Physicochemical Basis of the Recognition Process in Nucleic Acid Interactions. III. Proton Magnetic Resonance Studies on the Interactions of Polyuridylic Acid and Polycytidylic Acid with Nucleosides, 5′-Nucleotides, and Nucleoside Triphosphates*

Paul O. P. Ts'o and Martin P. Schweizer†

ABSTRACT: The technique of high-resolution proton magnetic resonance spectroscopy was applied to the study of monomer–polymer interactions of nucleic acids. This technique was first applied to the study of the adenosine–polyuridylic acid system which has been characterized before by other methods. Extensive broadening of the line widths of the adenosine and polyuridylic acid protons, especially the base protons, was found when conditions are favorable for the formation of the soluble complexes. Additional information about this interaction, such as specificity, stoichiometry, and dynamic states, was revealed from these proton magnetic resonance spectral studies. Then this technique was applied to the study of interactions of adenine nucleotides with polyuridylic acid and guanine nucleotides with polycytidylic acid. The spectral data confirm the observations reported in the preceding paper that *soluble* complexes can*not* be found in the mixtures of mononucleotides and the polynucleotides, though *insoluble* complexes will form in these same mixtures at temperatures 10–20° lower than those for the spectral measurement. Apparently, the interaction of the mononucleotides with the complementary polynucleotides is generally accompanied by a phase transition.

The rationale and the background of our research on monomer–polymer interactions in the field of nucleic acids have been described in our previous publication (Huang and Ts'o, 1966) and in our preceding paper (Ts'o and Huang, 1968).

Recently, certain successes have been achieved in using high-resolution proton magnetic resonance for the study of the binding of small molecules by proteins (Fischer and Jardetzky, 1965; Jardetzky and Wade-Jardetzky, 1965; Hollis, 1967). The basis of the method is that line widths in proton magnetic resonance spectra are sensitive to the degree and kinds of molecular motion present in the sample. Increase in line widths can be correlated with diminished rate of motion of the protons, particularly rotational motion. This loss of motional freedom usually takes places when a small molecule binds to a large one, or when an ordered, rigid complex is formed. The physical chemical basis for this phenomenon has been described in a review by Jardetzky (1964).

In this paper, the proton magnetic resonance technique was first applied to the study of the adenosine–$(U)_n$ system which has been characterized before by other methods (Huang and Ts'o, 1966; Howard *et al.*, 1966). Extensive broadening of the line widths of adenosine and $(U)_n$ protons was found when conditions were favorable for the formation of the soluble complex. Additional information about this interaction was revealed from these proton magnetic resonance spectral studies. Then, this technique was applied to the study of interactions of adenine nucleotides with $(U)_n$ and guanine nucleotides with $(C)_n$. The spectral data confirm the observations reported in the preceding paper that *soluble* complexes cannot be found in the mixtures of mononucleotides and the polynucleotides, though *insoluble* complexes will form in these same mixtures at temperatures 10–20° lower than those employed for the spectral measurements. Apparently, the interaction of the mononucleotides with the complementary polynucleotides is generally accompanied by a phase transition.

Materials and Methods

$(U)_n$ and $(C)_n$ were purchased from the Miles Laboratories, Elkhart, Ind. Nucleosides and nucleoside monophosphates or triphosphates were purchased from Sigma Chemical Co., St. Louis, Mo.

Proton magnetic resonance spectra were recorded with a Varian Associates HA-100 spectrometer. Probe temperature was 28–30° or controlled at lower temperatures as specified. A Varian C-1024 computer of average transients was used to enhance the signal intensity for dilute solutions. Chemical shifts were measured in

* From the Department of Radiological Sciences, The Johns Hopkins University, Baltimore, Maryland. *Received April 12, 1968.* This material was also submitted by M. P. S. as part of a Ph.D. thesis to The Johns Hopkins University, Feb 1968. This work was supported in part by grants from the National Science Foundation (GB-5483 and GB-767) and by a program project grant, National Institutes of Health (GM-10802-04, 05).

† Present address: Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, N. Y.

TABLE I: Proton Magnetic Resonance Studies on Binding of Nucleoside, Monophosphate, and Triphosphate to $(U)_n$[a] (Unless Otherwise Noted Temperature Is 28°).

