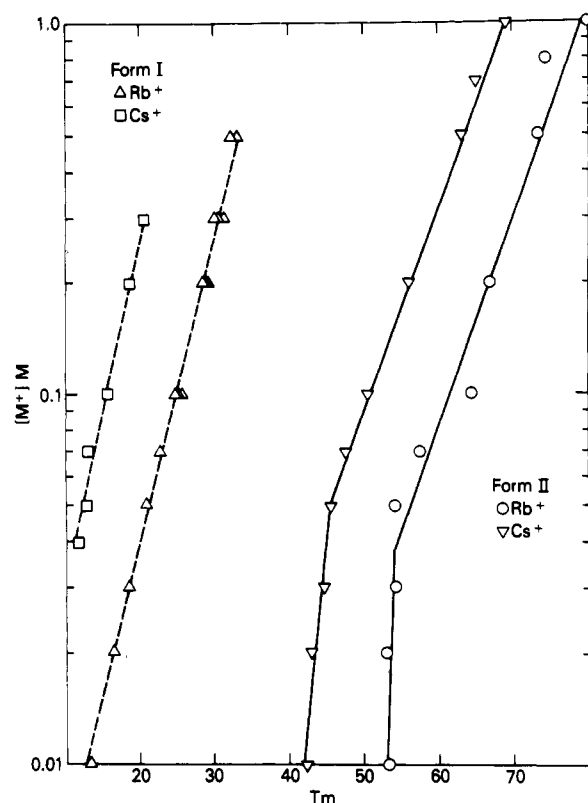


FIGURE 6: Salt dependence of melting of poly(I) complexes with Rb^+ and Cs^+ . The two curves on the left (Δ , \square) show the cation dependence of form I transitions and the two on the right (\circ , ∇) show those of form II transitions. Temperature profiles of the form I structures [left of the diagram (Δ , \square)] are not equilibrium melting curves, though they are reproducible. Temperature profiles of form II [right of the diagram (\circ , ∇)] are considered to be equilibrium melting curves for reasons given in the text. There is some variability of the Rb^+ form II curve because of aggregate formation (cf. Figure 2, supplementary material). This problem did not occur with the Cs^+ -poly(I) solutions. Least-squares lines were fitted by computer to the data. $d T_m / d \log [\text{Rb}^+] = 10.9 \pm 0.7 \text{ °C}$ (form I) and $17.4 \pm 1.5 \text{ °C}$ (form II, $[\text{Rb}^+] > 0.05 \text{ M}$; $2.1 \pm 1.6 \text{ °C}$ for form II at $[\text{Rb}^+] < 0.05 \text{ M}$). $d T_m / d \log [\text{Cs}^+] = 10.5 \pm 0.5 \text{ °C}$ (form I) and $17.8 \pm 0.3 \text{ °C}$ (form II, $[\text{Cs}^+] > 0.05 \text{ M}$; $4.5 \pm 1.0 \text{ °C}$ for form II at $[\text{Cs}^+] < 0.05 \text{ M}$). We note that these values give the statistical standard error and are not intended to imply experimental error limits.

for form II in Figure 6. No form I was present. An aliquot of the dialyzed gel before addition of any salt was analyzed for Rb^+ and found to have approximately 0.7 Rb^+ per polymer residue. The pH of the unbuffered solution was 4. The result indicated roughly one Rb^+ per phosphate, but the analysis was not accurate enough to provide information on the distribution of Rb^+ on the helix or on internal site occupancy. In a separate experiment, the gel obtained by dialysis against distilled water was dialyzed against three changes (4 L each) of 0.05 M Li^+ . Within 0.5 h of commencing dialysis, the gel disappeared and the solution flowed freely within the dialysis bag. The solution was then diluted ($8 \times 10^{-5} \text{ M polymer}$) in 0.1 M Li^+ . A melting curve showed the polymer was entirely in random coil form. Li^+ can therefore displace Rb^+ from a preformed helical Rb^+ -poly(I) complex but, as noted above, cannot itself support helix formation under these conditions. Other melting experiments in the presence of both Li^+ and excess Rb^+ show that form II persists at ambient temperature and is not displaced by Li^+ . Finally, another aliquot of the Rb^+ -poly(I) gel obtained by dialysis against distilled water was diluted with 0.1 M Li^+ and melted immediately. The melting curve indicated a mixture of form I and form II. The Li^+ - Rb^+ displacement reaction evidently requires an appreciable time to

Table I: Rb⁺ and Cs⁺ Titrations of Poly(I)

complex	conditions	[cation] at half-titration
Rb ⁺ -poly(I)	10.0 °C	2.40×10^{-2} M
Rb ⁺ -poly(I)	15.0 °C	3.63×10^{-2}
Rb ⁺ -poly(I)	15.0 °C	3.63×10^{-2}
		(at equilibrium)
Rb ⁺ -poly(I)	20.0 °C	6.92×10^{-2}
Rb ⁺ -poly(I)	25.0 °C	0.186
Rb ⁺ -poly(I)	10.0 °C; 0.1 M Li ⁺	5.89×10^{-2}
Rb ⁺ -poly(I)	15.0 °C; 0.1 M Li ⁺	8.32×10^{-2}
Rb ⁺ -poly(I)	20.0 °C; 0.1 M Li ⁺	0.140
Rb ⁺ -poly(I)	25.0 °C; 0.1 M Li ⁺	0.209
Cs ⁺ -poly(I)	10.0 °C	7.33×10^{-2}
Cs ⁺ -poly(I)	15.0 °C	0.146
Cs ⁺ -poly(I)	20.0 °C	0.359
Cs ⁺ -poly(I)	10.0 °C; 0.1 M Li ⁺	7.94×10^{-2}
Cs ⁺ -poly(I)	15.0 °C; 0.1 M Li ⁺	0.166

go to completion. The generation of form I in this experiment may result from reduction of Rb⁺ site occupancy in some regions of the helix (see Discussion).

