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Physicochemical Basis of the Recognition Process in Nucleic Acid Interactions. II. Interactions of Polyuridylic Acid and Polycytidylic Acid with Nucleoside Mono- and Triphosphates*

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ABSTRACT: Insoluble complexes of adenine nucleotides (AMP, dAMP, ATP, and dATP) and polyuridylic acid as well as guanine nucleotides (GMP, dGMP, and GTP) and polycytidylic acid are formed reversibly when the concentrations of the polynucleotides and especially of the nucleotides are sufficiently high and the temperature is sufficiently low. At moderate concentration of the interactants (0.01–0.02 M), Mg^{2+} ion is usually required for the interaction. These insoluble complexes have a definite stoichiometry, generally 2U to 1A or 1C to 1G and, in one case, 2C to 1G was found. The base-pairing specificity is the same as that of the polynucleotide interactions. On the other hand, a soluble complex between the mononucleotides and their complementary polynucleotides cannot be detected by optical rotatory dispersion, sedimentation, and viscosity, techniques which were employed successfully before to characterize the adenosine–$(U)_n$ system. These data indicate that interaction in these mixtures only takes place when accompanied by a phase transition, such as precipitation or gelation. The following mechanism is proposed. When the conditions are sufficiently favorable, the monomer–polymer interaction occurs through hydrogen bonding between the base pairs and through the cooperative stacking of the mononucleotides along side the complementary polynucleotides. In the complex, mononucleotides become polymerlike. Thus, a phase transition occurs, since the polymer–polymer complexes are insoluble under these conditions. This transition of physical state provides the additional driving force needed for the interaction to proceed by removing the complex into another phase of the system.

For the past decade our laboratory has been actively engaged in studies concerning the physicochemical basis of nucleic acid conformation and interaction. A considerable amount of work has been done on the problem of monomer–monomer interaction of the bases, nucleosides, and nucleotides in aqueous solution, from our laboratory (Ts'o *et al.*, 1963; Ts'o and Chan, 1964; Chan *et al.*, 1964; Schweizer *et al.*, 1965, 1968; Broom *et al.*, 1967) and from others (see Discussion). The first paper of this series concerns the problem of nucleosides' interaction with polynucleotides (Huang and Ts'o, 1966a). This problem was also investigated independently with different emphasis in laboratories of National Institutes of Health (Howard *et al.*, 1966; Maxwell *et al.*, 1966).

The interaction of nucleoside monophosphates with the polynucleotides was first investigated by Howard *et al.* (1964) using the technique of infrared spectroscopy. Definitive information about the interaction between guanosine mononucleotides and $(C)_n$ was obtained. These authors then extended their investigation to the problem of guanosine triphosphate interaction with $(C)_n$ and adenosine triphosphate interaction with $(U)_n$ (Miles *et al.*, 1966). In our laboratory, the research on the interaction of nucleotides and nucleoside triphosphates

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with polynucleotides was initiated independently in early 1965 and part of the results have been presented previously (Huang and Ts'o, 1966b).

In this paper, we report the observation that the interaction between the nucleoside mono- and triphosphates and their complementary polynucleotides always takes place with a concomitant change of the physical state of the solution such as precipitation or gelation. Soluble complexes of the monomer and the polymer cannot be detected by a variety of techniques which were employed successfully to study the system adenosine–$(U)_n$. The variables governing the formation of this insoluble complex such as temperature, pH, and concentrations of the mononucleotides, polynucleotides, and Mg^{2+} ions are described. These insoluble complexes have a definite stoichiometry and have the same specificity as the polynucleotides interaction.

The investigation of these systems by the technique of proton magnetic resonance is reported in the following paper (Ts'o and Schweizer, 1968).

Materials and Methods

Polynucleotides. $(U)_n$ and $(C)_n$ were purchased from the Miles Laboratories, Elkhart, Ind. They were extensively dialyzed before use in either HMP[1] or 0.01 M Tris (pH 7–7.5) buffer until no ultraviolet-absorbing material was detected in the dialysate. [^{3}H]$(U)_n$ was prepared enzymatically from [^{3}H]UDP with polynucleotide phosphorylase extracted from *Micrococcus lysodeikticus* according to the method described by Steiner and Beers (1961).

Nucleosides and Nucleotides. Commercially available nucleoside mono- and triphosphates were first checked by paper chromatography for possible contamination, and were used directly without further purification. [^{3}H]UDP and [^{14}C]AMP were purchased from Schwarz BioResearch, Inc.

Analytical Velocity Sedimentation, Gradient Sedimentation, Viscosity, Ultraviolet Absorbance Measurement, Optical Rotations, and Determination of Radioactivity. All of these physical methods were described in our previous paper (Huang and Ts'o, 1966a). The following instruments were used: Spinco analytical ultracentrifuge, Model E; Spinco preparative ultracentrifuge, Model L 2; Ubbelohde viscometer in a constant-temperature bath; Cary 14 or Cary 15 spectrophotometers; Cary 60 recording spectropolarimeter; Tri-Carb (Packard Instrument Co.) and Nuclear-Chicago Model 722 liquid scintillation counters.

Determination of A/U Ratio. When the pH of the solution is changed from 7 to 12, the molar absorbance at 259 mμ of uridine nucleotide decreases drastically, *e.g.*, 33% for $(U)_n$, due to ionization, while that of the adenine residue remains unchanged. By measuring solutions containing known ratios of AMP (same for ATP) to $(U)_n$, a standard curve relating A/U ratio to r was constructed. Here r is defined as (absorbance at pH 7 − absorbance at pH 12)/absorbance at pH 12. This standard curve is most useful at the region of A/U ratios from 0 to about 1.1 (the range of the present experiments) and is not very sensitive at A/U ratios above 1.5.