No.	Sample (M)	Chemical Shifts (ppm) from TMS Capillary					Line Widths (cps)[a]				
		H-8	H-2	H-6	H-1′	H-5′, H-5″	H-8	H-2	H-6	H-1′	H-5′,[b] H-5″
1	Adenosine (0.028) and $MgSO_4$ (0.03)	8.71	8.58		6.46	4.30	1.2	1.1		1.3	6
2	$(U)_n$ (0.028), NH_4 salt			8.31					2.3		
3	$(U)_n$ (0.028)[a] and adenosine (0.028) (no salt)	8.71	8.58	8.30	6.46 (A), 6.38 (U)		1.1	1.0	2.3		
4	Adenosine (0.028),[a] $(U)_n$ (0.028), and $MgSO_4$ (0.033) (pD 7.6)	8.71	8.59	*c*	6.46 (A)	4.31 (A)	7–8	7–8	*c*	5 (A)	10
5	Adenosine (0.057),[a] $(U)_n$ (0.028), and $MgSO_4$ (0.03) (pD 8.2)	8.69	8.54	*c*	6.45 (A)		3.6	3.2	*c*	3.3 (A)	
6	Adenosine (0.014), $(U)_n$ (0.028), and $MgSO_4$ (0.033) (pD 7.9)		8.63[d]	8.31	*c*		20[d]		5		
7	Adenosine (0.029), $(U)_n$ (0.029), and NaCl (0.82) (pD 7.8)	8.69	8.56	8.30			8–10	8–10	5.6		
8	Inosine (0.028) and $MgSO_4$ (0.041)	8.75	8.64		6.52		1.1	1.2		1.1	
9	Inosine (0.029), $(U)_n$ (0.03), and $MgSO_4$ (0.04)	8.76	8.65	8.30	6.51 (I), 6.54 (U)		1.1	1.0	2.2	1.0 (I)	
10	Inosine (0.029),[e] adenosine (0.027), $(U)_n$ (0.03), and $MgSO_4$ (0.04) (pD 7.4)	8.74 (I)	8.62 (I)	*c*	6.50 (I)		1.2 (I)	1.2 (I)		1.3 (I)	*c*

11	5'AMP (0.02), mixed with 3'-UMP (0.02) (pD 7.4)	8.90	8.63	8.29	6.54 (A), 6.35 (U)	1.5	1.2	2.2	1.5 (A)	
12	5'-AMP (0.02), $(U)_n$ (0.01), and $MgCl_2$ (0.04) (pD 7.4)	9.01	8.63	8.29		1.5	0.9	2.6		
13	ATP (0.02) and $MgCl_2$ (0.04), 14° (pD 8.4)	8.86	8.54	6.49		2.2	1.3		1.4	
14	ATP (0.02),[a] $(U)_n$ (0.01), and $MgCl_2$ (0.04), 14° (pD 8.4)	8.86	8.54	8.29	6.50 (A)	1.7	1.4	2.7	1.7 (A)	

[a] In these solutions (3, 4, 5, and 14), the line width of HOD was found to be 1.2–1.5 cps. [b] Width of whole pattern. [c] Lines too broad to discern. [d] H-8 and H-2 protons merged. [e] H-8 and H-2 protons of adenosine broadened as in adenosine, $(U)_n$, and $MgSO_4$ solutions without inosine.

parts per million from an external tetramethylsilane capillary to an accuracy of ±0.01 ppm. Determinations of apparent pH values in D_2O solvent were made with a Model 22 pH meter from Radiometer, Copenhagen. Micro calomel and glass electrodes were used on 0.5-ml volumes. To obtain pD values, the equation pD = meter + 0.4 of Glasoe and Long (1960) was used.

Results

Binding of Nucleosides, 5'-Nucleotides, and Nucleoside Triphosphates to $(U)_n$. BINDING OF ADENOSINE TO $(U)_n$. Previous studies from our laboratory (Huang and Ts'o, 1966) indicated that in the presence of 0.4 M NaCl and HMP, 0.015 M $(U)_n$ and adenosine form a complex with a stoichiometry of 2U:1A and with a T_m of about 24°. In the presence of 0.02 M $MgCl_2$, the T_m of this 2U:1A complex increases to about 33°. Owing to the sensitivity requirements of proton magnetic resonance measurements and for the convenience in recording the spectra, the concentrations of $(U)_n$, adenosine, and the salt were approximately doubled. It is safely assumed that the complex formation should be comparable at these high concentrations (the T_m is likely to be even higher) to the well-characterized interaction at the lower concentrations of interactants.

Data in Table I and Figures 1 and 2 describe the features of the interaction between adenosine and $(U)_n$ as studied by proton magnetic resonance at 28°. The chemical shifts and the line widths of H-8, H-2, H-1', and H-5',5'' protons of adenosine (0.028 M) are the same in the presence or the absence of 0.03 M $MgSO_4$ (Table I-1 and Figure 1a). However, the protons of $(U)_n$ were broadened by 20–30% when 0.03 M $MgSO_4$ was added (Table I-2 and comparison between Figure 1b,c). This observation indicates that even at 28°, addition of Mg^{2+} causes the secondary structure of $(U)_n$ to become more ordered and less flexible.

The spectra of a mixture of 0.028 M (U_n) and 0.028 M adenosine without added salt were identical with the sum of the spectra from the two individual components, each free of salt (Table I-3). This result agrees with our previous observation that in the absence of salt, complex formation is undetectable (Huang and Ts'o, 1966). Upon addition of 0.033 M $MgSO_4$ to this mixture, however, evidence for interaction is clearly demonstrated (Table I-4 and Figure 2a). Line widths of the adenosine protons are extensively broadened, while the peaks for $(U)_n$ protons (H-6, H-5, and H-1' at 8.31, 6.35, and 6.38 ppm, respectively) could not be detected even after fivefold signal enhancement. Solution viscosity is not an important factor in this line broadening since the HOD solvent lines are not affected (see Table I, footnote). Two other observations on the adenosine are noteworthy. First, the H-8 and H-2 protons are broadened by a factor of 6–7, while H-1' by 3.8 and H-5', H-5'' only by 1.7 (Figures 1a and 2a and Table I-4). This relaxation gradient from the base protons (H-8 and H-2) to the 5'-methylene protons of the adenosine implicates the base as the binding site to the $(U)_n$, an expected result. Second, while there is extensive broadening of the line widths, the chemical shifts of the base protons of

