The Interactions of Adenosine and Adenine Heptanucleoside Hexaphosphate with Polyuridylic Acid*

Paula M. Pitha and Paul O. P. Ts'o

ABSTRACT: Studies on the interaction of the adenine heptanucleoside hexaphosphate ($A(pA)_6$) with polyuridylic acid show that there exists only one type of $A(pA)_6$–polyuridylic acid complex under the present experimental conditions (0.4 M salt, pH 7.5, 0–35°) with stoichiometry of 1A to 2U, regardless of the ratio of the oligo A to the polyuridylic acid in the solution. This conclusion was based on quantitative evaluation of the mixing curve and of the hypochromicity in the transition observed for solutions of varying oligo A/polyuridylic acid ratios, as well as on the fact that the transition can be observed simultaneously by absorbance at 260 and 280 mμ. The concentration dependence of the T_m of the oligo A–2-polyuridylic acid complex has been observed. In this system, the formation of the $A(pA)_6 \cdot 2$polyuridylic acid complex with the polyuridylic acid in excess of 10–20-fold is not completely cooperative but could be partially cooperative. The over-all binding of adenosine to the remaining polyuridylic acid appears to be enhanced slightly by the presence of $A(pA)_6$ in forming the $A(pA)_6$–polyuridylic acid complex. Furthermore, a new site for the binding of adenosine has been created in the $A(pA)_6$–polyuridylic acid complex with polyuridylic acid in large excess. The binding of adenosine to this new site is also cooperative in nature and is favored by about 0.8 kcal than the over-all binding of adenosine to the remaining sites of the polyuridylic acid. The standard free-energy change of the binding of the adenosine to the new site is about 3.5 kcal. We propose that these new sites in the complex are located between two strands of polyuridylic acid and at the end of the $A(pA)_6$, and are benefitted by the conformational restraint imposed by $A(pA)_6$ to the two polyuridylic acid strands in the complex and by the stacking energy of adenosine monophosphate residue at the end of the oligomer.

During the past decade, our laboratory has systematically investigated the interactions of nucleic acid and its components at various levels of complexities. Research was first conducted on the extensive association of bases, nucleosides, and nucleotides and has established that vertical stacking with partial overlap is the mode of interaction of these compounds in aqueous solution (Ts'o *et al.*, 1963; Ts'o and Chan, 1964; Chan *et al.*, 1964; Schweizer *et al.*, 1965, 1968; Broom *et al.*, 1967). Studies on the interaction of mononucleosides, mononucleotides, and nucleoside triphosphates with the polynucleotides were next undertaken (Huang and Ts'o, 1966; Ts'o and Huang, 1968; Ts'o and Schweizer, 1968; Pitha *et al.*, 1968). The results indicate that the binding of the monomers to a polymer is a highly cooperative process with the participation of both hydrogen bonding and stacking energy. In this paper, we report the findings concerning the interaction of an oligomer, adenine heptanucleoside hexaphosphate ($A(pA)_6$), with the poly U as well as the interaction of adenosine with the $A(pA)_6$–poly U complex. These physicochemical studies provide us with a better understanding of the recognition process in nucleic acids replication or transcription.

Materials

The synthetic polynucleotides were purchased from Miles Laboratory, Elkhart, Ind. The sedimentation coefficients, s_{20} of poly U and poly A were 4.5 and 9.3 S, respectively, in 0.1 M NaCl–0.01 M phosphate buffer. The polynucleotide solutions were prepared by dialyzing the sample at the concentration 10 mg/ml at 4° against 0.01 M Tris buffer, pH 7, until no ultraviolet-absorbing material was detectable in the dialysate. The exact concentration of the polymer in a solution was determined spectrophotometrically and it is reported in monomer units using 9.2×10^3 as the maximum molar extinction coefficient for poly U and 10.5×10^3 for poly A (Ts'o *et al.*, 1966).

The adenine heptanucleoside hexaphosphate $A(pA)_6$ was purchased from Miles Laboratory and was examined by paper chromatography on DEAE paper (DE 81 Scientifica) with 1-propanol–concentrated NH_4OH–water (55:10:35, v/v) as the developing system. The concentration of $A(pA)_6$ in nucleotide units was determined from the absorbance at 259 mμ, using 11.0×10^3 as the molar extinction coefficient (Cantor and Chin, 1968). Adenosine was the Sigma A grade and [^{14}C]adenosine (307 mCi/μmole) was obtained from Nuclear-Chicago.

All experiments were carried out at pH 7.0 in 0.01 M Tris-HCl buffer solution containing NaCl.

Procedures

Complex Formation. All complexes were made from the same homopolymer preparations and the mixtures were equilibrated at 5° for 12 hr before use.

Equilibrium Dialysis. The experimental technique of equilibrium dialysis was described previously (Huang and Ts'o, 1966). Poly U and $A(pA)_6$ were mixed at the ratio of 10:1 (1.5×10^{-2} M poly U, 1.5×10^{-3} M $A(pA)_6$), or 20:1 (1.5×10^{-2} M, 7.4×10^{-4} M $A(pA)_6$), in 0.4 M NaCl–0.01 M Tris

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buffer, pH 7, and dialyzed against varying amounts of [^{14}C]-adenosine (concentration range from 1×10^{-4} to 2.5×10^{-2} M; 3.4×10^4 cpm/mmole) at 5° for 10 days. After this period, the equilibrium has been reached and the nucleoside distribution outside and inside the dialyzing tube was assayed by radioactivity counting in a Nuclear-Chicago 722 ambient temperature liquid-scintillation counter. The concentration of the nucleoside outside the dialysis tubing was considered to be the concentration of the free nucleosides, and the differences in radioactivity between outside and inside of dialysis tubing divided by the total radioactivity gave the percentage of the nucleoside bound. When $A(pA)_6$ was complexed with poly U, no oligonucleotide was found to come through the dialyzing membrane. However, during the time of these experiments, about 2.5% of poly U went through the dialyzing membrane. Therefore, the correction for poly U concentration has been included in the final calculation. No correction for Donnan equilibrium was made as the salt concentration in our system was sufficiently high.

Ultraviolet Absorbance. A Cary 15 spectrophotometer equipped with thermostatically controlled cell compartments (±0.5° between 0 and 60°) was used for obtaining the absorbance at varying temperatures. Samples were degassed before use and the spectrum from 300 to 220 mμ was recorded at each change of temperature.

Optical Rotation. Measurements of optical rotation [α] as the function of temperature were made at 350 mμ on a Cary 60 recording spectrophotometer with a temperature controlled cell (±0.5°) of 1-cm light path connected to a circulating bath with a thermostat.

Results and Discussion

The research program can be separated into two phases. The first phase consists of a study on the properties of the oligomer–polymer complex; the second phase consists of a study on the interaction of adenosine with the $A(pA)_6$–poly U[1] complexes having A:U ratios of 1:2, 1:10, and 1:20.

The Properties of the $A(pA)_6$–Poly U Complex. The thermally induced transition of the $A(pA)_6 \cdot 2$ poly U complex[1,2] in 0.1 M NaCl–0.01 M Tris buffer (pH 7.0) is shown in Figure 1. The transition has been studied at two concentration levels. At a concentration of 10^{-5} M the transition was measured by optical density at 259 mμ, whereas at a concentration of 10^{-2} M the transition was measured by optical rotation at 350 mμ. Under this condition, there is about a 13° difference in T_m over a 1000-fold change in concentration of the complex. Uhlenbeck *et al.* (1968) had reported a 14° difference in T_m over a 100-fold change of the concentration of the pentamer, $A(pA)_4$; the experimental conditions, however, were not described in their paper. The transition of the poly A·2poly U, the polymer complex, is also shown in Figure 1 for comparison. Within the experimental error, the polymer–polymer complex transition and the oligomer–polymer complex transition both appear to be about equally sharp; and yet the observed difference in T_m (about 3°[3]) between the optical density measurement (10^{-5} M) and the optical rotation measurement (10^{-2} M) for the polymer–polymer complex is far smaller than that for the oligomer–polymer complex (13°). Thus, though both transitions are highly cooperative, the transition of the oligomer–polymer is concentration dependent while the transition of the polymer–polymer is not. The concept emerged from the above studies can be qualitatively described as follows.