Determination of C/G Ratio. The spectrum of $(C)_n$ in 0.01 M Tris (pH 7.0) (Helmkamp and Ts'o, 1962) showed a characteristic ratio at 270–250 mμ of 1.35, while the value of this ratio for GMP or GTP is 0.7. Thus, the C/G ratio of a solution containing $(C)_n$ and guanosine nucleotides can be determined by the formula $C/G = (13.7K - 9.65)/(6.6 - 4.9K)$, where K is the ratio of the absorbance at 270 and 250 mμ, 6.6 and 4.9 $\times 10^3$ are the molar extinction coefficients of $(C)_n$ at 270 and 250 mμ, respectively, and 9.65 and 13.7 $\times 10^3$ are the molar extinction coefficients of guanosine nucleotides at the corresponding wavelengths. It should be noted that, as it will be shown later, at the level of concentration less than 1 OD and in the absence of divalent ion, there is no interaction between the mononucleotides and the complementary polynucleotides. The change of C/G ratio from 1 to 2 corresponds to a change of K value from 0.875 to 0.966. Therefore, an effort has been made to ensure that the measurements of K values were accurate to the second decimal place.

Results

Interaction of $(U)_n$ and AMP. THE FORMATION OF $(U)_n$–AMP INSOLUBLE COMPLEX. When $(U)_n$ and AMP of moderate concentration are mixed in the presence of Mg^{2+} at low temperature, precipitation occurs. Such precipitation is not observed when the same concentration of Mg^{2+} is added to either AMP or $(U)_n$ alone at the same temperature. The formation of the insoluble complex of AMP and $(U)_n$ has a definite stoichiometry and is critically dependent upon temperature, pH, and the concentrations of AMP and Mg^{2+} for a given concentration of $(U)_n$.

Specificity. The interactions of $(U)_n$ and various nucleotides in the presence of Mg^{2+} at 0° have been studied (Table I). The results show that all the adenosine monophosphates, regardless of the position of the phosphate attachment, are able to form an insoluble complex with poly U at a stoichiometric ratio of 2U to 1A. But the requirement of the $MgCl_2$ concentration differs, with 2′-AMP requiring the highest, then 3′-AMP, and 5′-AMP requiring the least. The concentration dependence of the chemical shifts of these nucleotides as studied by proton magnetic resonance indicates that the molecules associate in aqueous solution to form vertical stacks; the extent of the self-association can be ordered as 5′-AMP > 3′-AMP > 2′-AMP (Schweizer *et al.*, 1968). This observation is interpreted as the result of an adverse effect on the stacking of the adenine ring exerted by the negatively charged phosphate group. This adverse effect is a function of the distance between the adenine ring and the phosphate. The shorter the distance, the less the compound stacks. Since the formation of insoluble complexes of $(U)_n$ and AMP in Mg^{2+}, as considered in the Discussion, is due to the stacking of AMP along $(U)_n$, the primary function of Mg^{2+} ions is to reduce the electrostatic repulsion for the stacking and the

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: HMP, 0.0025 M Na_2HPO_4, 0.005 M NaH_2PO_4, and 0.001 M EDTA.

TABLE I: Specificity of the Interaction of $(U)_n$ (0.01 M) and Nucleotides at 0°, pH 5.7–6.5.

	M	$MgCl_2$ (M)	Precipitation: Occurrence	Precipitation: A/U
3′-AMP	0.02	0.03	−	
	0.04	0.03	+	0.53
2′-AMP	0.02	0.03	−	
	0.02	0.05	−	
	0.02	0.07	+	0.56
5′-AMP	0.02	0.02	+	0.50
5′-TuMP	0.02	0.03	−	
	0.02	0.07	−	
5′-CMP	0.04	0.05	−	
5′-IMP	0.04	0.05	−	
5′-GMP	0.04	0.05	+	No $(U)_n$
5′-ATP[a]	0.02	0.04	+	0.52
5′-CTP	0.02	0.05	−	
5′-ITP	0.02	0.05	−	

[a] pH 8.5.

TABLE II: The Stoichiometry of $(U)_n$–AMP Insoluble Complex.[a]

$(U)_n$ (M)	5′-AMP (M)	$MgCl_2$ (M)	A/U: Supernatant	A/U: Precipitate
0.01	0.02	0.03	∞[b]	0.50
0.01	0.04	0.05	∞	0.50
0.01	0.06	0.07	∞	0.58
0.005	0.06	0.08	∞	0.50
0.010	0.06	0.08	∞	0.48
0.016	0 06	0.08	∞	0.58
0.021	0.06	0.08	∞	0.67
0.02	0.01	0.06	0.6	0.55

[a] Experiments were done in 0.01 M Tris (pH 7.5) at 0°. [b] Within experimental error, infinity of A/U ratio means the solution contains only A and no U.

binding to occur. More Mg^{2+} ions will be required for the interaction of a system having less stacked species. This is indeed observed in the complex formation between $(U)_n$ and these three different AMP's. The Mg^{2+} concentration requirement for the precipitation (2′-AMP > 3′-AMP > 5′-AMP; Table I) increases in accordance with the decrease in the extent of self-association of these nucleotides.

The failure of tubercidin monophosphate (7-deaza-AMP) to form an insoluble complex with $(U)_n$ (Table I) indicates the involvement of the N_7 position of the adenine ring in the interaction. This is expected in view of the failure of $(U)_n$ to form a soluble complex with tubercidin as reported in our previous publication (Huang and Ts'o, 1966a). 5′-CMP, 5′-IMP, and 5′-GMP all failed to form insoluble complexes with $(U)_n$ (Table I). Thus, it is indicated that insoluble complex formation is specific in base pairing.