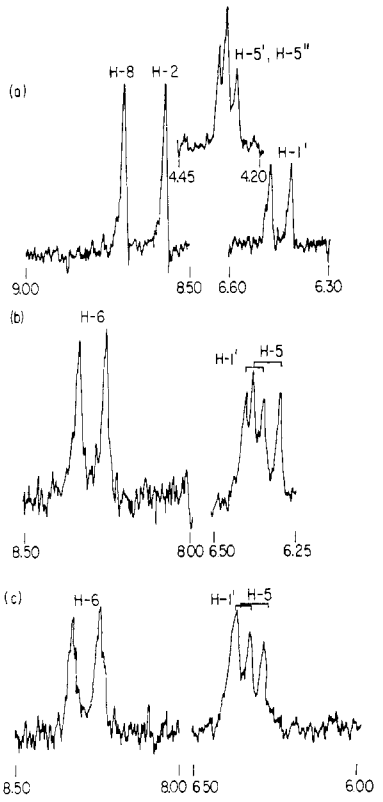

FIGURE 1: Proton magnetic resonance spectra. (a) 0.028 M adenosine and 0.03 M $MgSO_4$ in D_2O; identical spectrum was obtained in the absence of $MgSO_4$; (b) 0.028 M $(U)_n$, ammonium salt, pD 6.9; (c) 0.028 M $(U)_n$ with $MgSO_4$ added to 0.03 M, pD 8.

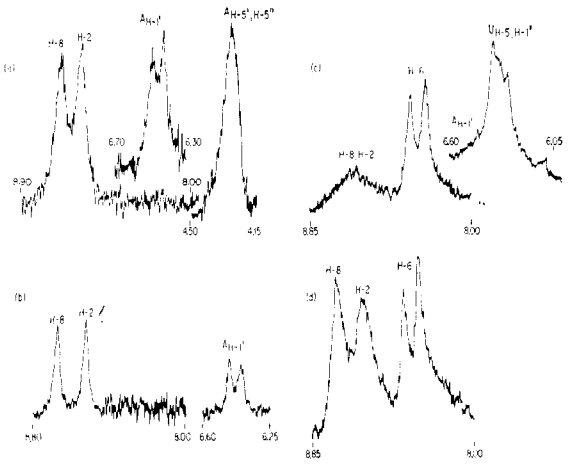

FIGURE 2: Proton magnetic resonance spectra. (a) Mixture of adenosine and $(U)_n$, 0.028 M each, 0.033 M $MgSO_4$, pD 7.6, 25 scans; (b) mixture of 0.028 M $(U)_n$, 0.057 M adenosine, 0.033 M $MgSO_4$, pD 8.2, no signal enhancement; (c) 0.028 M $(U)_n$, 0.014 M adenosine, 0.03 M $MgSO_4$, pD 7.9, 100 scans; (d) 0.029 M $(U)_n$, 0.029 M adenosine, 0.82 M NaCl, pD 7.8, 36 scans.

the adenosine are unchanged in the presence of $(U)_n$ (Table I).

When the input concentration of adenosine was increased to 0.057 M in the mixture of 0.028 M of $(U)_n$ and 0.03 M $MgSO_4$, the maximal amount of the input adenosine bound to $(U)_n$ could be only 25% because of the stoichiometry of 1A to 2U. Therefore, the broadening of the line widths of the base protons (3-fold) and H-1′ proton (2.5-fold), as shown in Table I-5, was not as large as in the case of 1:1 mixture of adenosine and $(U)_n$, 0.028 M each. As expected, the protons of the $(U)_n$, such as the H-6, at 8.30 ppm were undetectable in this high concentration of adenosine. On the other hand, when the input concentration of adenosine was reduced to 0.014 M in a mixture of 0.028 M $(U)_n$ and 0.03 M $MgSO_4$, the protons of the $(U)_n$, H-5, H-1′, and especially the H-6 (Figure 2c, Table I-6), became measurable after tenfold signal enhancement. The line width of H-6 was only 1.5-fold greater over that of the $(U)_n$ control in the absence of adenosine. The spectral lines of the H-2 and H-8 protons of adenosine in this case of low input (0.014 M), however, were much broadened and merged (Table I-6, Figure 2c). The result indicates while most of the adenosine is in the bound form under this condition, there is considerable freedom of movement for the $(U)_n$.