We should note, in connection with the thermal transitions, that when the temperature is held constant at intermediate points during the melting of form(II) of the Rb⁺ or Cs⁺ complexes, the absorbance remains constant (as does the infrared spectrum) until heating is resumed. Upon recooling the solution, we do not observe the same absorbances as during the heating cycle because of the slow kinetics of generating form II. Once the stable helices are regenerated, however, the same absorbance is observed at each temperature on the second heating cycle as on the first. We assume that these temperature profiles represent equilibrium melting curves, though we cannot demonstrate it directly by approaching each temperature from both directions. In contrast, when solutions of form I (Rb⁺ or Cs⁺) are held at intermediate temperature on their "melting curves", the spectrum continues to change as the I → II conversion takes place. These temperatures profiles obviously do not represent equilibrium melting curves. The salt-dependence curves are shown in Figure 6. While infrared and ultraviolet data suggest that form I is a favorable intermediate for the generation of form II, it is possible to show experimentally that it is not an obligatory intermediate. The temperature of an 8×10^{-5} M poly(I) solution (1 M Rb⁺) was raised sufficiently to dissociate any Rb⁺-poly(I) complex in either form. The temperature was lowered to 47 °C to be above T_m of form I but below T_m of form II and held for 18 h. A subsequent melting profile (beginning at 47 °C) showed that 28% of the poly(I) had been converted to form II under conditions in which form I does not exist.

Rb⁺ Titration Curves. Since there is a 32% reduction in molar extinction of poly(I) on formation of the metal ion complex, UV spectroscopy is well suited to determining the concentrations of helical and single-stranded poly(I). Although it is not possible to measure by UV spectroscopy the concentration of bound or free cation and hence the equilibrium constant, we have been able to determine the extent of helix formation as a function of concentration of Rb⁺. Titrations of poly(I) (8×10^{-5} M) with Rb⁺ were performed in the absence (Figure 7) and presence of 0.1 M Li⁺ (Table I). The concentration of Rb⁺ at half-titration of poly(I) increases with increasing temperature over the range 10–25 °C (Figure 7; Table I) in the presence or absence of Li⁺. Since both form I and form II are produced during the addition of Rb⁺ (see above), the titrated poly(I) consists of a mixture of the two, whose relative proportion varies with [Rb⁺]. At [Rb⁺] ≤ 0.2 M, form I is predominant. With [Rb⁺] > 0.2 M, an increasing

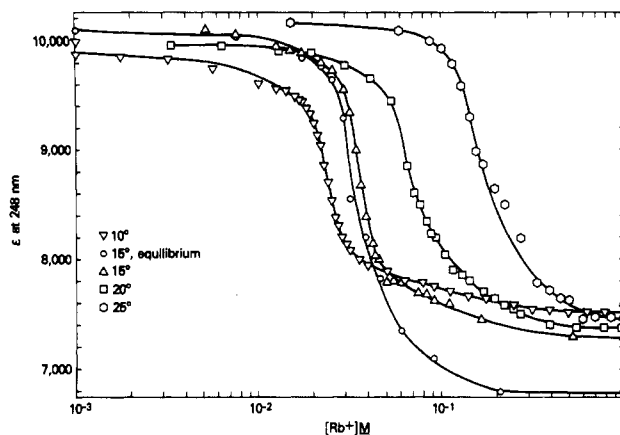


FIGURE 7: Spectrophotometric titration of poly(I) with Rb⁺. Spectral measurements were made 10 min after each addition of Rb⁺ for four of the curves (▽, △, □, and ○). For one case (○), measurements were made 24 h after preparation of separate solutions for each point. Conditions: 8×10^{-5} M poly(I); 0.002 M lithium cacodylate, pH 6.8; 1 cm path length; 10.0 (▽), 15.0 (○, △), 20.0 (□), and 25.0 °C (○).

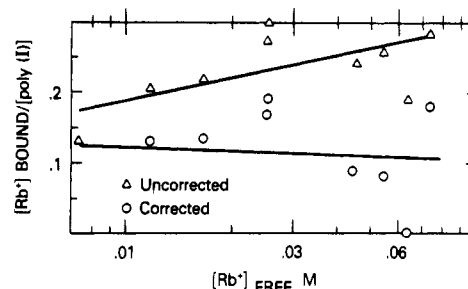


FIGURE 8: Binding curves for the interaction of poly(I) with Rb⁺. The value of $[Rb^+]_{\text{bound}}/[poly(I)]$ does not vary appreciably with $[Rb^+]_{\text{free}}$ for the corrected curve, allowing a binding ratio of approximately eight inosine residues for each Rb⁺ to be calculated. The corrected curve is derived from the corresponding uncorrected curve as described in the text. Conditions: 0.025 M poly(I); 0.1 M LiCl; 24 h at 5 ± 1 °C.

fraction of form II results, and at 1.0 M Rb⁺, nearly all of the complex produced is form II (Figure 4).

Owing to the appreciable time (5 h) required for completion of reaction, all but one of the curves in Figure 7 are nonequilibrium titration curves. Since measurements were made approximately 10 min after each addition of Rb⁺, spectral changes were incomplete. We find, however, that when an equilibrium titration curve is measured (24 h for each point, Figure 7), the [Rb⁺] at half-titration is identical with that observed with rapid titration but the total absorbance change is somewhat less (92% rather than 100% helix formation). The more rapid method of titration with Rb⁺ using a single sample of poly(I) is thus justified for most purposes. As we have seen above, however, the same conclusion does not apply to K⁺.