Stoichiometry. The stoichiometry of the $(U)_n$–AMP insoluble complex was examined by analyzing the A/U ratio of the precipitate and the supernatant at varying input ratios of $(U)_n$ to 5′-AMP. When the concentration of $(U)_n$ was held constant at 0.01 M, varying concentrations of 5′-AMP (0.02–0.06 M) with comparable amounts of Mg^{2+} caused a complete precipitation of $(U)_n$. Within experimental error, the A/U ratio of the precipitate is invariably 0.5 (Table II). The excess AMP remained in the supernatant. On the other hand, when the concentrations of AMP and Mg^{2+} were held at 0.06 and 0.08 M, respectively, and varying concentrations of $(U)_n$ (0.005–0.02 M) added, similar results were again obtained, *i.e.*, the stoichiometry is 2U to 1A for the insoluble complex (Table II). In all the above cases, AMP was present in excess amounts. When stoichiometric amounts of $(U)_n$ (0.02 M) and AMP (0.01 M) were mixed in the presence of sufficient $MgCl_2$ at 0°, the precipitate and the supernatant both had a ratio of 2U to 1A (Table II). These results show that the interaction of $(U)_n$ and AMP is driven mainly by the chemical potential of the monomers, similar to the $(U)_n$–adenosine system. A certain amount of free AMP will always be present as a result of the equilibrium established between the free and the bound nucleotides.

The completeness of the $(U)_n$ interaction with sufficient AMP and Mg^{2+} was also confirmed by using radioactive compounds. [^{3}H]$(U)_n$ (0.01 M) and [^{14}C]5′-AMP (0.02 M) in $MgCl_2$ (0.04 M) at pH 6 and 0° formed an insoluble complex containing 95% of the ^{3}H counts, while 75% of the ^{14}C counts (and ^{14}C alone) was found in the supernatant.

pH Dependence. The formation of the insoluble complex is critically dependent on pH. In a mixture of $(U)_n$ (0.01 M), 5′-AMP (0.02 M), and Mg^{2+} (0.03 M) the formation of the insoluble complex at pH values above 5.2 is visible at 0°. As the pH is lowered, the precipitate redissolves. As shown in Figure 1, the critical pH at which this change of state occurs is governed by the concentration of $MgCl_2$. The higher Mg^{2+} concentration, the lower the critical pH becomes. These results are best explained on the basis that AMP is being protonated at the purine ring below the critical pH; hence it is unable to form hydrogen bonds with $(U)_n$. The pK'_a's for 5′-AMP and $(A)_n$ are about 3.6 and 6, respectively, in 0.1 M salt. The value for $(A)_n$ can be lowered when the ionic strength is increased (Steiner and Beers, 1961). This *increase* in the pK_a of AMP cannot be due to base-pairing formation which leads to the *lowering* of pK_a instead. The ease of protonation of a molecule is reduced when this molecule has been hydrogen bonded already. One supporting evidence for this notion is the well-known irreversible titration of the native DNA (Cox and Peacocke, 1956, 1957; Cavalieri and Rosenberg, 1957). At the acidic side, the pK_a of the titratable groups in the native DNA is lower than that of the denatured

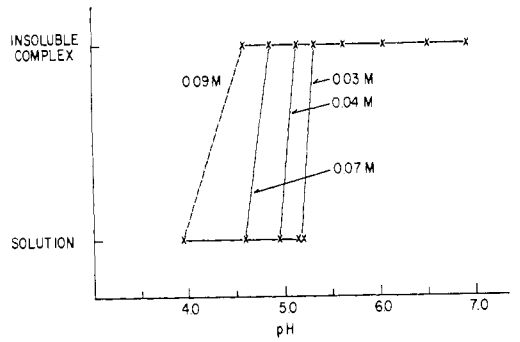

FIGURE 1: The pH dependence of the formation of $(U)_n$–AMP insoluble complex. $(U)_n$ (0.01 M) and 5′-AMP (0.02 M) were mixed with $MgCl_2$ at the indicated concentration. The pH was adjusted by the addition of HCl. At each pH interval the sample was allowed to stand at 0° for about 20 min. The appearance of the white precipitate indicated the formation of insoluble complex. The exact pH for the solution of the precipitate in 0.09 M Mg^{2+} was less certain and was, therefore, indicated by a broken line.

DNA. Therefore, the most reasonable interpretation for the present data is that the bound 5′-AMP may acquire a polymerlike property due to its stacking along $(U)_n$, for the observed value of the critical pH is near the pK'_a of $(A)_n$ and is much higher than that of the monomeric 5′-AMP.

Thermostability. The insoluble complex of $(U)_n$ and 5′-AMP can be dissociated by elevation of the temperature. These dissociation (melting) and association (precipitating) processes can be followed conveniently by the disappearance and reappearance of the white precipitate as the temperature is increased or decreased. Figure 2 shows a melting profile of a sample consisting of $(U)_n$ (0.01 M), 5′-AMP (0.01 M), and $MgCl_2$ (0.04 M) at pH 5.8. The temperature at the midpoint of the two states is designated as the precipitation temperature, T_p. An hysteresis of about 4° is observed for the T_p's of the forward dissociation curve and the reverse association curve.