When monovalent Na^+ ions were used to replace the divalent Mg^{2+}, an interesting situation was observed. In a mixture of 0.029 M adenosine, 0.029 M $(U)_n$, and 0.82 M NaCl (Table I-7, Figure 2d), though the line widths of H-2 and H-8 of the adenosine are broad (8–10 cps) in comparison with that (7–8 cps) in 0.033 M $MgSO_4$ mixture (Table I-4), the H-6 proton of $(U)_n$ is clearly detectable (line width 5.6 cps) after sixfold signal enhancement, while in the 0.033 M $MgSO_4$ solution containing 0.029 M adenosine, the H-6 proton doublet of $(U)_n$ is not visible at all after fivefold signal enhancement. In other words, in the same mixture of adenosine and $(U)_n$ and at the same temperature, the amount of adenosine bound, as indicated by the line-width broadening, appears to be the same (or slightly more) in the case of 0.82 M NaCl as compared with that of the solution of 0.033 M $MgSO_4$ (Figure 2a *vs.* 2d), yet the resonance line of $(U)_n$ is clearly visible in 0.82 M NaCl solution but not in 0.033 M $MgSO_4$ solution. This point will be further discussed in a later section.

Interaction of Inosine with $(U)_n$. Our previous studies by equilibrium dialysis and optical rotatory dispersion (Huang and Ts'o, 1966) clearly indicated that there is no interaction between inosine and $(U)_n$ even in 0.4 M NaCl and near 0°. It is of interest to study by proton magnetic resonance a mixture of inosine, $(U)_n$, and Mg^{2+}, and this system with adenosine added. These data not only can reconfirm previous studies, but also can serve as an additional control for the interacting systems by assessing the influence of polymer structure and structural alterations upon resonance line widths of a monomer species which is not bound to the polymer.

The spectrum of a solution containing 0.03 M $(U)_n$, 0.029 M inosine, and 0.04 M $MgSO_4$ clearly shows that there is no interaction between the nucleoside and the polymer (Figure 3a, Table I-8,9). The spectrum of the mixture is the same as the sum total of the two spectra of each individual component. When adenosine (0.027 M) was added to this mixture of inosine and $(U)_n$ (Figure 3b, Table I-10), the proton lines of the $(U)_n$ totally

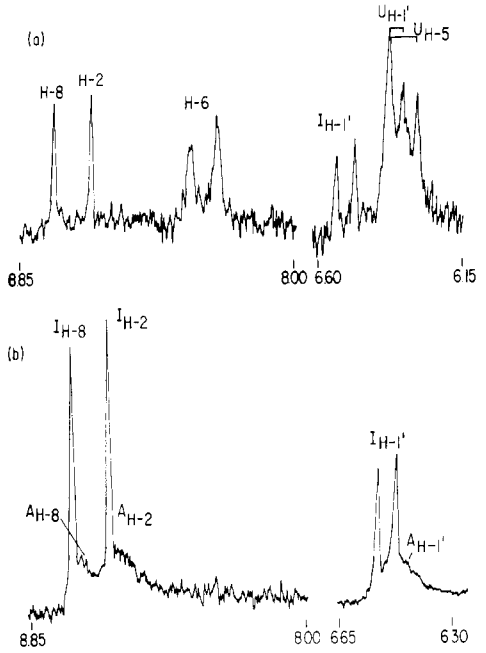

FIGURE 3: Proton magnetic resonance spectra. (a) Mixture of 0.03 M $(U)_n$, 0.029 M inosine, and 0.04 M $MgSO_4$; (b) 0.03 M $(U)_n$, 0.029 M inosine, 0.027 M adenosine, and 0.04 M $MgSO_4$, pD 7.4, 9 scans.

disappeared and the proton lines of adenosine were extensively broadened, but there was very little change in either line width or chemical shifts of the inosine protons in this interacting mixture.

INTERACTION OF 5′-AMP AND 5′-ATP WITH $(U)_n$. Our preceding paper (Ts'o and Huang, 1968) indicated that no soluble complex between 5′-AMP (0.01 M) and (U_n) (0.01 M) can be detected by the methods of viscosity, sedimentation, and optical rotation in the presence of 0.02 M Mg^{2+} even around 0°. Insoluble precipitates with definite stoichiometry were formed, however, when AMP concentrations were increased. It is important, therefore, by the use of the proton magnetic resonance technique to reach a definite conclusion about the existence of any interaction between AMP and $(U)_n$ in the soluble state. As shown in Table I-12, at 28°, a mixture of 0.02 M 5′-AMP, 0.01 M $(U)_n$, and 0.04 M $MgCl_2$ shows no line broadening of the proton resonances of monomer or polymer compared with controls. It must be concluded, therefore, that no interaction occurs in solution between 5′-AMP and $(U)_n$ under this condition.

Because of the lack of success in demonstrating an interaction between 5′-AMP, $(U)_n$, and Mg^{2+} in solution, we did not investigate the interaction of ATP and $(U)_n$ in Mg^{2+} solution by optical rotatory dispersion or by hydrodynamic methods in our preceding paper (Ts'o and Huang, 1968). Nevertheless, in a mixture of 0.02 M ATP, 0.01 M $(U)_n$, 0.04 M $MgCl_2$ (pH 8.4), a precipitate with a stoichiometry of 2U:1A was formed in the region of 2–7° (Table IV of Ts'o and Huang, 1968). Therefore, it was of interest to study this same mixture (0.02 M ATP, 0.01 M $(U)_n$, and 0.04 M $MgCl_2$ in D_2O, pD 8.4) at 14°, just above the precipitation temperature