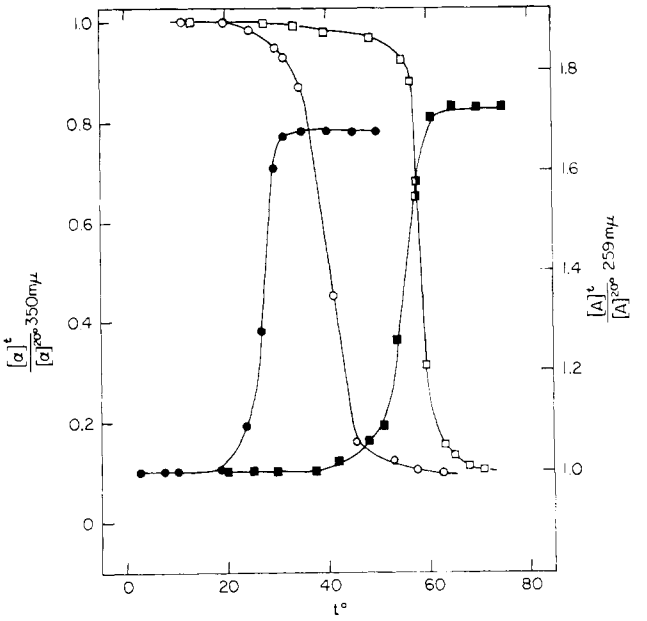

FIGURE 1: The thermal dissociation of complexes of poly A·2poly U (□—□) and $A(pA)_6$·2poly U (○–○) in 0.1 M NaCl–0.1 M Tris buffer (pH 7.0). Open symbols represnt the optical rotation measurements at 350 mμ and the full symbols represent the optical density measurements at 259 mμ.

In an interaction between two polymers of sufficient chain length, the concentrations of the monomeric units in the immediate surroundings are high. Under these conditions, the extent of the complex formation is not dependent on the bulk concentration of the polymers in solution, since an increase in the bulk concentration of the polymers may have little influence on the local concentration of the monomeric units in the vicinity of the two interacting polymers due to their excluded volumes. In an interaction between the oligomers and a polymer, however, the local concentrations of the monomeric units of the oligomer are subjected to the influence of the bulk concentration of the oligomers in solution to a certain degree. Therefore, the extent of complex formation between oligomers and a polymer is dependent upon the bulk concentration of the oligomers in solution as governed by the mass

[1] Two types of designations for the oligomer–polymer complexes are adopted: (1) $A(pA)_6$–poly U is used to designate the existence of a complex between $A(pA)_6$ and poly U without any specific reference to the nature of the complex. Often in this case, the input A:U ratio in the mixture is also given; (2) $A(pA)_6$·2poly U is used to designate a three-stranded complex of $A(pA)_6$ and poly U in stoichiometry of 1A to 2U. It does not mean that the complex has one molecule of $A(pA)_6$ to two molecules of poly U. Similarly, the commonly used designation of poly A·2poly U only means that the stoichiometry of the complex is 1A to 2U, and does not imply that the polymer complex contains one molecule of poly A to two molecules of poly U, since these polynucleotides all have different chain lengths.

[2] The stoichiometry of the complex in mixture of $A(pA)_6$ and poly U with ratio of 1A to 2U is shown to be 1A and 2U in a subsequent section.

[3] The difference between the temperature measurement in Cary 15 and 16 is estimated to be about 1°.

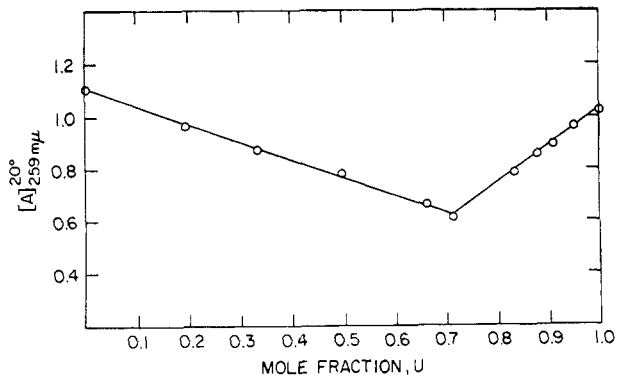

FIGURE 2: The mixing curve for A(pA)$_6$ and poly U at 20° in 0.4 M NaCl–0.01 M Tris (pH 7.0).

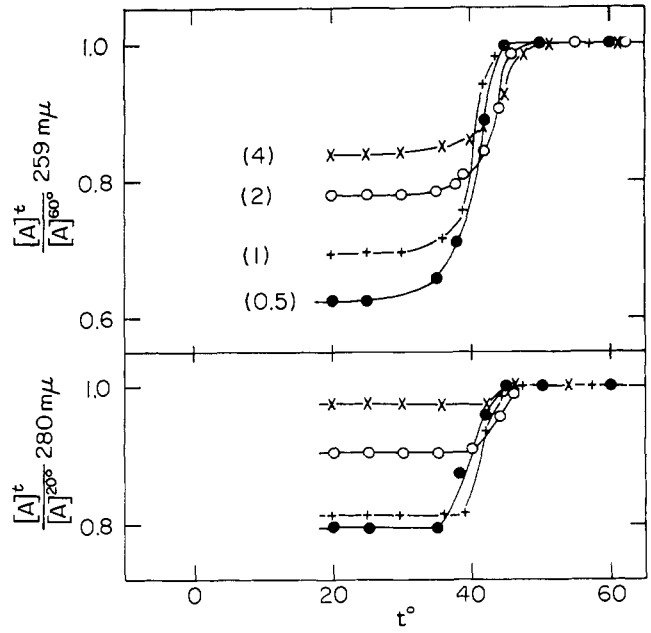

FIGURE 3: The absorbance–temperature profiles of the A(pA)$_6$·2 poly U complex in solutions of A:U ratios of 4 (x-x), 2 (O–O), 1 (+–+), and 0.5 (●–●) in 0.4 M NaCl–0.01 M Tris (pH 7.0). The upper portion represents the absorbance measured at 259 mμ and the lower portion represents the absorbance measured at 280 mμ.

action law, even though the interaction process is a cooperative one. The concentration dependence of the monomer–polymer complex formation is even larger, as may be expected from this concept. Only a threefold change in concentration of adenosine brings about a difference in T_m of 13° for the transition of the adenosine·poly U complex (Huang and Ts'o, 1966). In summary, although these three processes of complex formation (polymer–polymer, oligomer–polymer, and monomer–polymer) are all cooperative in nature, the local concentration of the monomeric units at the immediate vicinity of the interaction is not dependent upon the bulk concentration in the case of the polymer–polymer interaction; the local concentration is mildly dependent upon the bulk concentration in the case of the oligomer–polymer interaction; and the local concentration is directly related to the bulk concentration in the case of the monomer–polymer interaction.

Recently, Cassani and Bollum (1969) have investigated the concentration dependence of the T_m of the deoxyadenine oligomer–poly T complex and of the deoxythymine oligomer–poly dA complex with oligomers having 8 or 10 units in length. A linear relationship between the $1/T_m$ and the logarithm of the concentration of the oligomer was established in their investigation.

The T_m of the A(pA)$_6$·2poly U[2] complex is lower than that of the poly A·2poly U complex by about 28° at 10^{-5} M concentration and by about 17° at 10^{-2} M concentration. Experimental observations of Lipsett *et al.* (1960) and of Cassani and Bollum (1967, 1969), as well as the theoretical formulations by Magee *et al.* (1963, 1965), all indicate a linear relationship existing between the reciprocal of the T_m ($1/T_m$) and the reciprocal of the oligonucleotide chain length ($1/n$). However, Michelson and Monny (1967) reported that the plot of log n *vs.* T_m (or $1/T_m$) gives a better fit to the data of a variety of oligonucleotide–polynucleotide interaction.