At a given concentration of $MgCl_2$ (0.13 M) and $(U)_n$ (0.01 M), T_p increases with increasing concentrations of

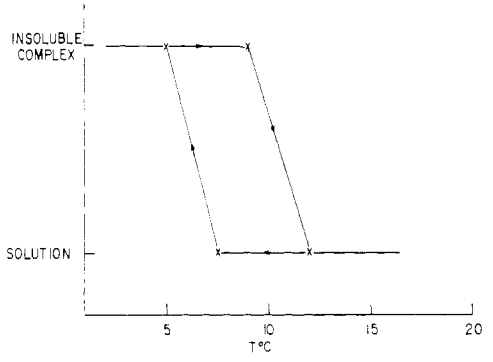

FIGURE 2: The melting profile of an insoluble complex of $(U)_n$ (0.01 M), 5′-AMP (0.01 M), and $MgCl_2$ (0.04 M) at pH 5.8 as determined visually. The temperature at which the melting began and ended was recorded for the forward dissociation as well as the reverse association reactions. The arrow designates the direction of the temperature changes.

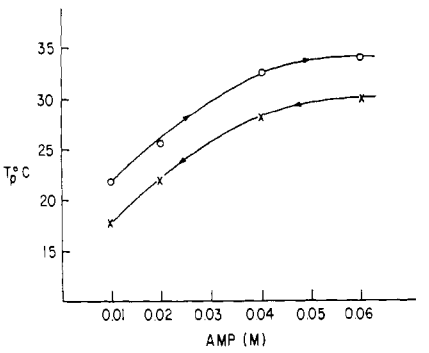

FIGURE 3: The dependence of T_p upon the 5′-AMP concentration at pH 6. The concentration of $(U)_n$ was invariably 0.01 M and that of $MgCl_2$ was 0.13 M. The upper curve is for the dissociation reaction and the lower one is for the association reaction.

5′-AMP until it reaches a plateau level at about 0.04–0.06 M of AMP (Figure 3). The T_p value is also dependent upon the Mg^{2+} concentration for a given $(U)_n$ and 5′-AMP mixture as shown in Figure 4. T_p's at all three levels of AMP concentration increase in response to the increasing concentration of $MgCl_2$.

THE SOLUTION PROPERTIES OF THE MIXTURE OF $(U)_n$ AND AMP. As shown in the above section, when the concentration of AMP or Mg^{2+} is not sufficiently high, or the temperature is not sufficiently low, no precipitation occurs in the mixed solution containing the polynucleotide and the monomer.

Possible interaction between $(U)_n$ and 5′-AMP in forming a soluble complex in solution was studied by the same hydrodynamic and spectrometric methods previously used to characterize the $(U)_n$–adenosine interacting system (Huang and Ts'o, 1966a). The interpretation of the present data, however, is complicated by the strong interaction of the added 5′-AMP with the Mg^{2+}, since the Mg^{2+} also has a large effect on the physical properties of the polynucleotides. This situation did not occur in our former studies on the nucleosides which did not interact with the divalent ions.

Sedimentation and Viscosity Measurements. The sedimentation coefficient and the specific viscosity of var-

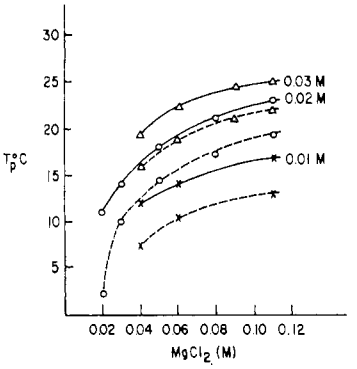

FIGURE 4: The dependence of T_p upon $MgCl_2$ concentration. The concentration of $(U)_n$ was 0.01 M and that of 5′-AMP as indicated. The curves in broken lines are for the association reactions while those in solid lines are for the dissociation reactions.

TABLE III: Sedimentation and Viscosity Measurements of $(U)_n$ and AMP Mixture in Mg^{2+} (pH 7) and 3.5°.

$(U)_n$	5′-AMP (M)	$MgCl_2$ (M)	$s_{3.5}$ (S)	η_{sp}
0.01	0	0.01	3.4	0.232
0.01	0	0.02	4.5	0.123
0.01	0.01	0	2.3	
0.01	0.01	0.01	3.3	
0.01	0.01	0.02	4.5	0.154

ious mixtures of $(U)_n$, AMP, and Mg^{2+} were determined at 3.5° (Table III). When $(U)_n$ (0.01 M) was sedimented in different $MgCl_2$ concentrations, the s value increased with increasing Mg^{2+} concentration. Furthermore, the schlieren pattern was progressively more diffuse at higher concentrations of Mg^{2+}. On the other hand, concurrently the specific viscosity of $(U)_n$ became lower. The addition of AMP (0.01 M) to the solution of $(U)_n$ (0.01 M) containing 0.01 or 0.02 M $MgCl_2$ had no effect on the sedimentation property of $(U)_n$. This is in contrast with the 37% increase in s value resulting from the presence of adenosine in a $(U)_n$ solution (Huang and Ts'o, 1966a). A small increase in specific viscosity (from 0.123 to 0.154) was observed when AMP was added to the poly U solution containing 0.02 M $MgCl_2$. This is again in contrast with the 85% increase in viscosity upon addition of adenosine (adenosine–$(U)_n$ interaction was studied in 0.4 M NaCl at 5°; Huang and Ts'o, 1966a). The competition of AMP for the Mg^{2+} ions may account for the small increase in specific viscosity observed, since this value (0.154) is intermediate between those of the $(U)_n$ solution containing 0.01 and 0.02 M Mg^{2+}. Thus, the ultracentrifugation and viscosity data provide no indication that $(U)_n$ and AMP interact in solution to an extent detectable by these techniques under these conditions.

Gradient Centrifugation. A mixture of [^{3}H]$(U)_n$ and [^{14}C]5′-AMP (0.01 M each) in 0.02 M $MgCl_2$ was sedimented in a 5–20% sucrose gradient at 7°. The rate and the profile of the $(U)_n$ sedimentation were essentially unchanged in the presence or absence of AMP (0.005 M) in the medium. They were again the same as those obtained in a control experiment in which [^{3}H]$(U)_n$ alone was similarly centrifuged. These results contrast to those obtained for the $(U)_n$–adenosine system (Huang and Ts'o, 1966a), and further indicate that $(U)_n$ and 5′-AMP do not interact in solution under these conditions.