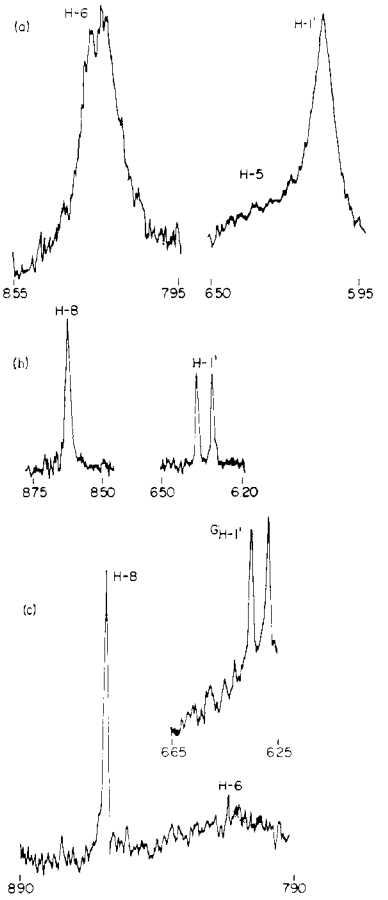

FIGURE 4: Proton magnetic resonance spectra. (a) 0.055 M $(C)_n$, 0.1 M NaCac, pD 7.7, 9 scans: (b) 0.05 M 5′-GMP, 0.1 M NaCac, pD 7.6; (c) 0.01 M 5′-GMP, 0.01 M $(C)_n$, 0.02 M NaCac, pD 7.5, 5°, 16 scans.

for formation of the insoluble complex. Data in Table I-14 show that there is no interaction of these two components detectable by proton magnetic resonance either in terms of the line width or in terms of the chemical shifts.

Binding of Nucleosides and Monophosphates to $(C)_n$. In our preceding paper (Ts'o and Huang, 1968), optical rotatory dispersion and sedimentation studies failed to show that a soluble complex forms between 0.01 M 5′-GMP (or 5′-dGMP) and 0.01 M $(C)_n$ in 0.4 M NaCl, either at 0 or at 27°.

The line widths of the protons of $(C)_n$ (0.055 M) in 0.1 M sodium cacodylate pD 7.7 buffer are considerably greater than those of $(U)_n$ (comparison between Figures 1b,c and 4a, as well as between Tables I and II). The line width of H-6, for instance, is broadened even further when the temperature is lowered from 28 to 8°, or when 0.02 M $MgCl_2$ is used in place of the 0.1 M Na^+ buffer (Table II-3). These observations suggest that the $(C)_n$ has less conformational mobility than $(U)_n$, and, therefore, the $(C)_n$ should have more ordered secondary structure than the $(U)_n$. This conclusion is in accord with previous studies (Helmkamp and Ts'o, 1962; Richards *et al.*, 1963; Fasman *et al.*, 1964; Ts'o *et al.*, 1966).

The line widths and chemical shifts of the protons in the mixture of 0.01 M 5′-GMP and 0.01 M $(C)_n$ in 0.02

TABLE II: Proton Magnetic Resonance Studies on Binding of Nucleoside, Monophosphate, and Triphosphate to $(C)_n$ (Temperature Is 28°, Unless Otherwise Specified).

		Chemical Shifts (ppm) from TMS Capillary					Line Widths (cps)						
No.	Sample (M)	H-8	H-2	H-6	H-5	H-1′	H-8	H-2	H-6[a]	H-5[a]	H-1′	HOD	CH_3[b]
1	$(C)_n$ (0.055) and NaCac (0.1), pD 7.7			8.21	6.32	6.06			16	20	9	1.3	
2	$(C)_n$ (0.055) and NaCac (0.1), pD 7.7, 8°			8.13	*d*	*c*			22	*d*	*c*	1.5	
3	$(C)_n$ (0.01)[e] and $MgCl_2$ (0.02), pD 8.5, 19°			8.18	*d*	*d*			20	*d*	*c*		
4	5′-GMP (0.05) and NaCac (0.1), pD 7.6	8.60				6.34	1.8				1.4	1.2	
5	5′-GMP (0.01), $(C)_n$ (0.01), and NaCac (0.02), pD 7.5	8.62		8.23	6.23	6.40(G), 6.09(C)	1.7		17	13	1.7(G), 4.5(C)		
6	5′-GMP (0.01), $(C)_n$ (0.01), and NaCac (0.02), pD 7.5, 5°	8.57		8.11	*d*	6.31(G)	1.5		30	*d*	1.8(G)	1.0	1.1
7	5′-GMP (0.01) and $MgCl_2$ (0.05)	8.64				6.38	2.8			*d*	2.9		
8	5′-GMP (0.01),[e] $(C)_n$ (0.01), and $MgCl_2$ (0.05)	8.63		8.16	*d*	6.36, *d*(C)	4.5		17	*d*	3.2(G), *d*(C)		
9	Adenosine (0.03), $(C)_n$ (0.027), and NaCac (0.8), pD 7.3	8.72	8.59	8.22	6.23	6.47(A), 6.09(C)	1.5	1.4	17	11	1.3(A)	0.8	1.2
10	Adenosine (0.03), $(C)_n$ (0.027), and NaCac (0.8), pD 7.3, 8°	8.63	8.44	*d*	*d*	6.38(A)	1.3	1.5	*d*	*d*	1.4	1.4	1.2
11	5′-AMP (0.03), $(C)_n$ (0.027), and NaCac (1.0), pD 7.2	8.98	8.65	8.21	6.23	6.09(C)	1.2	0.9	13	13–14	1.3(A)	0.7	0.8

[a] Doublet line widths. [b] Methyl protons of the buffer, sodium cacodylate (dimethylarsinous acid, sodium salt, abbreviated NaCac). [c] Obscured by HOD spinning side band. [d] Some resonances of $(C)_n$ too broad to be discerned. [e] Spectrum of $(C)_n$ (0.01 M) in 0.05 M $MgCl_2$ is essentially the same as reported in this row, Table II-3.