The next question is the stoichiometry of the complex of A(pA)$_6$ and poly U. The mixing curve of the heptamer and poly U in 0.4 M NaCl–0.01 M Tris buffer (pH 7.0) is shown in Figure 2. Within the experimental error, the result indicates that the stoichiometry of the complex is one A from A(pA)$_6$ to two U from poly U. This finding is in agreement with the conclusion of Cantor and Chin (1968) who had studied the complex of A(pA)$_{1-5}$ with poly U in 0.5 M salt. They considered that even in the 1:1 mixtures of oligo A and poly U, the solutions consist of 1:2 complexes plus free oligomer. Cassani and Bollum (1969) have found also a stoichiometry of 1A:2T in the mixing curve of d(pA)$_6$–poly dT. However, the mixing curve will not give definite information about the stoichiometry of the complex, unless only one type of complex exists in the solution. If one type of complex with a stoichiometry of (A·mU) exists in the region of excess of A(pA)$_6$, and another type of complex with stoichiometry of (A·nU) exists in the region of excess poly U, then the observed intersection of the mixing curve is governed not only by the stoichiometry of the two complexes (m and n), but also by the molar extinction coefficients of A(pA)$_6$, poly U, and the two complexes. The complete analytical expression is given in the Appendix (eq 1). The general expression can be readily converted to a specific equation for the case with $m = 1$ and $n = 2$ (eq 2 in Appendix) or for the case with $m = 2$ and $n = 1$ (eq 3 in Appendix). The molar extinction coefficients at 259 mμ for the A(pA)$_6$·poly U complex and A(pA)$_6$·2poly U were taken to be identical with those for the (poly A·poly U) complex ($7.0 \pm 0.1 \times 10^{-3}$) and (poly A·2poly U) complex ($6.2 \pm 0.1 \times 10^{-3}$), respectively. The interaction points in the mixing curve (0.34 for $m = 1$, $n = 2$; and 0.56 for $n = 1$, $m = 2$) calculated from these equations together with these extinction coefficients differed from the experimental value of 0.69 ± 0.02 (Figure 2). Thus, these calculations do not indicate the existence of two kinds of complexes with different stoichiometry at the two starting zones of the mixing curve. It is most likely, therefore, that only one type of complex exists with a stoichiometry of 1A:2U throughout the mixing curve.

In order to probe this question further, the thermal transition of mixtures of A(pA)$_6$ and poly U at the varying ratios of A:U in the mixing curve has been studied. The thermal transition profiles of eight mixtures of A(pA)$_6$ and poly U observed at 259 mμ are shown in Figures 3 and 4. These profiles are pre-

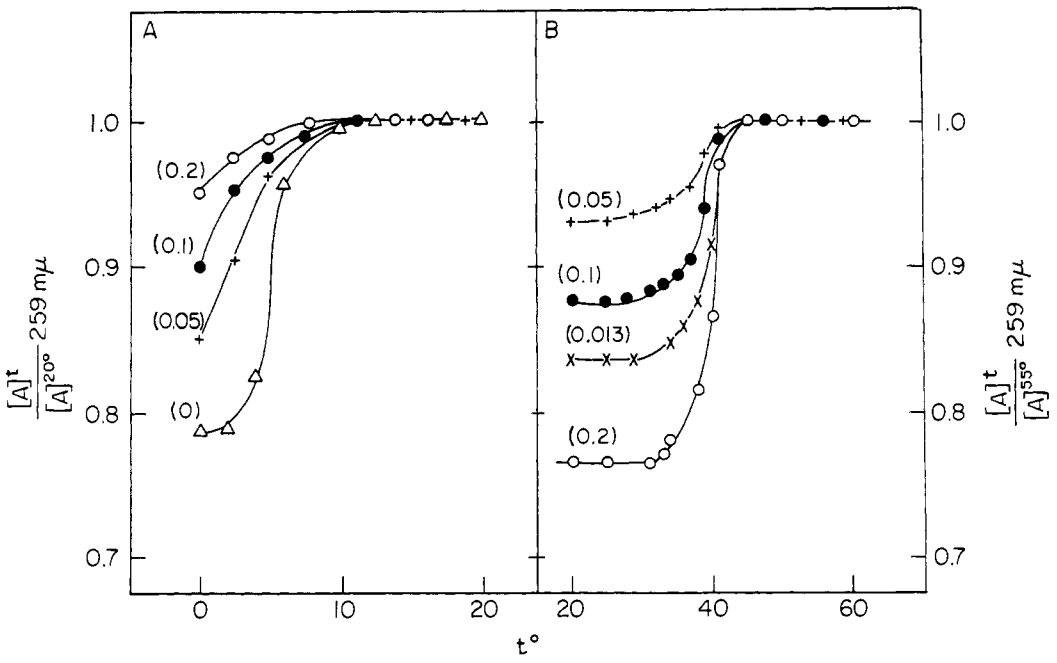

FIGURE 4: The absorbance–temperature profiles of solutions of A:U ratios of 0.2 (○-○), 0.1 (●-●), 0.05 (+-+), and 0 (△-△) measured at 259 mμ in 0.4 M NaCl–0.01 M Tris (pH 7.0). Part A represents the transition of poly U–poly U helix between 0 and 10°. Part B represents the transition of A(pA)$_6$·2poly U complex between 30 and 50°.

sented for hypochromicity analyses with the high temperature plateau normalized to unity. As can be seen from Figures 3 and 4B, there is only one transition located near 40° for the melting of the A(pA)$_6$–poly U complex. In solutions containing excess poly U, another transition can be detected near 5°, which is the transition of the poly U helix formed by the fraction of the poly U left in the solution which did not interact with A(pA)$_6$. In addition, a transition was also observed at 280 mμ which occurred at the same temperature as the transition observed at 260 mμ (Figure 3). The optical change observed at 280 mμ can be readily measured in solutions having A:U ratio higher than 0.5 (excess A) or in solutions having a large fraction of A–U complex. In the poly A–poly U system, the transition observed at 280 mμ has been shown to indicate the melting of the triple-stranded poly A·2poly U complex to single-stranded form (Stevens and Felsenfeld, 1964). It is most reasonable to assume that the optical properties of A(pA)$_6$–poly U complex are similar to those of the poly A–poly U complex as shown in other oligo A–poly U complexes (Cantor and Chin, 1968). Therefore, this thermal transition observable both at 259 and 280 mμ on the solutions of A(pA)$_6$ and poly U most likely also indicate the melting of the triple-stranded complex (2A:1U) to the single-stranded form. Since only one thermal transition is observed (Figures 3 and 4), this fact shows that the triple-stranded A(pA)$_6$·2poly U complex dissociates directly into the single-stranded state in 0.4 M NaCl without passing through a double-stranded intermediate. This observation is in harmony with that of the polymer complexes reported by Stevens and Felsenfeld (1964) and by Blake *et al.* (1967). These authors observed that at concentrations of 0.2 M salt and higher, the triple-stranded poly A·2poly U dissociates directly to the single-stranded state. All these experimental findings, therefore, indicate that even in solutions containing excess A(pA)$_6$, the A-(pA)$_6$–poly U complex still has a stoichiometry of 1A:2U.

The question of stoichiometry can be further investigated by a quantitative examination of the hypochromicity observed between 55 and 20°, which is defined as $(1 - A^{20°}_{259\ m\mu}/A^{55°}_{259\ m\mu}) \times 100$. The hypochromicity so defined and observed is a total sum of the hypochromicity of each component in the solution where each component contributes in accordance to its mole fraction. The equation for the calculation of hypochromicity for solutions containing 1A:2U complex is

$$H_{obsd} = (F_U H_U) + (F_A H_A) + (F_{A:2U} H_{A:2U}) \qquad (1)$$

where F is the mole fraction (in monomeric unit) and H is the hypochromicity of poly U, A(pA)$_6$ and the A(pA)$_6$·2poly U complex as denoted by the corresponding subscript. Similarly, the equation for the calculation of hypochromicity for solutions containing 1A:1U complex is

$$H_{obsd} = (F_U H_U) + (F_A H_A) + (F_{A:U} H_{A:U}) \qquad (2)$$

where $F_{A:U}$ and $H_{A:U}$ are the mole fraction and the hypochromicity, respectively, of the 1A:1U complex. H_U and H_A were determined experimentally to be 0 and 7%, respectively (Table I). Values of $H_{A:U}$ (35%) and $H_{A:2U}$ (40%) were taken from measurements for the poly A·poly U complex and for the poly A·2poly U complex (Stevens and Felsenfeld, 1964; Blake *et al.*, 1967). The observed hypochromicity for solutions containing varying ratios of A(pA)$_6$/poly U are shown in Table I together with the hypochromicity for these solutions calculated both from eq 1 and 2 based on the above H values. The calculated values from the eq 1 for the 1A:2U complex

TABLE I: Interaction of $(Ap)_6A$ and Poly U as Studied by the Hypochromicity[a] of the Helix–Coil Transition between 20 and 55° (0.4 M NaCl Figures 3 and 4).