Optical Rotation Measurements. The optical rotation at 350 mμ of the $(U)_n$ and 5′-AMP mixture (0.01 M each) in 0.02 M $MgCl_2$ (pH 6.5) was determined at varying temperatures from 0.5 to 26°. Since the conformation and, hence, the rotation of $(U)_n$ is very sensitive to Mg^{2+} ions, the effect of the phosphate of the added nucleotide in complexing with Mg^{2+} must be compensated. For this reason, a mixture of 5′-IMP and $(U)_n$ under identical conditions was similarly studied as a control for the noninteracting case. This is justified, since inosine does not form a soluble complex with $(U)_n$ (Huang and Ts'o, 1966a) nor does 5′-IMP form an insoluble complex with $(U)_n$ as reported in Table I. The rotation *vs.* temperature profile of the two mixtures, 5′-AMP with $(U)_n$ and 5′-IMP with $(U)_n$, *were identical.* The failure of AMP to promote ordered structure formation of $(U)_n$ was again taken to mean that $(U)_n$ does not interact with 5′-AMP in solution.

Similar measurements were also performed using 0.4 M NaCl (pH 7.4) in an equal molar mixture of $(U)_n$ and 5′-AMP (0.015 M). Again, the rotation *vs.* temperature profile of the mixture was essentially the same as that of the $(U)_n$ alone in 0.4 M NaCl. Under these identical conditions, interaction of adenosine with $(U)_n$ causes a very significant increase in rotation (Huang and Ts'o, 1966a).

Interaction of $(U)_n$ and ATP or dATP. Since the interaction of $(U)_n$, AMP, and Mg^{2+} cannot be demonstrated in solution, no further attempt was made to search for a soluble complex of $(U)_n$ and ATP in Mg^{2+}. However, the formation of a specific insoluble complex of $(U)_n$ and ATP in a mixture containing a sufficient concentration of Mg^{2+} was readily demonstrated in Table I.

In Table IV, some characteristics of the insoluble complex of $(U)_n$ and ATP or dATP are listed. Reported also are those of the complex of $(U)_n$ with 5′-AMP or 5′-dAMP for comparison. It is noted the T_p of the insoluble complex is lower while the requirement of $MgCl_2$ is higher for the nucleoside triphosphates as compared with those for the monophosphates. Furthermore, the deoxynucleotides form more stable complexes with $(U)_n$ as compared with their ribose counterparts. This is in contrast to the $(U)_n$–adenosine system, in which the T_m of the $(U)_n$–adenosine complex is the same as that of the corresponding $(U)_n$–deoxyadenosine complex as determined by optical rotation (Huang and Ts'o, 1966a). Moreover, $(A)_n \cdot (U)_n$ gives a higher T_m than $d(A)_n \cdot (U)_n$ (Riley *et al.*, 1966; Ts'o *et al.*, 1966). At present, no definite explanation can be assessed. Additional information concerning the comparative binding of ribo- and deoxyribonucleotides to Mg^{2+} seems highly desirable.

Interaction of $(C)_n$ with 5′-GMP or 5′-dGMP. FORMATION OF $(C)_n$–GMP INSOLUBLE COMPLEX. As shown in Table VA, when a sufficient amount of Mg^{2+} (0.04–0.05 M) is present in the mixture of 0.01 M $(C)_n$ and 0.01 M GMP, precipitation occurs at 0°. The precipitate has a definite C/G ratio of 1:1, regardless of the concentration of the Mg^{2+} present. The same stoichiometry was obtained also when the input ratio of $(C)_n$ and GMP was 2:1 instead of 1:1. No precipitation was observed when $(C)_n$ or GMP was present alone with the Mg^{2+}. Therefore, the precipitate formation with a definite stoichiometry is a result of interaction between $(C)_n$ and GMP in the presence of Mg^{2+}.

In 0.2 M sodium cacodylate (pH 7.0) heavy precipitation also occurs at 0° or even at room temperature when the concentrations of $(C)_n$ and 5′-GMP are sufficiently high (0.05–0.1 M), as shown in Table VB. Instead of precipitation, gel formation was observed when 5′-dGMP was added instead of 5′-GMP (Table VB). These are definite indications of interaction between the $(C)_n$

TABLE IV: Formation of Insoluble Complex of Adenosine Mono- and Triphosphates with $(U)_n$ (0.01 M).

Nucleotide (0.02 M)	$MgCl_2$ (M)	pH	T_p (°C) Dissocn	T_p (°C) Assocn	A/U Ppt
5′-AMP	0.03	7.0	17	12.5	0.46
5′-dAMP	0.03	7.0	24.5	20.5	0.53
5′-ATP	0.04	8.4	7	2	
	0.05	8.4	10	5	0.52
5′-dATP	0.04	8.4	15	10	
	0.05	8.4	18	13.5	0.57

TABLE V: Formation of Insoluble Complex of 5′-GMP or 5′-dGMP with $(C)_n$ at 0°.[a]

Concentrations (M)			Precipitate Formation: C/G Ratio		Precipitate Formation: Distribution (%) of $OD_{250\ m\mu}$	
$(C)_n$	GMP	Mg^{2+}	Precipitate	Supernatant	Precipitate	Supernatant
		A. In Mg^{2+} Solution (pH 7.1), 0.01 M Tris Buffer				
0.01	0.01	0.02		1.0		100
0.01	0.01	0.03		1.0		100
0.01	0.01	0.04	0.99	0.98	20	80
0.01	0.01	0.05	0.98	1.05	60	40
	0.01	0.04		0		100
0.01		0.04		∞		100
0.02[b]	0.01	0.08	1.05	3.60	40	60
0.02		0.08		∞		100
	0.01[b]	0.08		0		100