Ratio of Rb⁺ Ions Bound per Inosine Residue. A knowledge of the number of Rb⁺ ions bound for each inosine residue is important in establishing the structure of the helical complex. Since the spectroscopic methods described above measure helix formation rather than metal binding, we have sought direct measurements of binding by equilibrium dialysis.

Despite the theoretical advantage of equilibrium dialysis, the accuracy of the method is to some extent limited by the difficulty of determining the concentration of Rb⁺ in small volumes of dilute solutions. After a valinomycin K⁺-ion electrode (also sensitive to Rb⁺) proved to have inadequate precision, we took advantage of the 27.85% natural abundance of ⁸⁷Rb, a β emitter of energy close to that of ¹⁴C, to measure

Table II: Circular Dichroism Data

substance	λ_{\max} (nm)	$\epsilon_L - \epsilon_R$	λ_{\min} (nm)	$\epsilon_L - \epsilon_R$
Na ⁺ -poly(I) ^a	234	-0.32	208	-5.7
	252	2.01	274	-1.28
Na ⁺ -poly(I) ^b	251	4.72	208	-18.1
			276	-5.92
K ⁺ -poly(I) ^c	252	4.35	198	-25.2
			275	-5.82
NH ₄ ⁺ -poly(I) ^d	253	2.06	205	-9.6
	284	2.01	269	0.55
Rb ⁺ -poly(I)	220	2.82	208	-3.1
(form I) ^e	246	5.75	231	1.08
			265	-2.65
Rb ⁺ -poly(I)	216	1.28	204	-7.3
(form II) ^f	250	1.54	230	0.48
	278	2.16	265	0.74
Cs ⁺ -poly(I)	221	1.47	205	-7.4
(form I) ^g	245	5.62	231	-0.07
			267	-3.52
Cs ⁺ -poly(I)	250	1.52		
(form II) ^h	278 (sh)	0.52		

^a Conditions: 1×10^{-4} M poly(I), 0.001 M lithium cacodylate, pH 6.8, 1.0 M Na⁺, 17.4 °C, and path length 1 cm. The spectrum was measured 500 h after components were mixed. ^b Conditions: 1×10^{-2} M poly(I), 0.01 M lithium cacodylate, pH 6.8, 0.4 M Na⁺, 25 °C, and path length 1.00×10^{-2} cm. The spectrum was measured 6 days after sample preparation (Howard & Miles, 1982). ^c Conditions: 1×10^{-2} M poly(I), 0.025 M lithium cacodylate, pH 6.8, 0.1 M K⁺, 25 °C, and path length 1.00×10^{-2} cm. The spectrum was measured 21 days after sample preparation. ^d Conditions: 1×10^{-4} M poly(I), 0.002 M lithium cacodylate, pH 6.8, 1.0 M NH₄⁺, 25 °C, and path length 1 cm. The spectrum was measured after the sample was frozen in liquid N₂ and allowed to stand 2 months at room temperature (Howard & Miles, 1982). ^e Conditions: 8×10^{-5} M poly(I); 0.002 M lithium cacodylate, pH 6.8, 0.2 M Rb⁺, 10 °C, and path length 1 cm. The spectrum was measured after the sample had been kept 3.5 days at 5 °C. ^f Conditions: 8×10^{-5} M poly(I); 0.002 M lithium cacodylate, pH 6.8, 1.0 M Rb⁺, 17.5 °C, and path length 1 cm. The sample was annealed at 47 °C for 2 days prior to spectral measurements. ^g Conditions: 6.4×10^{-5} M poly(I), 0.002 M lithium cacodylate, pH 6.8, 0.1 M Cs⁺, 2 °C, and path length 1 cm. The spectrum was measured 10 min after sample preparation. ^h Conditions: 8×10^{-5} M poly(I), 0.002 M lithium cacodylate, pH 6.8, 1.0 M Cs⁺, 17.3 °C, and path length 1 cm. The sample was annealed at 40 °C for 4 days before spectral measurements.

[Rb⁺] with a liquid scintillation counter. Unfortunately, the method suffers from lack of sensitivity, owing to low specific activity [half-life of ⁸⁷Rb 5×10^{10} years (Kallman, 1961)], and measurement of [Rb⁺]_{free} at values less than 0.007 M was not practical. Complete binding isotherms could not be determined because [Rb⁺]_{free} was too low to be measured at values corresponding to partial saturation of poly(I). The curve in Figure 8 represents only that portion of the binding isotherm at which all binding sites are saturated. [Rb⁺]_{bound} was measured experimentally by counting an aliquot of poly(I) containing bound Rb⁺. The bound Rb⁺ includes two categories: Rb⁺ bound at size-specific sites within the helix cavity and that bound nonspecifically, as a counterion to phosphate charges. The positive slope of the uncorrected binding curve (Figure 8) results from nonspecific binding of Rb⁺ to phosphate. Li⁺ (0.1 M) was added as the phosphate counterion to reduce the fraction of Rb⁺ nonspecifically bound. An empirical correction was employed with separate experiments with poly(A) as a control in which size-specific binding of alkali metal ions does not occur to a significant extent. The extent of binding is independent of [Rb⁺] for these concentrations, as would be expected for the saturation region of a binding isotherm. The value of [Rb⁺]_{bound}/[poly(I)], moreover, is approximately 0.125 (lower curve) or about eight inosine