TABLE III: Comparison between Conditions of Proton Magnetic Resonance Measurement and Occurrence of Precipitation.[a]

Concentrations (M)			Temperature (°C)	
			Proton Magnetic Resonance Measurement	Precipitation
5′-AMP (0.02)	$(U)_n$ (0.01)	$MgCl_2$ (0.04)	28[b]	12–16[c]
5′-ATP (0.02)	$(U)_n$ (0.01)	$MgCl_2$ (0.04)	14[b]	2–7[d]
5′-GMP (0.01)	$(C)_n$ (0.01)	$MgCl_2$ (0.05)	28[e]	5[f]

[a] Concentrations of reactants were the same. The only difference was in temperature. No interaction was detected in the pmr spectra. [b] From Table I-12 and -14. [c] From Figure 4 in Ts'o and Huang, 1968. [d] From Table IV in Ts'o and Huang, 1968. [e] From Table II-8. [f] From Table V in Ts'o and Huang, 1968.

M sodium cacodylate, pD 7.5, at 28 or 5° are very similar to those of the individual components in separate solutions (Table II-5,6, Figure 4). These data clearly show that there is no interaction between 5′-GMP and $(C)_n$ under these conditions.

From our preceding paper (Ts'o and Huang, 1968, Table V), it is known that the formation of an insoluble complex will take place in a solution of 0.01 M 5′-GMP, 0.01 M $(C)_n$, and 0.05 M Mg^{2+} near 5°. Therefore, this same mixture was studied by proton magnetic resonance at room temperature in order to search for the soluble complex. As shown in Table II-3,7,8, there is a small increase (from 2.8 to 4.5 cps) in the H-8 line width of 5′-GMP when it is mixed with $(C)_n$; however, the H-6 line width of the $(C)_n$ is completely unchanged. We concluded, therefore, that this small increase is likely to be caused by the viscosity of the $(C)_n$ in a solution of 0.05 M magnesium. Thus, again, there is no interaction between 5′-GMP and $(C)_n$ under this condition.

As control experiments for the interaction studies between adenosine or 5′-AMP with $(U)_n$ described in a previous section, mixtures of adenosine or 5′-AMP and $(C)_n$ were studied. As shown in Table II-9–11, there is little change in line width or chemical shifts of both adenosine (0.03 M) and $(C)_n$ (0.027 M) in 0.8 M Na^+ buffer either at 28 or 8°, in sharp contrast to the extensive broadening observed for the resonances in the adenosine–$(U)_n$ mixture. There is no interaction detected between 5′-AMP and $(C)_n$ either; this interaction, however, is also undetected between 5′-AMP and $(U)_n$ as described above.

Discussion

When the spectral positions and the line widths of the base protons and H-1′ protons of the mononucleotides and the polynucleotides are the same in spite of whether they are dissolved together in a mixture or individually in separate solutions, it may be concluded safely that no appreciable interaction exists among these compounds in the mixture under these conditions. In the present study, there are several cases belonging to this category, in which the spectrum of the mixture is the same as the total sum of the separate spectrum of each individual compound. The most pertinent ones are summarized in Table III. It is clearly demonstrated in this table, though no interaction could be detected by proton magnetic resonance spectra in all these cases, precipitation did occur in these mixtures at temperatures 10–20° lower than those for the proton magnetic resonance measurement. These observations strongly suggest that prior to precipitation, no interaction takes place in solution. As soon as interaction proceeds, precipitation occurs. This all or none situation of the interaction between the mononucleotides and the corresponding complementary polynucleotides was discussed fully in the preceding paper and will not be repeated here. This is, nevertheless, one of the major findings in this paper.

There are other noninteracting mixtures observed, such as the mixture of adenosine with $(C)_n$ (Table II-9, 10). These experiments further confirm the previous findings that the monomer–polymer interaction system has the same specificity as the polymer–polymer interaction (Huang and Ts'o, 1966; Howard *et al.*, 1966).