A:U Ratio[b]	$r(U)_n$ Concn[b] (10^{-5} M)	$[A]^{55°}_{259\,m\mu}$	Obsd Hypochromicity (%)	Calcd Hypochromicity (A:U = 1:2)[c]	Calcd Hypochromicity (A:U = 1:1)[d]	T_m
0:1	8	0.81	0	0	0	
0.05:1	8	0.87	7(6)[e]	6	3.5	39.5
0.1:1	8	0.89	12(13)[e]	11	6	39.8
0.133:1	8	0.92	16.5	14.5	8	40.0
0.2:1	8	0.985	23(23)[e]	20	11.5	40.0
0.5:1	5	0.79	37(37)[e]	40	23	40.5
1:1	2.5	0.56	30(32)[e]	32	35	41.0
2:1	2.5	0.84	23(24)[e]	24	25.5	42.0
4:1	2.5	1.45	16(17)[e]	17	18	44.6
1[f]:0	0	0.84	7.2	7	7	

[a] Hypochromicity (per cent) is defined as $(1 - (A^{20°}_{259\,m\mu}/A^{55°}_{259\,m\mu})) \times 100$. [b] The accuracy of the concentration and the ratio is about ±3–4%. [c] Equation 1 was used for the calculation of hypochromicity at 1A:2U complex. $H_U = 0$, $H_A = 7.2$, and $H_{2U:A} = 40$ (see text). [d] Equation 2 was used for the calculation of hypochromicity at 1A:1U complex. $H_{A:U} = 35$ (see text). [e] Another set of hypochromicity obtained at the same A:U ratio but by slightly different concentrations of $(U)_n$ and $(A)_7$. [f] Concentration of $(A)_7$ was 7×10^{-5} M.

agree well with the experimental values throughout the whole range of A:U ratios. On the other hand, for solutions having an A:U ratio of 0.5 or less, the calculated values from eq 2 for the 1A:1U complex do not agree well at all with the experimental values. For the solutions having an A:U ratio of one or larger, the agreement between the experimental values and the calculated values from eq 1 (1A:2U complex) is still better than that from eq 2 (1A:1U complex). However, the disagreement between the experimental values and the calculated values from eq 2 for solution having an A:U ratio of 2 or larger may not be experimentally significant. Thus, the quantitative study and calculation on the hypochromicity again indicate that under the present experimental conditions only one type of $A(pA)_6$–poly U complex exists in solutions of varying A:U ratio and this complex has a stoichiometry of 1A:2U. For the poly A–poly U interaction, it has been shown that the formation of the double-stranded complex is more favored at lower ionic strength (Stevens and Felsenfeld, 1964; Blake *et al.*, 1967). This situation has not been adequately investigated in the oligomer–polymer interaction. Lipsett *et al.* (1961) showed that in 0.1 M NaCl, the mixing curve of $(pA)_4$ and poly U gives a stoichiometry of 1:1, while in 0.001 M $MgCl_2$, the mixing curve gives a stoichiometry of 1:2.

The next issue is the degree of cooperativeness in the interaction between the $A(pA)_6$ and the poly U when the poly U is in excess. In other words, in a solution containing a large amount of poly U and a small amount of $A(pA)_6$, will the oligo A interact exclusively with a small portion of poly U in a stoichiometric and cooperative manner, or will the oligo A interact with all poly U molecules equally and dispersively? The dispersive mode of interaction is favored by the chemical potential of all available sites on poly U, while the cooperative mode is favored by the nearest-neighbor interaction of the end base in the oligomer and possibly by the reduction of the energy required to complete a conformational unit in the complex (for instance, if two oligomers are needed to complete a conformational unit in the complex, such as a helical turn, the presence of the first oligomer will tend to induce the interaction of the second oligomer to complete the turn). Certain information pertinent to this important question can be derived from Figure 4A, though the present data is insufficient to provide a complete answer. The mole fractions of free poly U remaining per total amount of poly U in the solution are 60, 80, and 90%, respectively, for solutions containing $A(pA)_6$ and poly U in (A:U) ratios of 0.2, 0.1, and 0.05. Yet the percentages of hypochromicity remaining due to the formation of poly U helix in solution are only about 25, 50, and 75%, respectively, in solutions with A:U ratios of 0.2, 0.1, and 0.05. Therefore, the percentage of hypochromicity remaining appears to be considerably less than the mole fraction percentage of free poly U remaining. If the extent of hpyochromicity in the transition between 0 and 10° is proportional to the extent of formation of helix by free poly U, then the per cent of the poly U helix formed in the solution is less than the per cent of poly U not complexed with the oligo A. It appears, therefore, that the formation of a given amount of $A(pA)_6 \cdot 2$poly U complex in solution partially suppresses the formation of a helix by the remaining poly U. For instance, in a solution containing 50% $A(pA)_6 \cdot 2$poly U complex, the hypochromicity data of Figure 4A (curve in A:U ratio of 0.2) shows that only 40% of the remaining poly U can form some sort of self-complex. This observation strongly suggests that the interaction of oligo A with excess poly U cannot be totally cooperative because the self-association of the completely free poly U molecules into helices should not be hindered by the completely interacted oligo A–poly U complex. The simplest interpretation is that in the presence of excess poly U, the oligo A interacts with the poly U at least in a partially dispersive mode. Thus, certain segments of a poly U molecule may be interacted with the oligo A to form an $A(pA)_6 \cdot 2$poly U complex, while other segments in the same poly U molecule are not interacted. The noninteracted segments of the poly U chain,

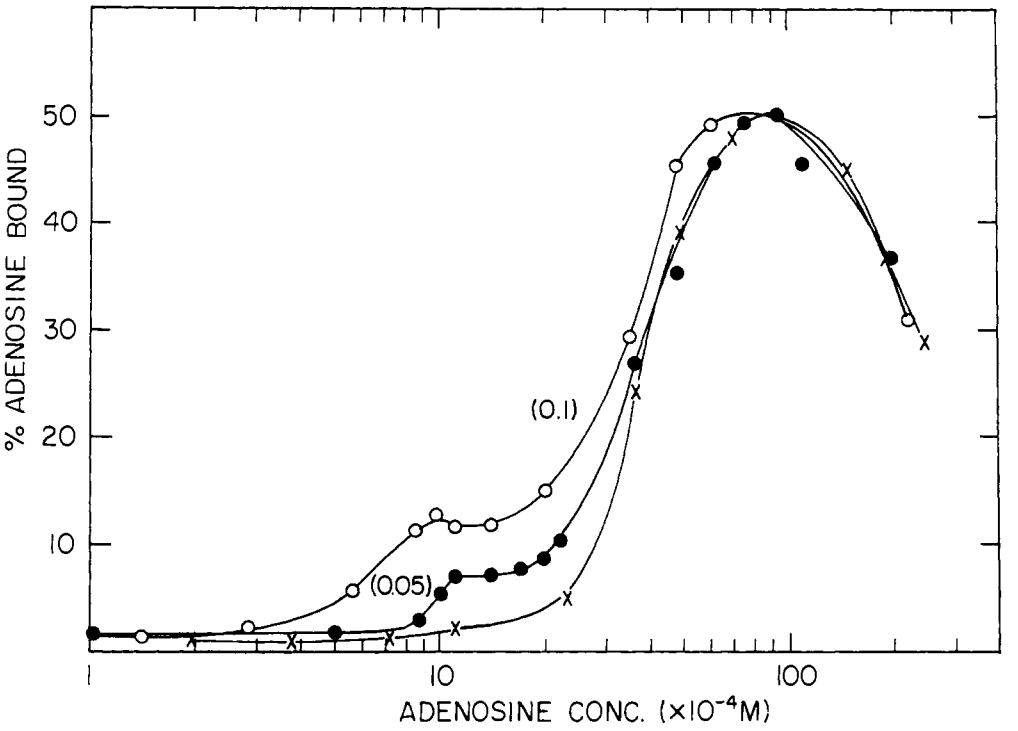

FIGURE 5: Percentage of adenosine bound to poly U (x-x) or to A(pA)$_6$–poly U complexes having A:U ratios of 0.05 (●-●) and 0.1 (○-○) *vs.* log input concentration of adenosine at 5° in 0.4 M NaCl–0.01 M Tris (pH 7.0) as measured by equilibrium dialysis. Concentration of poly U was 1.5×10^{-2} M and concentration of A(pA)$_6$ was 7.4×10^{-4} and 1.4×10^{-3} M, correspondingly, for the two complexes.

however, are hindered in forming poly U–poly U helical complexes by these interacted segments in the same molecule; alternatively, the noninteracted segments may form another type of poly U–poly U helical complex which has less hypochromicity relative to the normal complex formed by the completely free poly U. The conclusion that the interaction between oligo A and poly U in the presence of excess poly U is at least partially dispersive is supported by the results on the binding of adenosine to the A(pA)$_6$–poly U complex.