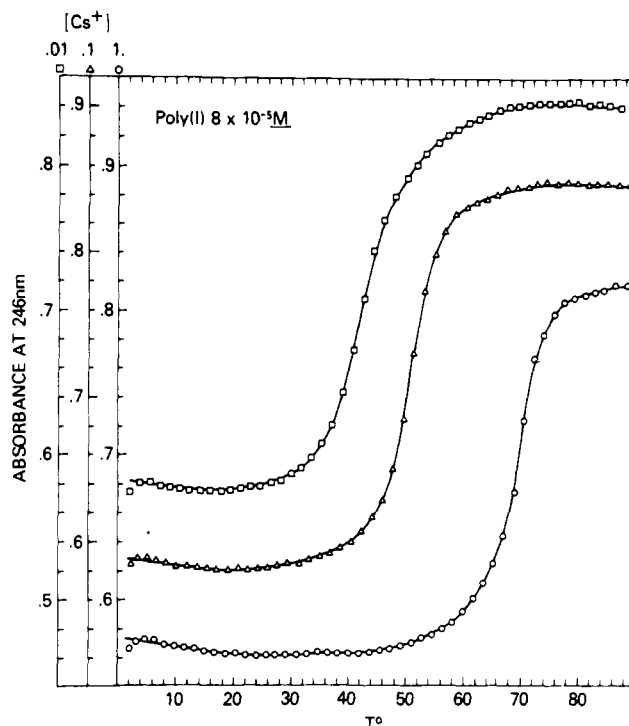


FIGURE 9: UV melting curves of form II of the Cs⁺-poly(I) complex, obtained as with Rb⁺ from a more concentrated solution of the polymer. Poly(I) (0.01 M) in 1.0 M CsCl was held at 40 °C for 45.5 h, converting all of the polymer to form II. Aliquots were diluted for UV observation to 8×10^{-5} M poly(I) in 0.01 (□), 0.1 (Δ), and 1.0 M (○) Cs⁺ and 0.002 M lithium cacodylate, pH 6.8, for measurement of melting profiles. Transition breadths were 12.6, 10.9, and 7.1 °C in 0.01, 0.1, and 1.0 M Cs⁺, respectively. No transition attributable to form I was observed throughout the range $0.01 \leq [\text{Cs}^+] \leq 1.0$ M.

residues per Rb⁺. Because of uncertainties noted above, the determination is not highly accurate. There are, nevertheless, more than four inosine residues and possibly as many as 12 per Rb⁺ specifically bound. The most probable value, eight, would correspond to one Rb⁺ bound at every other potential site in the axial channel, with a spacing ~ 6.8 Å. We have noted above that the Rb⁺-poly(I) helix under conditions of these experiments consists of a mixture of forms I and II. The accuracy of the measurements is not adequate to resolve the question of a possible difference in site occupancy of these forms, provided the difference is not large.

Interaction of Cs⁺ with Poly(I). Cs⁺, like Rb⁺, reacts with poly(I) to produce a metastable and stable helical complex, which we also designate as forms I and II, respectively. At low concentration of poly(I) (8×10^{-5} M), both forms may be detected in thermal profiles (supplementary material, Figure 3), the fraction of form II increasing with increasing [Cs⁺]. The I \rightarrow II conversion at a given cation concentration is more extensive with Cs⁺ than with Rb⁺. The two forms may also be distinguished by differences in their IR and CD spectra (Miles & Frazier, 1978; Table II), as in the case of the two forms of the Rb⁺-poly(I) complex.

The relative amounts of the two forms depends on the experimental conditions. Raising the concentration of both reactants [0.01 M poly(I), 1 M Cs⁺] and holding the temperature (40 °C) above the thermal transition of form I for long periods (46 h) results in complete conversion to the stable form, II. Once the complex is formed, the concentration of both complex (form II) and Cs⁺ may be greatly reduced [8×10^{-5} M poly(I), 0.01 M Cs⁺] without reformation of any form I (Figure 9). The Cs⁺-poly(I) complex is partially

insoluble at the initial high concentration of reactants, but unlike the Rb^+ -poly(I) complex, it dissolves completely on dilution to UV concentrations.

The decidedly higher stability of form II [the Cs^+ -poly(I) complex] compared to that of form I is shown in Figure 6. Cs^+ -poly(I) (form II) is less stable than Rb^+ -poly(I) (form II) (Figure 6), but the dependence of T_m upon $[\text{M}^+]$ is about the same for each complex.

We have measured the dependence of helix formation by poly(I) (8×10^{-5} M) on $[\text{Cs}^+]$ by the rapid method of spectrophotometric titration described above. Titrations were carried out in the presence (Table I) and absence (supplementary material, Figure 4) of 0.1 M Li^+ . As in the case of Rb^+ titrations, the concentration of Cs^+ required for half-titration increases with increasing temperature between 10 and 20 °C, in the presence and absence of Li^+ (Table I). A mixture of forms I and II is produced (supplementary material, Figure 3) during the titration, as was observed in the titration of poly(I) with Rb^+ . Rb^+ and Cs^+ titration curves are similar, but a higher concentration (3–5-fold) of Cs^+ is required for transforming half of poly(I) to the helical state.

Circular Dichroism. (1) CD Spectra of Na^+ -Poly(I) and K^+ -Poly(I) Complexes. Circular dichroism spectra of poly(I) have been reported by a number of workers (Brahms & Sadron, 1966; Formoso & Tinoco, 1971; Thiele & Guschlbauer, 1973; Cech & Tinoco, 1976), and while they are in qualitative agreement, they frequently show quantitative differences, especially in magnitudes of bands. All previously published spectra have had a negative first extremum. CD spectra of poly(I) have usually been measured at what have been termed low-salt conditions (0.1 M NaCl) where the polymer is reportedly single stranded and at high salt (1 M NaCl) where poly(I) exists in an ordered, helical form. Previously reported spectra generally have not been measured under precisely comparable conditions, making comparisons between them difficult. As we have seen above, the fraction of poly(I) in the helical state strongly depends upon a number of experimental variables. Since CD spectra are quite sensitive to the degree of helicity of the polymer, it is not surprising that discrepancies have occurred.