Concentrations (M)			
$(C)_n$	GMP	dGMP	Observations
		B. In 0.2 M Sodium Cacodylate (pH 7.0)	
0.05	0.05		Precipitation
0.1[c]	0.05[c]		Precipitation
0.1	0.1		Precipitation at room temperature and 0°
0.1[c]		0.05[c]	Very viscous gel
0.1[c]		0.10[c]	Completely immobilized gel
		0.05	Not a very viscous solution
0.1			Soluble and not viscous
	0.1		Viscous at 0° but not at room temperature

[a] No precipitation of all the mixtures listed in this table took place at room temperature, except the mixture of $(C)_n$ and 5′-GMP, 0.1 M each. [b] The experiment with 5′-dGMP gave essentially the same results: no precipitation for the 5′-dGMP in 0.08 M Mg^{2+} and the C/G ratio of the precipitate is near unity in the mixture of 0.02 M $(C)_n$, 0.01 M dGMP, and 0.08 M Mg^{2+}. Precipitates in the 5′-dGMP mixture, however, were more difficult to separate from the supernatant after centrifugation. [c] These conditions are essentially those specified by Howard *et al.* (1964) for the infrared study in D_2O, pD 7.0–7.8.

and the guanosine monophosphates. The conditions employed in Table VB were adapted from the previous study of Howard *et al.* (1964), since they have found evidence of interaction by using infrared techniques. These authors did not indicate the physical state of their solutions in D_2O. Data in Table VB support their finding of interaction under these conditions.

THE SOLUTION PROPERTIES OF THE MIXTURE OF $(C)_n$ AND GMP OR dGMP. Owing to the observations reported in Table V, attempts were made to search for a

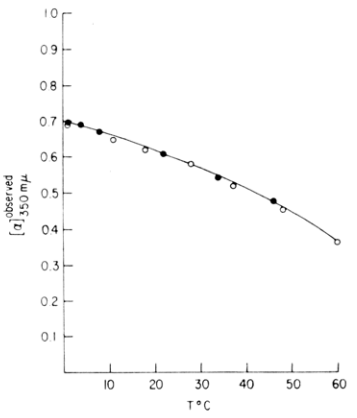

FIGURE 5: The observed rotation at 350 mμ of a mixture of $(C)_n$ (0.01 M) and 5′-GMP (0.01 M) in 0.2 M NaCl, —○—○—, and $(C)_n$ (0.01 M) with 5′-dGMP (0.01 M) in 0.4 M NaCl, —●—●—, at pH 7.

soluble complex between $(C)_n$ and 5′-GMP (or dGMP) at lower concentrations (0.01 M instead of 0.05 M) in NaCl (0.2–0.4 M) by the use of polarimetric and ultracentrifugation measurements.

The optical rotations at 350 mμ were determined at varying temperatures for two mixtures: $(C)_n$ (0.01 M) with 5′-GMP (0.01 M) in 0.2 M NaCl and $(C)_n$ (0.01 M) with 5′-dGMP (0.01 M) in 0.4 M NaCl (pH 7). The melting profiles for the $(C)_n$ in these two mixtures are identical, broad and without definitive transition (Figure 5). The absence of a sharp transition and the system's relative insensitivity to ionic strength indicate that $(C)_n$ and GMP (or dGMP) may not be interacting with each other under these conditions.

Optical rotatory dispersion curves from 310 to 230 mμ were determined with the mixtures of $(C)_n$ (0.01 M), dGMP (at 0.01 and 0.02 M), and NaCl (0.4 M) at 0 and at 27° with a 1-mm cell. These two curves both showed a single Cotton effect with a peak at 293 mμ and a trough at 265 mμ. These spectral positions are identical with those of the $(C)_n$ (Fasman *et al.*, 1964; Sarkar and Yang, 1965; Ts'o *et al.*, 1966). On the other hand, when $(C)_n$ interacts with $(G)_n$ to form a 1:1 complex, the peak and the trough of the optical rotatory dispersion curve are shifted to 275 and 244 mμ, respectively (Sarkar and Yang, 1965). Although it is not certain whether the optical rotatory dispersion curve of $(C)_n \cdot d(G)_n$ will be similar to that of the $(C)_n(G)_n$ or not (the optical rotatory dispersion curve of $d(A)_n \cdot (U)_n$ is not similar to that of $(A)_n \cdot (U)_n$ (Ts'o *et al.*, 1966)), the optical rotatory dispersion curve of the $(C)_n$–dGMP complex may be expected to be quite different from that of $(C)_n$. The close resemblance of the optical rotatory dispersion curves obtained for the mixture of $(C)_n$ and dGMP to that of $(C)_n$ alone again indicates that $(C)_n$ and 5′-dGMP do not interact with each other under this condition.

A mixture of $(C)_n$ (0.01 M) and 5′-dGMP (0.04 M) in 0.4 M NaCl (pH 7) was studied by velocity sedimentation at 5 and 25°. Since it is known that the polymer–monomer interaction becomes stronger at increasing concentrations of monomer, therefore a concentration of 5′-dGMP higher than that for the optical rotatory dispersion studies was used to force its interaction with $(C)_n$. The schlieren pattern (Figure 6a,b) and the rate of sedimentation of the mixture are very similar to those of $(C)_n$ sedimenting alone under identical conditions in a double-cell run. In view of the large effects of the adenosine on $(U)_n$ in an interacting system (Huang and Ts'o, 1966a), the failure of dGMP to cause any significant change in the sedimentation of $(C)_n$ is again taken as another indication that $(C)_n$ and dGMP do not interact in solution.