Binding of Adenosine to the A(pA)$_6$–Poly U Complex. The binding of adenosine to A(pA)$_6$–poly U complexes with A:U ratios of 0, 0.05, 0.1, and 0.5 have been studied by equilibrium dialysis at 5° in 0.4 M NaCl–0.01 M Tris buffer (pH 7.0). As shown in the plot of per cent of the adenosine bound *vs.* the input concentration of adenosine (Figure 5), the binding of adenosine to poly U alone (A:U ratio = 0) is essentially the same as that described previously (Huang and Ts'o, 1966). There is no binding of adenosine to poly U until a critical concentration of the input adenosine is reached. From this point, the binding increases rapidly over a narrow adenosine concentration until saturation is reached at the stoichiometry of 1A to 2U. The steep transition shown in the absorption isotherm (Figure 6; the amount of adenosine bound per nucleotide of poly U *vs.* the log free adenosine concentration) indicates the cooperative nature of this binding process. The slope at the midpoint of this transition has been estimated in our previous work, and indicates a stacking energy of −5 to −6 kcal/mole due to the nearest-neighbor interaction of adenosine upon pairing with 2U of poly U. Under the identical conditions, however, no binding of adenosine to the A(pA)$_6$·2poly U complex (A:U ratio of 0.5) was observed. This important observation confirms the stoichiometry of the A(pA)$_6$–poly U complex to be 1A to 2U under the present conditions of the dialysis experiment. If the stoichiometry of the complex under these conditions is 1A to 1U, then binding of adenosine to the remaining poly U should take place, which was not observed. This experiment also shows that no binding of adenosine can be detected unless there are available sites from poly U for base pairing.

In Figures 5 and 6, the binding of adenosine to A(pA)$_6$–poly U complexes having A:U ratios of 0.05 and 0.1 is also illustrated. The binding curves of adenosine to these A(pA)$_6$–poly U complexes in the presence of large poly excess (20U or 10U to 1A) show a biphasic character. The transition in the binding curves at high adenosine concentration (input adenosine concentration shown in Figure 5, or free adenosine concentration shown in Figure 6) observed for the A(pA)$_6$–poly U complexes is essentially the same as that observed for the poly U alone. At low adenosine concentration, a new transition in the binding curves is clearly shown in Figures 5 and 6. This binding of adenosine at low concentration (the first transition in the binding curve) must be due to the new binding sites created through the formation of the A(pA)$_6$–poly U complex. As shown in Figure 6, this new binding process levels at a ratio of bound adenosine:poly U of about 0.09 for the A-(pA)$_6$–poly U complex having 0.1 A:U ratio, and levels at a ratio of bound adenosine:poly U of about 0.08–0.07 for the A-(pA)$_6$–poly U complex having 0.05 A:U ratio. These results indicate that the number of new sites available is related to the amount of the A(pA)$_6$–poly U complex formed. In Figure 7, the ratio of adenosine bound:A(pA)$_6$ chain added (γ = A bound/A(pA)$_6$ chain) is plotted against the free adenosine concentration, measured from the first transition in the binding curve at low adenosine concentration. Within the limits of ex-

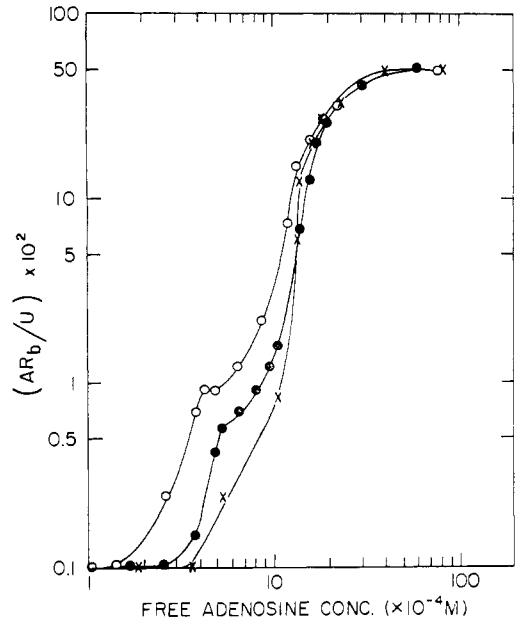

FIGURE 6: Adenosine bound per nucleotide unit of poly U *vs.* log concentration of free adenosine as calculated from the data shown in Figure 5. Binding curve to poly U alone (x–x), to $A(pA)_6$–poly U complex having A:U ratio of 0.05 (●-●), and to $A(pA)_6$–poly U complex having A:U ratio of 0.1 (○-○).

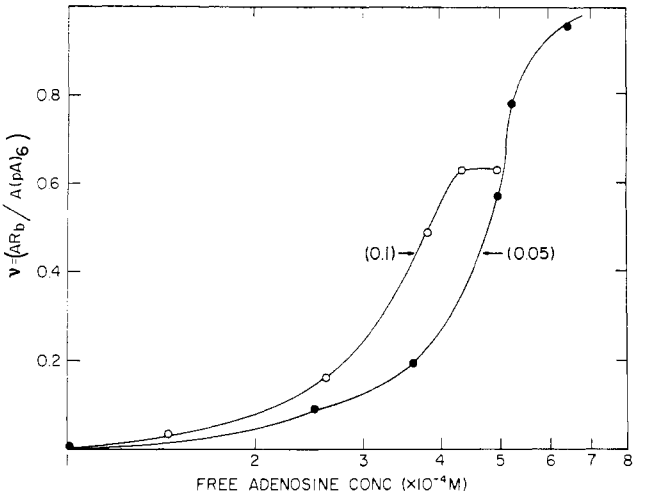

FIGURE 7: Ratio of adenosine bound per oligomer of $A(pA)_6$ ($\gamma = AR_b/A(pA)_6$) *vs.* free adenosine concentration. The original data were from Figures 5 and 6. Concentration of oligomer chain was obtained by dividing the molar concentration of $A(pA)_6$ in nucleotide unit by 7. (●-●) The complex having A:U ratio of 0.50 and (○-○) complex having A:U ratio of 0.1.

perimental error, these data indicate that the saturation of the binding process at the first transition took place at $\gamma = 1$ for $A(pA)_6$–poly U complex having A:U ratio of 0.05, and at $\gamma = 0.6$ for $A(pA)_6$–poly U complex having A:U ratio of 0.1. In other words, the number of sites available for binding of adenosine at the first transition of the binding curve is about 1/oligo A in the $A(pA)_6$–poly U complex of 1:20 ratio, and is about 0.6/oligo A in the $A(pA)_6$–poly U complex of 1:10 ratio. It is reasonable to propose that the binding of adenosine to the $A(pA)_6$–poly U complex takes place near the ends of the oligo A by base pairing to two UMP units of the two poly U strands and by base stacking to the AMP unit at the end of the oligo A. This proposed binding site, located between two strands of poly U and at the end of the oligo A, is benefitted by the conformational restraint imposed by $A(pA)_6$ on the two poly U strands in the complex and by the stacking energy of the AMP at the end of the oligomer. If this is indeed the new site for the binding of adenosine in the first transition at low concentration, then there should be two such sites per $A(pA)_6$ in the complex since there are two ends (3′ end and the 5′ end) in each oligonucleotide. However, only one site per oligomer was detected in the complex having A:U ratio of 0.05 and only 0.6 site per oligomer was detected in the complex having A:U ratio of 0.1. The fact that the complex having an A:U ratio of 0.5 (or 1A to 2U) does not bind adenosine at all suggests that a partial cooperativeness in the binding between oligo A and poly U may be responsible for the decrease in the number of sites available per oligomer upon an increase of A:U ratio in the complexes. First of all, the presence of the first transition in the binding curve immediately indicates that the interaction of oligo A and poly U cannot be completely cooperative. A completely cooperative interaction between oligo A and poly U in the presence of excess poly U will produce only the 1A:2U complex, which does not bind adenosine, and will leave the remaining poly U free. Therefore, no binding site different from that in free poly U can be created in a mixture of oligo A and poly U if the interaction is completely cooperative. This finding of new sites in the binding curve supports the conclusion in the first section that the interaction between oligo A and poly U in the presence of excess poly U is not completely cooperative. On the other hand, a partially cooperative interaction between oligo A and poly U in the complex will remove the newly created binding sites at the end of the oligomer. For instance, if two oligomers interact with the poly U next to each other, then the two sites between them (3′ end of one oligomer and 5′ end of the other) will not be available for binding of adenosine through this cooperative interaction. The net result will show that there is only one site per oligomer instead of two; the other site is removed by the adjacent oligomer through cooperative interaction. Similarly, if three oligomers interact with poly U cooperatively and stack along the polymer strands, then four sites will be removed by the oligomers being adjacent to each other in the stack (the 3′ end from one oligomer at one end, the 5′ end of the other oligomer at the other end, and both 3′ and 5′ ends of the oligomer in the middle of the stack). The net result is that only two sites are left for the three oligomers totally (one at each end of the stack), or 0.6 site/individual oligomer. On the basis of this argument, we propose that the partial cooperative interaction of $A(pA)_6$ with poly U removes some end sites for binding of adenosine by forming stacks of the oligomers. If this explanation is correct, then on the average there are about two $A(pA)_6$ oligomers per stack in an $A(pA)_6$–poly U complex having an A:U ratio of 0.05 (for $\gamma = 1$) and about three $A(pA)_6$ oligomers per stack in an $A(pA)_6$–poly U complex having an A:U ratio of 0.1 (for $\gamma = 0.6$). As observed, the increase in the number of unfilled sites of poly U as the consequence of decreasing the A:U ratio is expected to enhance the dispersive mode of interaction from the entropy consideration.