The only interacting system in solution observed by proton magnetic resonance is that of adenosine–$(U)_n$, a system previously studied in our laboratory (Huang and Ts'o, 1966) and by Howard *et al.* (1966). As anticipated, there is extensive broadening of the resonance lines. The major source of the line broadening must come from the anisotropic nuclear dipole–dipole interaction of the protons with their environment due to the loss of their freedom in motion when the binding occurs. This conclusion is supported by two lines of evidence. (1) As described in the results, there exists a gradient of line broadening among various protons of the adenosine starting from the base to the 5′ end of the ribose; see Table I-4. As compared with free adenosine, the base protons of adenosine in the interaction system have the highest degree of broadening, next the H-1′ proton, and least the H-5′′ protons. This relaxation gradient suggests that the base protons of adenosine lose more

freedom of motion in the binding process than the H-1′ protons, and in turn the H-1′ protons lose more motional freedom than the H-5′,H-5″ protons. This is the predicted result if the binding of adenosine to the $(U)_n$ takes place at the bases. (2) Within the range of ± 0.03 ppm, the chemical shifts of the adenosine protons are the same in the mixture as in its own individual solution. This observation is not unexpected in light of our previous work on the extensive association of adenosine in solution (Broom *et al.*, 1967). Thus, adenosine already partially self-aggregates into stacks, before binding. The chemical shifts of the H-2 and H-8 protons are already 0.1 ppm upfield from the values at infinite dilution. Furthermore, the hydrogen bonding which takes place in the complex formation should cause a downfield shift of the base protons, and, therefore, may compensate the additional shielding due to further stacking in the complex. A similar situation was found also for the $(U)_n$. Since the chemical shifts of the bound form and the free form appear to be very close, the broadening observed cannot be due to an exchange of two forms having very different chemical shifts. Therefore, we may conclude that the main cause of the line-width broadening comes from the dipolar interaction of the protons with their environment as a result of loss of motional freedom upon binding.

The line shapes of the resonances further suggest that there is a rapid exchange between the bound form and the free form. The line width observed for a proton is likely to be an average of line widths of various fractions of this proton in different states in accordance to

$$\Delta\nu(^1/_2)_{av} = \sum_{i=1}^{n} \rho_i \Delta\nu(^1/_2)_i$$

where $\Delta\nu(^1/_2)_{av}$ is the average line width at half-height and is proportional to $1/T_2$, ρ_i is the fraction of the proton in the ith state of motional freedom, therefore having a value of $\Delta\nu(^1/_2)_i$ as its line width. When only two forms are being considered in the simplest case, *i.e.*, the free form and the bound form, and $n = 2$, the observed line width can be considered as a simple number average of the two line widths. On the other hand, if there is no exchange between the free form and the bound form, then we should have a sharp line (free form) superimposed (if having the same chemical shifts, otherwise located nearby) on a broad curve (bound form), contrary to what was observed (Figure 2).

The above understanding provides the necessary basis for further discussion of the experimental observations. In the mixture of 0.028 M each of adenosine and $(U)_n$ together with 0.03 M Mg^{2+}, the motional freedom of the adenosine protons are greater than that of the $(U)_n$ protons which were too broad to be detected while the base proton line widths of adenosine were still measurable. This can be due either to the fact that bound adenosine has more motional freedom than the bound $(U)_n$, or, much more likely, the fraction of the bound adenosine in the mixture (maximum 50% of the stoichiometry of 2U:1A) is less than the fraction of the bound $(U)_n$. When the adenosine concentration was increased to 0.057 M, the line widths of the adenosine protons became narrower. This is due to the fraction of the bound adenosine becoming further diminished because of saturation. When the adenosine concentration was lowered to 0.014 M, the line width of the adenosine protons now became extensively broadened, while the $(U)_n$ proton resonances sharpened and became measurable. These interesting data indicate that now the fraction of the bound adenosine became much larger, but the concentration of the adenosine appeared to be too low to keep all the $(U)_n$ in the complex form at 28°. Our previous studies (Huang and Ts'o, 1966) showed that when the A/U ratio in the mixture was reduced to 0.4–0.5, the melting profile of the $(U)_n$–adenosine complex became much more broadened and less cooperative as compared to the sharp transition observed for the mixture having an A/U ratio of unity. It was interpreted that as the $(U)_n$ sites become excessive, the size of the adenosine stacks along the $(U)_n$ becomes more reduced and variable, and the distribution of the stacks along the $(U)_n$ becomes less dense. The present proton magnetic resonance study supports this interpretation. With an A/U ratio of 0.5, while most of the adenosines are bound, however, a substantial fraction of the protons of $(U)_n$ under these conditions does have considerable motional freedom. Again, the line shape of H-6 proton of $(U)_n$ indicates rapid exchange (Figure 2c), and the line width represents an average of the line widths of the various forms.

Another interesting observation was from the mixture of 0.029 M adenosine, 0.029 M $(U)_n$, and 0.82 M NaCl (Table I-7, Figure 2d). As stated in the results, though the line widths of H-2 and H-8 of the adenosine are broad (8–10 cps) in comparison with that (7–8 cps) in 0.033 M $MgSO_4$, the H-6 proton of $(U)_n$ is clearly detectable in the NaCl solution (line widths 5.6 cps), but *not* in the Mg^{2+} solution. These data suggest that the fraction of adenosine bound in 0.8 M NaCl is slightly *larger* than that in 0.033 M Mg^{2+}, but the fraction of $(U)_n$ bound in 0.8 M NaCl is substantially *less* than that in 0.033 M Mg^{2+}. The only reasonable interpretation which can be offered at this moment is that there is a change of stoichiometry of 2U:1A in 0.033 M Mg^{2+} solution to 1U:1A in 0.8 M NaCl. Therefore, for about equal amounts of adenosine bound, more $(U)_n$ in the free form was observed in 0.8 M NaCl than in 0.033 M $MgCl_2$. Our previous paper (Huang and Ts'o, 1966) did report a change of stoichiometry from 2U:1A to 1U:1A based on solubility and hydrodynamic measurements when the temperature was raised from 5 to 20° for mixtures in 0.4 M NaCl. More investigation along this line appears to be of value.