The most common high-salt condition used for observing the CD spectrum of multistranded poly(I) may not be a suitable condition for observations. In 1 M NaCl poly(I), even at low concentration, not only undergoes complex formation but also slowly aggregates, and the solution becomes increasingly opalescent with time.

Measurements at somewhat lower NaCl concentration show no opalescence and presumably more reliably reflect solution properties of the helix. The CD spectrum of 10^{-2} M poly(I) in 0.4 M NaCl (Table II; Howard & Miles, 1982) was recorded at 20 °C after changes monitored by UV spectroscopy had ceased (6 days; $\epsilon_{247} = 7460$). The CD spectrum is similar in shape and location of bands to published spectra (Brahms & Sadron, 1966; Formoso & Tinoco, 1971; Thiele & Guschlbauer, 1973; Cech & Tinoco, 1976), but the intensity of the negative band at 276 nm ($\epsilon_L - \epsilon_R = -5.80$) is somewhat greater. Brahms & Sadron (1966) measured values of -2.5 and 4.1 for extrema at 275 and 250 nm, respectively [$\sim 10^{-3}$ M poly(I), 0.5 M NaCl, 0 °C, time not specified], while Thiele & Guschlbauer (1973) reported -4 and 4 for the same wavelengths [8.8×10^{-5} M poly(I), 0.3 M NaCl, 4 days at 4 °C].

A change from Na^+ to K^+ results in little change in the CD spectrum [Figure 5 of Howard & Miles (1982); Table II]. Extrema observed for 0.01 M poly(I) in 0.1 M K^+ (18 days,

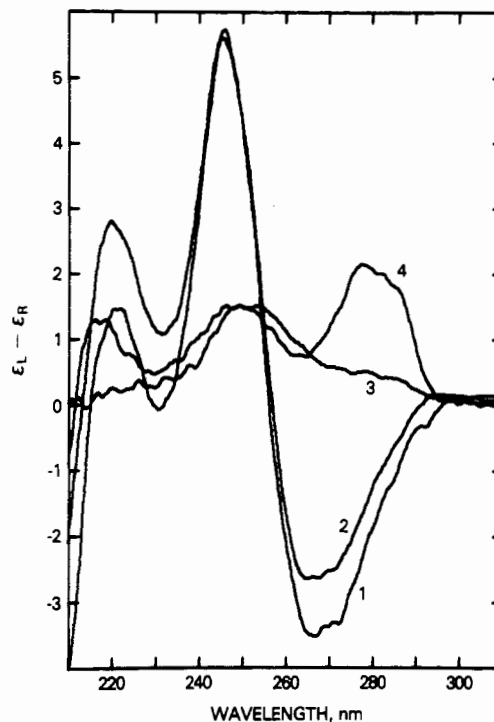


FIGURE 10: CD spectra of 6.4×10^{-5} M poly(I) (form I) in 0.1 M Cs^+ (curve 1), 8×10^{-5} M poly(I) (form I) in 0.2 M Rb^+ (curve 2), 8×10^{-5} M poly(I) (form II) in 1.0 M Cs^+ (curve 3), and 8×10^{-5} M poly(I) (form II) in 1.0 M Rb^+ (curve 4). Curve 1 was measured at 2 °C, 10 min after sample preparation. Curve 2 was measured at 10 °C after the sample had been kept at 5 °C for 4 days. Curve 3 was measured at 17.3 °C after the polymer had been annealed at 40 °C for 4 days to convert it entirely to form II. Curve 4 was measured at 17.5 °C after the polymer had been annealed at 47 °C for two days to convert it entirely to form II. All solutions contained 0.002 M lithium cacodylate, pH 6.8. The path length of the cell was 1 cm. Each curve is a computer average of three spectra and was drawn by a Cal-Comp plotter.

complex formation complete) at 276 nm ($\epsilon_L - \epsilon_R = -5.82$) and 252 nm ($\epsilon_L - \epsilon_R = 4.35$) are the same as values observed at 0.4 M Na^+ .

(2) CD Spectra of Rb^+ -Poly(I) and Cs^+ -Poly(I) Complexes. A striking change in the CD spectrum of ordered poly(I) occurs when the cation is changed from Na^+ or K^+ to NH_4^+ , Rb^+ , or Cs^+ . This marked dependence of the CD spectrum on the nature of the cation occupying the central cavity of the multistranded helix is reported below for Rb^+ and Cs^+ .

The spectrum of the less stable (form I, see above) of the two forms of the Rb^+ -poly(I) or Cs^+ -poly(I) complex (Figure 10; Table II) is similar to the spectra of the poly(I) complexes with Na^+ and K^+ , but with significant changes in wavelength. The negative first band of the Rb^+ -poly(I) complex has shifted to lower wavelength (265 nm, $\epsilon_L - \epsilon_R = -2.65$) by 10 nm and the positive band (246 nm, $\epsilon_L - \epsilon_R = 5.75$) by 6 nm. A resolved band at 220 nm ($\epsilon_L - \epsilon_R = 2.82$), which occurs only as a shoulder in the spectrum of poly(I) with Na^+ or K^+ , has also shifted about 10 nm to lower wavelength, increasing in intensity and becoming positive.

The more stable form II of the Rb^+ -poly(I) complex has a CD spectrum totally different from that of form I or of the Na^+ and K^+ complexes (Figure 10; Table II). There is now a positive first extremum at 278 nm ($\epsilon_L - \epsilon_R = 2.16$), together with second and third bands which are also positive. The first band has undergone a reversal in sign and a shift (from the form I spectrum) to higher wavelength by 13 nm. The second and third bands show only small shifts in maxima but have

been reduced in intensity.