As shown in Figures 5 and 6, the second transition in the

binding curves of the A(pA)$_6$–poly U complexes and the single transition in the binding curve of the free poly U are both steep and similar in shape. In addition, both transitions take place at approximately the same concentration of adenosine; the second transition occurs at a slightly lower adenosine concentration (5–10%). Apparently, after the binding of the first adenosine to the new site at the A(pA)$_6$–2poly U complex (the first transition), subsequent binding of adenosine to the remaining sites of this complex (the second transition) is essentially similar to, but perhaps slightly more energetically favored than, the binding of adenosine to the free poly U. In this binding process, the adenosine molecules bind to the two poly U strands cooperatively in filling up the sites in the polymer within a narrow concentration of adenosine to give a 1A to 2U complex (Huang and Ts'o, 1966). In the condition of the binding study (0.41 M salt, pH 7.5 and 5°) poly U does have some residual secondary structure due to self-association (Huang and Ts'o, 1966, and Figure 4). The effect of the secondary structure of poly U on the binding process is not known. Binding of adenosine to poly U does take place, however, even at lower concentration of adenosine (and the resultant complex even has a higher T_m) in 0.02 M $MgCl_2$, 5° conditions where poly U exist in helical form. In our experiments, the free poly U has more extensive secondary structure than the "unpaired segment" of the poly U partially complexed with A(pA)$_6$ at an A:U ratio of 0.1 or 0.05. This conclusion was derived from the hypochromicity studies discussed above (Figure 4). Since the adenosine binding processes to the free poly U and to the "unpaired segment" of the poly U in the A(pA)$_6$–poly U complex appear to be very similar to each other, the effect of the residual secondary structure of poly U on the binding process cannot be very large.

As shown in Figures 6 and 7, the midpoint of the first transition in the binding curve of A(pA)$_6$–poly U complex is about 3–4.5 $\times$ 10^{-4} M free adenosine, while the midpoint of the second transition (or the single transition in the binding curve of poly U) is about 12–13 $\times$ 10^{-4} M. Thus, the two free adenosine concentrations at which these two binding processes take place differ from each other by a factor of 3–4. An approximate estimation by the term RT ln 3.5 yields a difference in standard chemical potential of these two binding processes of 0.7–0.8 kcal.

The binding process of adenosine to the newly created sites in the A(pA)$_6$–poly U complex (the first transition) is also cooperative. The association constant, K, estimated from the standard procedure (Tanford, 1961) increases with increasing values of θ, the degree of saturation of available sites. A formal approach based on the assumption that the sites are identical but interact with one another is adopted as follows (Tanford, 1961):

$$\Delta F^\circ = \Delta F^\circ_{\text{intrinsic}} + RT\phi(\theta)$$

thus

$$K = K_{\text{intrinsic}}\, e^{-\phi(\theta)}$$

or

$$\ln K = \ln K_{\text{intrinsic}} - \phi(\theta) \qquad (3)$$

The application of eq 3 to the analyses of the data in Table II is shown in Figure 8. The points of ln K values are approximately linear with θ except at very high value of θ. The slope in Figure 8 is the value for the function $\phi(\theta)$, and the term $RT\phi(\theta)$ represents the average free energy of interaction between the filled sites. At $\theta = 0$, the value of ln K was extrapolated to be about 5.6–5.8. Based on this number, the $K_{\text{intrinsic}}$ is about 300 and the $\Delta F^\circ_{\text{intrinsic}}$ of the adenosine binding to the new sites in the complex is about 3.3–3.5 kcal. The nature of the cooperativeness as shown in the slope of the line in Figure 8 is not well understood. These interactions between the binding sites should be an interaction between several sites in the same two strands of poly U and not an interaction between sites located at different strands of poly U. If this is the case, then the binding of adenosine to these sites along the same two strands of poly U probably increases the stability of these complexes and, therefore, increases the binding affinity of the remaining sites.

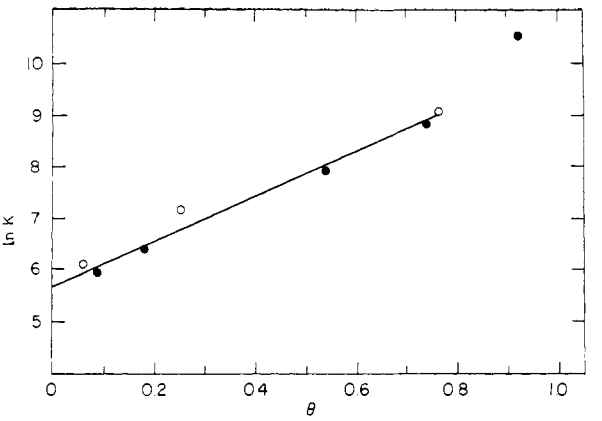

FIGURE 8: Ln K *vs.* θ where K is the association constant and θ is the degree of saturation of the available site in the first transition. Data are from Table II. (●-●) The complex having A:U ratio of 0.05 and (○-○) the complex having A:U ratio of 0.1.

The thermal stability of the adenosine–A(pA)$_6$–poly U complex has also been investigated by measuring the optical rotation at 350 mμ (Figure 9). The optical rotation *vs.* temperature profile of the adenosine–A(pA)$_6$–poly U complex (A(pA)$_6$:poly U = 0.1) shows two transitions. In the biphasic curve, the transition at the high temperature (T_m = 52.5°) represents the dissociation of the (A(pA)$_6$·2poly U complex, while the transition at the low temperature (T_m = 19.5°) represents the dissociation of the adenosine from the A(pA)$_6$–poly U complex. The T_m value of the dissociation of adenosine from the poly U shown in the monophasic transition is 15°. This small but significant increase (4.5°) in the T_m value indicates the presence of A(pA)$_6$ in the A(pA)$_6$–poly U complex tends to increase the stability of the adenosine–poly U complex in the adenosine A(pA)$_6$–poly U complex. This is in agreement with the slight increase in affinity reflected in the binding curve discussed above (Figure 6).

Significance. The experiments reported here are part of our general effort to understand the recognition process in the replication or transcription of nucleic acids on a physicochemical basis. Studies concerning the interaction of mononucleotides with polynucleotides (Huang and Ts'o, 1966; Howard *et al.*, 1966) show that the interaction is a highly cooperative process requiring the participation of both hydrogen

TABLE II: Analyses on the Binding of Adenosine to the $A(pA)_6$–poly U Complexes at Low Concentration of Adenosine (the First Transition in the Binding Curve) in 0.4 M NaCl–0.01 M Tris Buffer, pH 7.0, 5°.