Interaction of $(C)_n$ *and GTP.* Since the interaction of $(C)_n$ and GMP (or dGMP) could not be demonstrated in solution, no extensive effort was made to search for the soluble complex of $(C)_n$ and GTP. Nevertheless, the effect of GTP on the sedimentation of $(C)_n$ (0.01 M) has been studied in 0.02 M $MgCl_2$ at 3° (Figure 6c). The sedimentation coefficient of the $(C)_n$ actually was found to decrease (about 10%) in the presence of GTP in comparison to that in the absence of GTP. This is probably due to the binding of Mg^{2+} by the GTP which may affect the conformation of $(C)_n$ indirectly. Since the formation of soluble complex was shown to *increase* (about 40%) the *s* value of the polynucleotide (Huang and Ts'o 1966a), these sedimentation data also indicate that there is no demonstrable interaction between the $(C)_n$ and GTP in solutions of 0.02 M $MgCl_2$ at 3°.

At higher Mg^{2+} concentrations (0.03–0.05 M) the formation of an insoluble complex similar to the ATP–$(U)_n$ system was also observed for the $(C)_n$ and GTP mixture. The insoluble complex is formed at low temperature (near 0°) and is dissociated reversibly upon elevation of temperature. At the level of low $(C)_n$ concentration (0.01–0.02 M), Table VI shows that the precipitate has a stoichiometry of 1C:1G regardless of the ratio of the $(C)_n$ and GTP initially present. In the presence of excess amounts of GTP (0.02 M) the $(C)_n$ (0.01 M) was found to be precipitated completely, leaving mainly GTP in the supernatant. When the concentration of $(C)_n$ was initially greater with the ratio of 2C to 1G, the stoichiometry of the precipitate was still 1C:1G. The remaining portion of the $(C)_n$ stays in the supernatant which, therefore, has a C/G ratio slightly higher than the initial value of 2. The stoichiometry of the precipitate is also independent of the Mg^{2+} concentration (Table VI). However, at

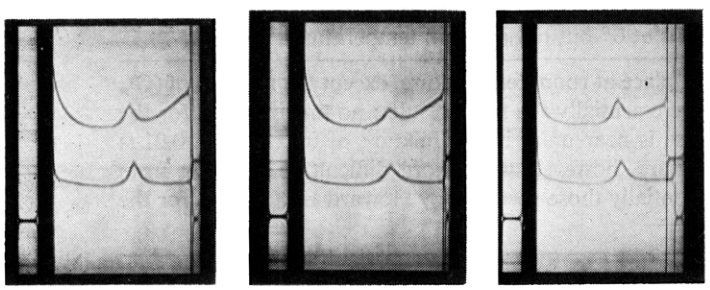

FIGURE 6: Schlieren patterns of $(C)_n$ (lower) and $(C)_n$–guanosine nucleotide mixtures (upper) in a double-cells ultracentrifugation experiments. Rotor speed was 56,100 rpm. (a) $(C)_n$ (0.01 M) and $(C)_n$ (0.01 M)–dGMP (0.04 M) in 0.4 M NaCl (pH 7) at 3°. (b) The same mixtures as (a) at 25°. (c) $(C)_n$ (0.01 M) and $(C)_n$ (0.01 M)–GTP (0.01 M) in 0.02 M $MgCl_2$ (pH 8) and at 3°.

high concentrations of the interactants, 0.04 M $(C)_n$, 0.02 M GTP, and 0.08 M Mg^{2+}, the precipitate was found to have a ratio of 2C to 1G. This condition is that reported by Miles *et al.* (1966). In this mixture, they reported the formation of a three-stranded C_2G complex in D_2O having a T_m of 25° as studied by infrared spectroscopy. Our observation here in H_2O solution supports this previous finding.

TABLE VI: Formation of $(C)_n$ and 5′-GTP Insoluble Complex at pH 8 and 0°.

$(C)_n$ (M)	GTP (M)	$MgCl_2$ (M)	C/G Ratio[a] Supernatant	C/G Ratio[a] Precipitate
0.01	0.01	0.02	1.0	*b*
0.01	0.01	0.03	1.0	1.0
0.01	0.01	0.05	1.0	1.0
0.01	0.02	0.03	0.08	0.9
0.02	0.01	0.03	2.3	1.1
0.04[c]	0.02[c]	0.08[c]	*d*	2.1[d]

[a] See Materials and Methods for the equation to calculate C/G from A_{270}/A_{250}. [b] No precipitation occurs. [c] In Tris buffer (0.01 M, pH 7.1) heavy precipitation occurs at room temperature. This condition is essentially that specified by Miles *et al.* (1966) for infrared studies in D_2O, pD 6.1. In 0.2 M cacodylate (pH 7.0) the precipitation does not take place at room temperature but occurs at 0°. The C/G ratio of the precipitate is also 2.0. [d] Over 90% of the material was found in the precipitate.