The Cs⁺-poly(I) helix (form II) also has a CD spectrum differing markedly from those observed in the presence of Na⁺ and K⁺ (Figure 10; Table II). The first negative band at 275 nm has reversed in sign, though the new band is so weakly positive ($\epsilon_L - \epsilon_R = 0.6$) that it is incompletely resolved from the maximum at 249 nm ($\epsilon_L - \epsilon_R = 1.52$).

We have recently described (Howard & Miles, 1982) a stereospecific complex between poly(I) and NH₄⁺ ion which undergoes a similar reversal in sign of the first CD extremum at 285 nm on formation of the four-stranded complex.

The apparently conservative first pair of CD bands of the Na⁺ and K⁺ (Table II) as well as of the Rb⁺ and Cs⁺ form I complexes (Figure 10, curves 1 and 2) may suggest an origin in exciton splitting, but a simpler interpretation appears more probable. Two $\pi-\pi^*$ transitions have been assigned in this region in several reports: 246 and 266 nm (Cech & Tinoco, 1976); 250 and 276 nm (Sprecher & Johnson, 1977), 249 and 260–270 nm (Clark & Tinoco, 1965), 242 and 263 nm (Voelter et al., 1968). The first and second bands of the Rb⁺ and Cs⁺ form I complexes (246 and 265 nm, Figure 10) and of the Na⁺ and K⁺ complexes (252 and 275 nm, Table II; Howard & Miles, 1982) are assigned to these two $\pi-\pi^*$ transitions. These assignments are confirmed by the spectrum of the Rb⁺ (form II) complex (Figure 10), in which both long-wavelength bands are positive, ruling out exciton splitting of a single transition as the origin of both. The CD spectrum of Cs⁺-poly(I) (form II) is similar to that of the Rb⁺ complex (form II) (Figure 10) except that the longest wavelength band at ~ 273 nm is present only as an unresolved shoulder having only one-quarter the intensity of the Rb⁺ complex, and the positive band of the later at 216 nm is not detectable.

In concluding this section, we should point out that the specific alkali metal ion effects previously observed by infrared spectroscopy at high poly(I) concentration (e.g., 0.04 M) are also observed by UV and CD in this report at much lower polymer concentration ($\sim 10^{-4}$ M). The poly(I) concentration has no discernible effect on either ion specificity or helix stability, in contrast to the marked effect of concentration on rate of complex formation.

Discussion

In previous reports we have proposed a model for ordered poly(I) in which alkali metal or NH₄⁺ ions form specific complexes with carbonyl oxygens in the axial channel of the helix (Miles & Frazier, 1978; Howard & Miles, 1982). The reasons leading to formulation of the model may be summarized as follows: (a) A striking and unique selectivity of the poly(I) helix for the different alkali metal ions is observed. (b) The observed stability of the poly(I) helix cannot be accounted for on the basis of the accepted four-stranded structure, with only one hydrogen bond between bases and with a large axial hole. (c) Ammonium ion forms with poly(I) a specific complex which must satisfy additional constraints of NH₄ symmetry, geometry, and hydrogen bonding. These points are developed in the following paragraphs, and consistency of the model with the data is examined. Several predictions of the model are discussed.

Li⁺ does not support formation of a poly(I) helix in solution, but Na⁺ forms a complex of moderate stability ($T_m = 29^\circ\text{C}$ in 0.1 M Na⁺). A dramatic increase in T_m occurs on going to the next alkali metal ion, K⁺ ($T_m = 56^\circ\text{C}$ in 0.1 M K⁺). Rb⁺ and Cs⁺ form complexes with poly(I) similar in stability to that of K⁺. These specific effects are the more striking when compared with the interaction of alkali metal ions with other polynucleotides. Thus, double helical polynucleotides exhibit

a T_m difference of only 1 or 2 $^\circ\text{C}$ between adjacent alkali metal ions and of 5 $^\circ\text{C}$ or less between Li⁺ and Cs⁺ (Gruenwedel et al., 1971; Krakauer & Sturtevant, 1968; Schildkraut & Lifson, 1965). These very large differences from other polynucleotides indicate the specificity observed with poly(I) is not due to variation in electrostatic screening at the phosphates. The only apparent precedent for such selectivity is that of such complexes as the crown ethers, antibiotic ionophores, and certain peptides [for a review of alkali metal coordination, see, for example, Truter (1973)]. The specificity of these latter complexes depends upon a grouping of electronegative atoms (usually oxygen) which forms a cavity that precisely fits the ion for which the complex is selective. Any departure from the preferred M–O ligand distance results in reduced selectivity, in a less stable complex, or in exclusion of the ion. The problem in the poly(I) case then becomes one of identifying groupings of oxygen atoms in the four-stranded helix, providing geometries, coordination numbers, and ligand distances consistent with the observed pattern of alkali metal ion specificity. We find such groupings in the carbonyl oxygens lining the axial cavity of the helix (Figure 11; for consideration of possible alternative sites, see below). In the proposed model for alkali metal ion complexes of poly(I), the metal occupies one of two distinct binding sites on the helix axis, depending on ion size. Na⁺ has the proper M–O ligand distance, ~ 2.3 Å, to fit in the center of the planar array of inosine residues and interact with the four carbonyl oxygens. Li⁺, with a ligand distance of 2.0–2.2 Å, is evidently too small to fit effectively in this space and hence cannot support complex formation. K⁺ and Rb⁺ are too large for this site. A second site, on the helix axis midway between the planar arrays of inosine, is 2.8 Å from each of eight carbonyl oxygens. This distance is appropriate for K⁺ and Rb⁺ with M–O distances of 2.7–3.1 Å, respectively, and the coordination number of eight is favorable for these ions.