Poly U ($\times 10^{-2}$ M)	A(pA) ($\times 10^{-3}$ M)	Input[a] Adenosine ($\times 10^{-4}$ M)	Adenosine[a] Bound ($\times 10^{-5}$ M)	Adenosine[a] Free ($\times 10^{-4}$ M)	γ[b] ($\times 10^{-1}$)	θ[c]	K[d] ($\times 10^{2}$ M^{-1})
1.5	0.74	1.0	0.2	0.5	0.2		
		4.5	0.9	2.5	0.84	0.084	3.6
		7.6	1.9	3.7	1.8	0.18	6.0
		10.5	5.8	4.9	5.4	0.54	27.0
		11.0	7.9	5.2	7.4	0.74	68.0
		13.6	9.8	6.3	9.2	0.92	380.0
1.5	1.4	1.4	0.21	0.70	0.1		
		2.8	0.75	1.4	0.4	0.06	4.5
		5.6	3.3	2.6	1.6	0.25	12.0
		8.5	9.9	3.8	4.9	0.76	88.0
		9.9	13.0	4.3	6.5	1.0	
		11.3	13.0	4.9	6.5		

[a] Because of the design of the dialysis experiment (Huang and Ts'o, 1966), within the experimental error, the following relationship should hold: $A_{\text{input conen}} = A_{\text{bound}} + 2A_{\text{free}}$. [b] $\gamma = A_{\text{bound}}$/chain of $A(pA)_6$. Concentration of $A(pA)_6$ chain = molar concentration of $A(pA)_6$ in nucleotide/7. [c] θ = degree of saturation of available sites. For $A(pA)_6$–poly U complex having A:U ratio of 0.05, the number of sites per $A(pA)_6$ chain in the complex is assumed to be extrapolated to 1 (see Figure 7 and text). For complex having A:U ratio of 0.1, the number of sites per $A(pA)_6$ chain in the complex is measured to be 0.65 as shown in the table. Therefore $\theta = \gamma/1$ or $\gamma/0.62$ for the two complexes, respectively. [d] K (the association constant) $= \theta/(1 - \theta)A_{(\text{free})}$.

bonding which is more related to specificity, and the stacking energy which is more related to stability. These studies also show that the interaction of purinyl mononucleoside to the pyrimidinyl polynucleotide is far stronger than the pyrimidinyl mononucleoside to the purinyl polynucleotides, as may be expected from the findings that the tendency of self-stacking of the purine derivatives is much larger than that of the pyrimidinyl derivatives (Ts'o *et al.*, 1963; for review, see Ts'o, 1968). Our research (Ts'o and Huang, 1968; Ts'o and Schweizer, 1968) on the interaction of nucleoside monophosphate and triphosphate (A and G) with the complementary polynucleotides (U and C) have confirmed the occurrence of this interaction (Howard *et al.*, 1964; Miles *et al.*, 1966). In addition, we have observed that the interaction is always accompanied by a phase transition. The polymer–polymer complexes are insoluble under these conditions. In the complex, mononucleotides become polymerlike. Since transition of physical state removes the complex into another phase of the system, it provides an additional driving force needed for the interaction to proceed. Similarly, in the process of nucleic acid synthesis, the nucleoside triphosphate substrates are continuously being polymerized into a polynucleotide at the catalytic site, and thus are removed from the dynamic equilibrium with the remaining nucleoside triphosphates in solution. In this respect, the polymerization process in an enzymic reaction may have a similar influence on the monomer–polymer interaction as the precipitation process in a physiocochemical reaction.

The highly cooperative nature of the monomer–polymer interaction indicates the importance of the nucleation process in the interaction (Huang and Ts'o, 1966). Therefore, we have investigated the interaction of the monomers with an oligomer–polymer complex, in which the polymer is in excess and provides the available sites for binding of monomers while the oligomer serves as an initiator in a nucleation process. However, before the interaction of monomers–oligomers–polymers can be investigated meaningfully, adequate knowledge must be obtained about the oligomer–polymer complex formed in the presence of excess polymer. The results in the first section show that for the present system only one type of $A(pA)_6$–poly U complex exists with the stoichiometry of 1A:2U regardless of the input ratio of A to U in solution. The formation of the $A(pA)_6 \cdot 2$poly U complex with the poly U in excess of 10–20-fold is not completely, but could be partially, cooperative. The results given earlier suggest that there is an average of two $A(pA)_6$ chains in a stack for an input oligo A:poly U ratio of 0.05 and there are three $A(pA)_6$ chains in a stack for an input oligo A:poly U ratio of 0.1.

The overall binding of adenosine to the remaining poly U appears to be enhanced slightly by the presence of $A(pA)_6$ (Figures 6 and 9). Moreover, a new site for the adenosine binding has been created in the $A(pA)_6$–poly U complex in the presence of excess poly U; 1 new site/$A(pA)_6$ chain is formed in oligo A:poly U complex of 0.05 ratio and about 0.6 new site/$A(pA)_6$ chain is formed in oligo A/poly U complex of 0.1 ratio as shown in Figure 7 and discussed above. The binding of adenosine to these new sites is also cooperative and is favored by about 0.8 kcal than the over-all binding of adenosine to the remaining sites of the poly U. The intrinsic free-energy change ($\Delta F°$ intrinsic) of the adenosine binding to the new site in the $A(pA)_6$–poly U complex at 5°, 0.4 M NaCl, is about 3.5 kcal. We propose that these new sites are located between two strands of poly U and at the end of the $A(pA)_6$, and are benefitted by the conformational restraint imposed by the

$A(pA)_6$ on the two poly U strands in the complex and by the stacking energy of the AMP residue at the end of the oligomer.

Knowledge of these new sites created in the oligomer–polymer complex is pertinent to our understanding of enzymic synthesis of nucleic acids. The importance of oligonucleotides which serve as primers in the enzymic process has been noted for DNA polymerase with single-stranded homopolymer template systems, such as poly dA plus oligo dT (Bollum *et al.*, 1964; Bollum, 1967; Jovin and Kornberg, 1968). In the reaction catalyzed by calf thymus DNA polymerase, oligodeoxyribonucleotides with a chain length of 6 or larger are required for initiation of the replication process on the complementary template chain. More recently, the requirement for oligodeoxyribonucleotide primers in the enzymic synthesis of the biologically active ϕX-174 DNA duplex circle from the single-stranded circular DNA has been clearly demonstrated for the *Escherichia coli* DNA polymerase system (Goulian, 1968). For *E. coli* RNA polymerase, Niyogi and Stevens (1965) demonstrated that with single-stranded poly A or poly U templates, the oligonucleotides complementary to the template enhance the incorporation rates and are incorporated into the product. A similar stimulatory effect by the complementary oligomer on the incorporation rate with the single-stranded template has been observed also with the *Micrococcus luteus* RNA polymerase system (Steck *et al.*, 1968; Straat and Ts'o, 1969). The present physicochemical studies on the monomer–oligomer–polymer complex clearly demonstrate that the binding site of substrate to the template can be enhanced by the presence of a primer in forming a primer–template complex. The primer can impose a restraint on the template, forcing it to assume a conformation more favorable for binding. Furthermore, the base moiety at the end of the primer can provide the stacking energy for the oncoming substrate. If the contribution of the enzyme is temporarily ignored, the energy difference between the binding of the substrate to the template, with and without the primer, represents the energy difference between the initiation step and the elongation step in the enzymic process. In the system presently studied, the binding of the adenosine to the new site in the $A(pA)_6$–poly U complex is favored by about 0.8 kcal over the binding of adenosine to the noninteracted poly U. It is interesting to note that the free-energy change in the association of adenosine to form stacks in aqueous solution is about 1 kcal. Though the apparent agreement between these two values may be fortuitous it is not unreasonable, since the binding of the adenosine to the end of the oligomer in the $A(pA)_6$–poly U should utilize the base-stacking energy more effectively than the binding process to the poly U in the absence of oligo A. It should be emphasized, however, unless the initiation step is the rate-limiting step in the enzymic process of polynucleotide synthesis, the stacking interaction will have little effect on the kinetics of the incorporation. Indeed, recent research from our laboratory has indicated that under certain conditions of homopolymer-directed polynucleotide synthesis "product release" rather than "chain initiation" may be the limiting step (Straat *et al.*, 1969; Straat and Ts'o, 1969). In this situation, the incorporation kinetics was found not to be influenced by the stacking interaction and the observed effect of the complementary oligomer on the incorporation kinetics could be due to functions of the oligomer other than serving as primer (Straat and Ts'o, 1969).

As mentioned above, the intrinsic free energy change of the binding adenosine to the new site in the $A(pA)_6$–poly U complex is about 3.3–3.5 kcal at 0.4 M NaCl, 5°. Without assistance from the enzyme, this value of 3.5 kcal is probably the upper limit of the free energy available for the binding of substrate to the growing chain in the polymerase reaction with a single-stranded template. The binding of ATP to such a site in an enzymatic reaction at 30–40° should have considerably less free-energy change, because of both the electrostatic repulsion of negative charges of the triphosphate group and the exothermic nature of the interaction. It is likely, therefore, that the enzyme in the polymerase-template-growing chain complex may make a significant contribution to the binding of the substrate to this complex during the polynucleotide synthesis.