Discussion

The formation of insoluble complexes due to the interaction of $(U)_n$ and $(C)_n$ with their respective complementary nucleoside mono- and triphosphates is readily demonstrable. When the concentrations of the interactants are sufficiently high and the temperature is sufficiently low, precipitates are formed with definite stoichiometry. At moderate concentration of the interactants (0.01–0.02 M), Mg^{2+} ion is usually required for the precipitation. On the other hand, a *soluble* complex between $(U)_n$ and adenine nucleotides or between $(C)_n$ and guanine nucleotides *cannot* be detected by a variety of techniques such as optical rotatory dispersion, viscosity, and sedimentation, reported above, or by proton magnetic resonance spectroscopy, reported in the following paper (Ts'o and Schweizer, 1968), though all these techniques have been employed successfully to study the soluble complex of the adenosine–$(U)_n$ system (Huang and Ts'o, 1966a; Ts'o and Schweizer, 1968). Complex formation of (C_n) with guanine nucleotides and $(U)_n$ with adenine nucleotides has been studied by infrared spectroscopy, a technique which requires the presence of the interactants in fairly high concentration (Howard *et al.*, 1964; Miles *et al.*, 1966). Representative experiments in these previous studies were repeated in our study. In every case, the interaction occurs as previously reported, but apparently also with a concomitant change in the physical state of the solution such as precipitation or extensive gelation. This situation of all or none phenomenon is best demonstrated in experiments listed in Table III of the following paper (Ts'o and Schweizer, 1968). In all of these cases, no interaction exists as shown conclusively by the proton magnetic resonance techniques, yet precipitation does occur in these same mixtures at temperatures 10–20° lower than that for the proton magnetic resonance measurement. All these experiments indicate that prior to the change in physical state, no detectable interaction takes place in solution. As soon as interaction proceeds, precipitation or gelation occurs. This is the major difference between the system of adenosine–$(U)_n$ and the systems of nucleotide–polymer reported in this paper. In the system of nucleoside–polymer interaction, soluble complex can be found and characterized. Furthermore, though the nucleoside system is also highly cooperative as shown in its absorption isotherm (Huang and Ts'o, 1966a), nevertheless, in solutions of low adenosine/$(U)_n$ ratio, the distribution of adenosine stacks along the $(U)_n$ strand can become less dense and variable as shown by the profile of the melting curve and the proton magnetic resonance spectrum (Huang and Ts'o, 1966a; Ts'o and Schweizer, 1968). Apparently, when the monomer possesses the charged phosphate group, additional driving force is required for the interaction which can be provided by the phase transition.

In Figures 3 and 4, the dependence of the precipitation temperature (T_p), which is an index of the stability of the insoluble complex, on the concentration of the mononucleotide in the AMP–$(U)_n$ system is clearly illustrated. Sedimentation equilibrium study, osmotic coefficient measurement, and proton magnetic resonance data conclusively show the extensive association of adenine nucleotides to form stacks in aqueous solution (Rossetti and Van Holde, 1967; Schweizer *et al.*, 1968). Furthermore, the order of the extent of stacking of various adenine nucleotides as measured by proton magnetic resonance, *i.e.*, 5′-AMP > 3′-AMP > 2′-AMP (Schweizer *et al.*, 1968), is the same as the order of their comparative ease of forming insoluble complexes with $(U)_n$, as measured by the Mg^{2+} concentration requirement listed in Table I. Association of GMP or dGMP to form a helix was first studied by Gellert *et al.* (1962). Subsequently, this problem was investigated by Miles and Frazier (1964) and by Sarkar and Yang (1965). At a sufficiently high concentration of GMP, and sufficiently low temperature, helical structure is formed involving both stacking and intermolecular hydrogen bonding. It should be noted that this solution of helical GMP is very viscous and is probably in a gel state. Association of adenine nucleosides in water to form stacks has been studied by vapor pressure osmometry and proton magnetic resonance techniques (Broom *et al.*, 1967) and association of isoguanosine to form an ordered structure has been studied by viscosity, ultraviolet and infrared spectroscopy, and optical rotation (Ravindranathan and Miles, 1965).

All of these observations suggest to us the following mechanism of interaction between the mononucleotides and the polynucleotides. When the conditions are sufficiently favorable, the monomer–polymer interaction takes place through hydrogen bonding between the base pairs and through the cooperative stacking of the mononucleotides along side the complementary polynucleotides. In the complex, mononucleotides become polymerlike. In doing so, a phase transition occurs. This transition of physical state provides the additional driving force needed for the interaction to proceed by removing the complex into another phase of the system. Two lines of evidence support this notion that the stacked mononucleotides in the insoluble complex behave like their corresponding polynucleotides. First, $(U)_n + (A)_n$ (0.01 M each) does precipitate in 0.02 M Mg^{2+} even at room temperature while the individual polynucleotides are soluble in separate solution under this condition. Second, as shown in Figure 1 and discussed in Results, the critical pH for the dissociation of the AMP–$(U)_n$ insoluble complex can be as high as pH 5.2 in 0.02 M Mg^{2+}. It appears that the pK_a of 5′-AMP in the insoluble complex may be much higher than that of the free 5′-AMP, and closer to that of the $(A)_n$. It should be noted also that the specificity of the insoluble complex formation is the same as that of the polynucleotide interaction. In the cooperative process of insoluble complex formation, the forces of hydrogen bonding and hydrophobic stacking work together in unison. The hydrogen bonding provides the specificity of this interaction, and most likely the strength of the bonding between the base pairs increases substantially in the insoluble complex. Inside the complex, the hydrogen-bonding sites of the base pairs probably are much more shielded from the water molecules in the solution which weaken the bonding by competition. In chloroform, the ΔH° for the association of a mixed dimer of substituted adenine and uracil was found to be -6 kcal/mole, or -2 kcal/mole over the self-association of adenine dimer and uracil dimer (Kyogoku *et al.*, 1967). The base pairs in the insoluble complex may have an environment more analogous to that in chloroform than in water. On the other hand, the stacking force must play the predominant role in supplying the chemical potential for the interaction. As calculated from the slope of the steep transition of the adsorption isotherm of the adenosine–$(U)_n$ system, the stacking energy of the adenosine upon pairing with 2U of $(U)_n$ was found to be around 5–6 cal/mole. Therefore, the stacking energy is the primary source of the cooperative effect observed.

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