The usual range of Cs–O distances is 3.0–3.6 Å, though a few anomalously short distances have been reported [crystallographic references for M–O distances are cited in Miles & Frazier (1978)]. The most common Cs–O distances are too large for the proposed binding site, though the lower end of the range is only slightly so. It is relevant that the dianion of 5'-GMP forms ordered structures with Na⁺, K⁺, and Rb⁺ but fails to do so with Cs⁺ or Li⁺ (Pinnavaia et al., 1978). We suggest that the stable Cs⁺-poly(I) helix (form II) may be slightly expanded in the axial direction to accommodate the large Cs⁺ ion while maintaining a close similarity to the other four-stranded structures. Bouhoutsos-Brown et al. (1982) have proposed that the Na₂-5'-GMP ordered structure in neutral solution is stabilized by 2'-OH hydrogen bonds on the periphery of the complex. It is possible that the slight increase in planar separation necessary to accommodate the Cs⁺ ion would prevent formation of these H bonds and so prevent complex formation. In the helix formed by a polymer rather than a monomer, such hydrogen bonds would not be necessary for stability of the ordered structure. Though there is no direct evidence on this hypothesis, it is consistent with the contrast in structure-directing ability of Cs⁺ in the two cases.

While the four-stranded structure for the poly(I) helix (Arnott et al., 1974; Zimmerman et al., 1975) is stereochemically satisfactory, it does not account for the energetics of helix formation. There is only a single hydrogen bond between bases, Figure 11, an arrangement not known in other instances to support helix formation. The axial hole should further add to instability. The model we have proposed, however, can explain the apparently anomalous stability by providing en-

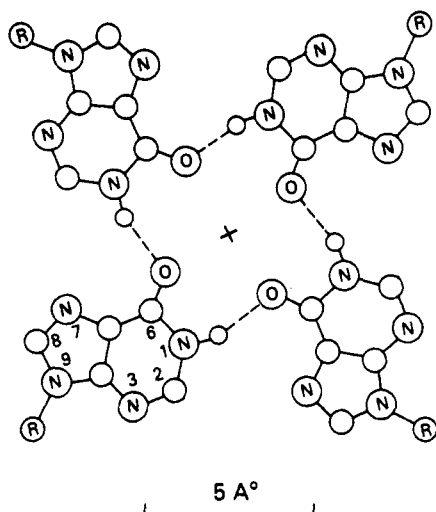


FIGURE 11: Hydrogen bonding in poly(I) helix. There is only a single hydrogen bond between adjacent bases.

energetically favorable metal-oxygen interactions and by filling the hole. The cations can contribute as well to electrostatic stabilization of the helix by providing attractive interactions between the axial core and the ribose-phosphate chains and by partial screening of repulsions among these chains. Failure of the small Li^+ ion to support helix formation is consistent with these ideas.

Strong support for the foregoing model has been provided by a recent study of the interaction of NH_4^+ with poly(I) (Howard & Miles, 1982). NH_4^+ was shown to support helix formation, and equilibrium dialysis demonstrated specific binding to poly(I). In this case, the problem is more complex than with the spherically symmetrical metal ions. We show in that report that the same model is mutually consistent with the tetrahedral symmetry of NH_4^+ , the symmetry of the helix, the formation of four $\text{NH}\cdots\text{O}$ hydrogen bonds of acceptable length and bond angle, and the observed stability of the helix. The larger number and different nature of the constraints in the NH_4^+ case make it more difficult to locate specific binding sites and greatly increase the probability that a model which accommodates both these constraints and those of the metal ions is correct. The evidence clearly demonstrates specific binding of and control of structure by the ions indicated above.

If it is assumed that the accepted three-dimensional structure of the poly(I) helix (Arnott et al., 1974; Zimmerman et al., 1975) is at least approximately correct, our binding model shows that internal sites having the correct dimensions and geometry to account for observed cation specificity exist in the axial cavity of the four-stranded helix. Extensive studies of alkali metal ion complexes of small molecules [see, for example, reviews by Lehn (1973), Truter (1973), Simon et al. (1973), and Izatt et al. (1973)] have shown that when a cavity exists (or can be induced through conformational changes) having electronegative atoms of appropriate geometry, the cavity will always be filled by alkali metal ions for which the preferred ligand distances are present in the complex. We therefore conclude on these grounds as well as on the specific evidence cited above that the axial cavity of the poly(I) helix must contain appropriate cations as proposed. The question remains: are there also alternative binding sites which are as important or more important for structure formation? We maintain that there are not for the following reasons.

All other sites would be on the outside of the helix. While a few electronegative atoms might be arranged to provide

favorable ligand distances to a metal, there would be no possibility of a cavity surrounding the ion. The affinity of such a site would therefore be much less (see reviews cited above), and its significance for poly(I) structure formation slight. The absence of marked specific ion effects in other polynucleotide structures implies that our negative view of alternative binding sites is correct.