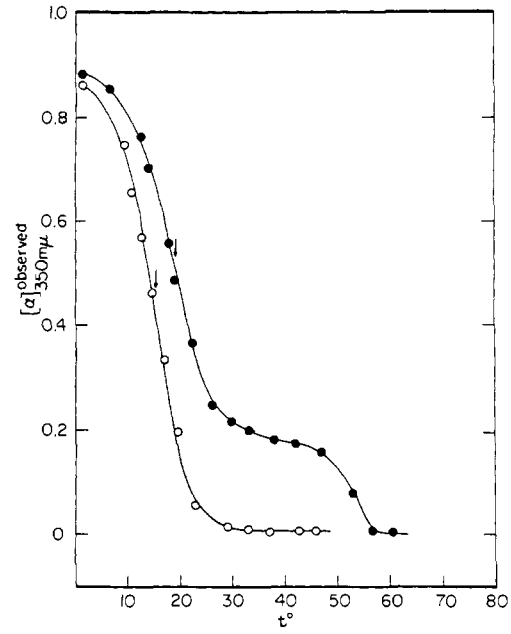

FIGURE 9: The thermal dissociation of adenosine–poly U complex and the adenosine–$A(pA)_6 \cdot 2$poly U complex in 0.4 M NaCl–0.01 M Tris (pH 7.5) measured by optical rotation at 350 mμ. (○-○) Adenosine–poly U complex and (●-●) adenosine–$(pA)_6 \cdot 2$ poly U complex.

Appendix

We wish to expand the formal approach previously formulated by Felsenfeld and Rich (1957) on the analysis of the mixing curve of the complementary polynucleotides as assayed by hypochromicity. In the previous approach, an unambiguous answer to the question of stoichiometry can be obtained only when both starting zones of the mixing curve have the same type of complex. In other words, unless there is only one common type of complex existing throughout the mixing curve, the stoichiometry of the complex cannot be determined. A general expression is developed here which allows the evaluation of the intersection in the mixing curve even though the complex in each starting zone may have different stoichiometry. The intersection can be obtained either from the actual measurement or from the extrapolation of data obtained at the two starting zones. The implicit assumptions in the formulation are: (1) the interaction essentially reaches completion; (2) the interaction is reversible to allow maximal pairing (Felsenfeld, 1958); (3) there exists only one type of

complex at each starting zone and these complexes may have different stoichiometry. If both types of complexes can coexist without conversion into each other at or near the intersection, then the points from the starting zones to the intersection would be in a straight line. On the other hand, if there is extensive conversion between these complexes as governed by their relative stabilities, then the data points from the starting zones to the experimental interaction would not be in a straight line. In this situation, the calculated intersection from these equations should correspond to that obtained from the straight-line extrapolation of data points near the origins of both starting zones. The symbols and definitions are essentially the same as those used by Felsenfeld and Rich (1957), which are for the system of poly A–poly U interaction. These symbols are defined as follows: $a \equiv$ total sum of poly A and poly U in solution, an invariant quantity; $Q_1 \equiv$ molar extinction coefficient (or optical density per unit nucleotide) of poly A; $Q_2 \equiv$ molar extinction coefficient of poly U; $Q_3 \equiv$ molar extinction coefficient of (poly $A \cdot m$poly U) at the starting region of poly A excess; $Q_4 \equiv$ molar extinction coefficient of poly $A \cdot n$poly U at the starting region of poly U excess; $U \equiv$ the total amount of poly U in the solution (in all forms).

At the region of excess poly U, addition of poly A will replace an equal amount of poly U and will form an equal amount of (poly $A \cdot n$poly U) complex. Thus, the total optical density contributed by all the components, D, is given as

$$D = [Q_3 - (n + 1)Q_2](a - U) + Q_2a$$

or

$$\frac{D}{a} = [Q_3 - (n + 1)Q_2]\left(1 - \frac{U}{a}\right) + Q_2$$

At the region of excess poly A, addition of poly U will replace an equal amount of poly A and will form an equal amount of (poly $A \cdot m$poly U) complex. Thus, the optical density, D, is given as

$$D' = \left[\frac{Q_4}{m} - \left(1 + \frac{1}{m}\right)Q_1\right]U + Q_1a$$

or

$$\frac{D'}{a} = \left[\frac{Q_4}{m} - \left(1 + \frac{1}{m}\right)Q_1\right]\frac{U}{a} + Q_1$$

At the intersection, $D/a = D'/a$, then the location of the intersection, U/a, is given by

$$\frac{U}{a} = \frac{Q_3 - nQ_2 - Q_1}{\frac{Q_4}{m} + Q_3 - (n + 1)Q_2 - \left(1 + \frac{1}{m}\right)Q_1} \quad (1)$$

If there exists one type of complex at both starting zones, then the stoichiometry may be obtained without the knowledge of the extinction coefficients.

For instance, let $m = n = 1$, $U/a = 1/2$; and let $m = n = 2$, $U/a = 2/3$. If the complex at each starting zone has a different stoichiometry, for instance, for $m = 1$ and $n = 2$

$$\frac{U}{a} = \frac{Q_3 - 2Q_2 - Q_1}{Q_4 - 2Q_1 + Q_3 - 3Q_2} \quad (2)$$

For $m = 2$, and $n = 1$

$$\frac{U}{a} = \frac{Q_3 - Q_2 - Q_1}{\frac{Q_4}{2} - \frac{3}{2}Q_1 - Q_3 - 2Q_2} \quad (3)$$

For the present experimental system of $A(pA)_6$ and poly U interaction, the following quantities were used in the calculation: $Q_1 = 11 \times 10^3$, $Q_2 = 9.2 \times 10^3$, $Q_{(A+U)} = 7.0 \pm 0.1$, and $Q_{(A+2U)} = 6.2 \pm 0.1$ (Felsenfeld and Rich, 1957; Blake *et al.*, 1967). With these quantities, the U/a from eq 2 was calculated to be 0.34, and from eq 3 was 0.56. These calculated results are substantially different from the observed intersection of 0.68 ± 0.02 (Figure 2) and, therefore, do not indicate that a different type of complex exists at each starting zone.

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Further Studies of the Molecular Weight in Aqueous Solution of the Low-Sulfur Wool Protein Component 8 and a Reinterpretation of Previous Results*

P. D. Jeffrey

ABSTRACT: In a previous sedimentation equilibrium study in 0.01 M sodium tetraborate it was found that at low concentration the measured molecular weight of the reduced and alkylated low-sulfur wool protein, component 8, increased with increasing protein concentration. The variation of molecular weight was satisfactorily explained as the consequence of a rapid reversible equilibrium between a monomer of mol wt 23,000, dimer, and trimer. The present paper gives the results of frontal analysis gel filtration on Sephadex G-200 and osmotic pressure experiments which show that component 8 is almost certainly *not* involved in such an equilibrium in 0.01 M sodium borate. These results together with those from more high-speed sedimentation equilibrium experiments and the use of two-species plots indicate that the component 8 used in these studies is a mixture of more than 95% of a protein of mol wt 45,000 and less than 5% of one of mol wt 14,000. Calculations using these figures show that the results of the high-speed sedimentation equilibrium experiments can indeed be explained on this basis and it is concluded that the molecular weight of component 8 is 45,000.

In a previous paper (Jeffrey, 1968) I presented the results of a sedimentation equilibrium study in 0.01 M sodium tetraborate of the reduced and alkylated low-sulfur wool protein termed component 8. The molecular weight *vs.* concentration data were explained in terms of thermodynamic nonideality and a rapid, reversible equilibrium between a monomer of mol wt 23,000 and the dimer and trimer of this unit. The present paper gives the results of an extensive investigation of component 8 in the same solvent by gel filtration, sedimentation equilibrium and osmometry, and shows that the protein is almost certainly not involved in such an equilibrium and that the results presented in the previous paper can be explained in a different way.

Gel filtration on Sephadex was used both as a method of fractionation and as a technique for examining an interacting system by the method of frontal analysis. It was the latter application which originally indicated that component 8 was not an associating system in sodium tetraborate solution and made it necessary to carry out more sedimentation equilibrium experiments and to reinterpret the results obtained before. A determination of the molecular weight of component 8 by osmotic pressure also reported in this paper provides additional support for the explanation of the high-speed sedimentation equilibrium results advanced here.

Materials

Buffer. The buffer salt was analytical reagent grade sodium tetraborate and 0.01 M solutions were made with glass-distilled water. The pH was measured and if necessary adjusted to pH 9.20.

Component 8. The component 8 used in the experiments reported here came from three different preparations. In all of these the component 8 was fractionated from the soluble low-

* From the Division of Protein Chemistry, CSIRO, Parkville (Melbourne), Victoria 3052, Australia. *Received June 16, 1969.*