Finally, the proton magnetic resonance results on the 0.029 M inosine, 0.027 M adenosine, and 0.03 M $(U)_n$ mixture in 0.04 M $MgSO_4$ provided the most direct demonstration of base-pairing specificity (Table I-10, Figure 3b). In this mixture, the spectra indicated that extensive interaction between adenosine and $(U)_n$ occurs, while the inosine is left untouched in solution.

Application of the proton magnetic resonance technique does provide valuable information about the monomer–polymer interactions of nucleic acids not obtainable by other methods.

Acknowledgment

We wish to thank Dr. Norman Kondo and Miss Helen Holmes for obtaining several spectra in this work, and we also appreciate the consultation and assistance of Dr. Wai-Mun Huang.

References

Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Am. Chem. Soc. 89*, 3612.
Fasman, G. D., Lindblow, C., and Grossman, L. (1964), *Biochemistry 3*, 1015.
Fischer, J. J., and Jardetzky, O. (1965), *J. Am. Chem. Soc. 87*, 3237.
Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Helmkamp, G. K., and Ts'o, P. O. P. (1962), *Biochim. Biophys. Acta 55*, 601.
Hollis, D. P. (1967), *Biochemistry 6*, 2080.
Howard, F. B., Frazier, J., Singer, M. F., and Miles, H. T. (1966), *J. Mol. Biol. 16*, 415.
Huang, W. H., and Ts'o, P. O. P. (1966), *J. Mol. Biol. 16*, 523.
Jardetzky, O. (1964), *Advan. Chem. Phys. 7*, 499.
Jardetzky, O., and Wade-Jardetzky, N. G. (1965), *J. Mol. Pharm. 1*, 214 (1965).
Richards, E. G., Flessel, C. P., and Fresco, J. R. (1963), *Biopolymers 1*, 431.
Ts'o, P. O. P., and Huang, W. H. (1968), *Biochemistry 7*, 2954 (this issue; preceding paper).
Ts'o, P. O. P., Rapaport, S. A., and Bollum, F. J. (1966), *Biochemistry 5*, 4153.

Role of the Net Electrical Charge of the Complete Antigen in Determining the Chemical Nature of Anti-*p*-azobenzenearsonate Antibodies*

Erwin Rüde,† Edna Mozes, and Michael Sela

ABSTRACT: Poly-ε-*N*-trifluoroacetyl-L-lysyl ribonuclease was prepared from ribonuclease and ε-*N*-trifluoroacetyl-α-*N*-carboxy-L-lysine anhydride. The free amino groups of the polypeptidyl protein were trifluoroacetylated, and the resulting molecules were conjugated through their tyrosine and histidine residues with the diazonium salt derived from arsanilic acid. After removal of the trifluoroacetyl groups, the basic *p*-azobenzenearsonate-poly-L-lysyl ribonuclease was used for immunization of rabbits. Most of the anti-*p*-azobenzenearsonate antibodies formed were found in the second fraction upon chromatography on DEAE-Sephadex under conditions which separate antibodies according to the net electrical charge of the antigens. In contrast, antibodies of the same specificity induced with the acidic *p*-azobenzenearsonate conjugates of rabbit serum albumin and hexa-L-tyrosine chromatographed mainly in the first fraction. Thus, the antibodies formed reflected the over-all net charge of the molecule, rather than the charge within the limited area around the *p*-azobenzenearsonate determinant in the immunogen, an area similar in its charge properties to similar areas in other *p*-azobenzenearsonate conjugates of proteins. It is concluded that the antigenic control of the antibody type formed (DEAE-Sephadex chromatography detection) occurs at the level of the complete antigenic molecule.

Antibodies from different rabbit antisera distribute unequally among two immunoglobulin G fractions obtained upon chromatography on DEAE-Sephadex (Sela *et al.*, 1963a). From a detailed study of rabbit antisera to lysozyme, obtained under a variety of conditions, it was concluded that the distribution of antibodies among the two chromatographic fractions is controlled by the chemical properties of the antigen, rather than by the course of immunization, the span of time elapsed between the immunization and the collection of the antisera, or the genetic makeup of the animals (Sela and Mozes, 1966; Sela, 1966). The antibodies to lysozyme were found predominantly, or exclusively, in the second chromatographic immunoglobulin G fraction on DEAE-Sephadex, and immunospecifically purified antilysozyme antibodies were found only under the second peak (Sela, 1967).

The extension of this investigation to antibodies produced by a variety of natural and synthetic antigens led to the conclusion that there is an inverse correlation

* From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel. *Received April 8, 1968.*

† Visiting scientist from the Max-Planck-Institute for Immunobiology, Freiburg, Germany.