One of the factors considered in evaluating any model is whether it leads to testable predictions. The present model has led to several. The structures proposed for the ordered form of neutral poly(X) (Roy et al., 1979) and the interaction product of poly(U) and poly(X) (Roy & Miles, 1982) result from predictions of the poly(I) model. In both cases a previously unsuspected specificity of alkali metal ion interaction was sought and confirmed by experiment, leading to new structures for the complexes. In the poly(X) case, this result led to a solution of a long-standing problem: how a stable helix could be formed by base pairing between xanthine residues having the same negative charge. By placing specifically bound cations in the center of a four-stranded helix, similar to that of poly(I), the model provides attractive electrostatic interactions as well as partial screening of electrostatic repulsion of the negatively charged bases. The minimum combining ratio of poly(X) and poly(U) was known to be 1:1. The observed alkali metal ion specificity strongly suggests that the correct ratio is the 2:2 multiple of the minimum value and that the structure is again four stranded with axial binding of the metal ions. A further prediction is that the four-stranded, extensively hydrogen bonded poly(G) helix (Zimmerman et al., 1975) binds metal ions in the central cavity, but here confirmation has not yet been obtained. The very high stability ($T_m > 100^\circ\text{C}$) prevents use of thermal transitions to detect specific ion effects as with poly(I). A published experiment with poly(G), however, may be reinterpreted in a way consistent with the model. Poly(G) was converted to the $^+\text{N}(\text{Et})_4$ salt with the objective of lowering the T_m below 100°C by use of a large cation known to be inefficient in screening interstrand phosphate repulsion (Howard et al., 1977). Poly(G) was indeed caused to melt completely below 100°C , in part presumably for the reason suggested. It now seems likely, however, that replacement of internally bound metal ions also made a significant contribution to the observed destabilization. Further support for specific ion complexing of poly(G) is provided by the helical specific alkali metal ion complexes of the dianion of 5'-GMP (Pinnavaia et al., 1978; Bouhoutsos-Brown et al., 1982). In these cases, even extensive hydrogen bonding of the G tetramers is insufficient to cause GMP helix formation without specific ion complexing.

The model predicts an upper limit at saturation for selectively bound metals ions ($\text{I}/\text{M}^+ = 4$), but without auxiliary hypotheses cannot predict the site occupancy in any particular case. In the present report, we have determined the stoichiometry of interaction with Rb^+ , chosen because of its rapid reaction and relatively sensitive analytical method. Equilibrium dialysis shows a selective binding at saturation of approximately one Rb^+ per eight inosine residues, a result fully consistent with the model. If one assumes that electrostatic repulsion would prevent occupation of adjacent sites, the prediction would be $\text{I}/\text{Rb}^+ = 8$, as observed. The result is, in any event, generally confirmatory of the model.

There is no reason to believe that a stable helix is associated with a unique site occupancy. Presumably there is a continuum of values, depending on experimental conditions, with the stability decreasing as the site occupancy decreases from an optimum value, which may be 6.8 \AA , or some larger spacing.

K⁺ dilution experiments using a high poly(I) concentration and a constant concentration of Li⁺ counterion, follow a pattern that supports this view (Miles & Frazier, 1978). Specific NH₄⁺ binding has been demonstrated for the lower part of the binding curve and similarly indicates a variation in site occupancy with [NH₄⁺]. In the case of Rb⁺ and Cs⁺, however, there may be a point of metal ion occupancy of the axial channel below which the structure of the helix changes (see below).

One of the more puzzling findings of the study is the existence of two discrete forms of the Rb⁺ and Cs⁺ complexes of helical poly(I) over a wide range of polymer concentrations. To account for these multiple forms, we suggest the following hypothesis, which is consistent with the mode of formation and higher stability of form II, as well as with the relative rates of formation of I and II. The observation that formation of the stable (form II) complexes is favored by high Rb⁺ or Cs⁺ concentration suggests that they may differ from form I in having a higher site occupancy by metal ions. The greater stability could result from a larger number of favorable metal-carbonyl interactions and more effective screening of chain-chain electrostatic repulsion, as well as more complete filling of the destabilizing axial cavity. The kinetic differences in formation of I and II also appear to be consistent with this hypothesis. The very rapid reaction of antibiotic ionophores with alkali metal ions is attributed to a flexibility of the carrier molecules sufficient to allow stepwise substitution of solvent molecules, resulting in a low activation barrier and fast complexation rates (Simon et al., 1973; Winkler, 1972). The relatively rapid rate of formation of the metastable form I complexes of Rb⁺ and Cs⁺, compared to form II, may similarly reflect a greater flexibility of partially formed helices having nonregular looped regions or longer runs of helix without complexed ions. Since the formation of any poly(I) helix evidently depends upon internal cation complexation, those regions with low cation density would be more flexible, more readily melted, and more accessible to reaction with further Rb⁺ or Cs⁺ ions. As the cation density (and the rigidity of the macromolecule) increases, the rate would be expected to become much slower. The transient formation of form I from II by a Li⁺-Rb⁺ displacement in the absence of added Rb⁺ has been reported above. This may arise when the displacement reaction has reduced the Rb⁺ occupancy below a level which can support form II but not yet low enough to produce the random coil.

Despite the marked differences in CD spectra [Figure 10; Howard & Miles (1982), Figure 5], we conclude, for the reasons detailed above, that all the cation-poly(I) complexes are structurally similar to the extent of being four-stranded helices with N1H...O6 hydrogen bonding and size-specific (and, for NH₄⁺, stereospecific) binding to sites in the axial channel. We suggest that the sign changes may result from relatively small changes in some geometrical parameters, such as the tilt of the bases. There is nothing to exclude small variations of structure, and minor accommodations for optimum binding with different ions or with different occupancies may well occur. The general structural model we have proposed allows such variability and accounts quite well for a large body of experimental data.

Supplementary Material Available

Figures showing the time course of conversion of Rb⁺ and poly(I) to form I of the Rb⁺-poly(I) complex, UV melting

curves of the form II Rb⁺-poly(I) complex and the Cs⁺-poly(I) complex, and the spectrophotometric titration of poly(I) with Cs⁺ (4 pages). Ordering information is given on any current masthead page.

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