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44. Teisuke Okano,*[1] Takao Iwaguchi,*[2] and Satoshi Mizuno*[3] :

Pairing of Monomeric Units of Nucleic Acids. 1. Stoichiometric Interactions of Complementary Nucleosides in Methanol. Detection through Conductance Measurements (Studies on Heterocyclic Compounds. V.*[4]).

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With the object of obtaining informations about stoichiometry and specificity of interactions of unit monomers of nucleic acids in solution, specific conductance of mixed methanol solutions of nucleosides of various molar ratios was measured. Maximum interactions were indicated by the minimum or the inflection point in the conductance-molar ratio relationships, and equimolar interactions were detected in the two component systems of uridine-adenosine, cytidine-adenosine, cytidine-guanosine, deoxythymidine-deoxyadenosine, and deoxycytidine-deoxyguanosine, and also in the three component systems of uridine-cytidine-guanosine. No interaction was observed between uridine and guanosine, deoxythymidine and guanosine, or uridine and inosine.

The whole experimental results were explainable consistently in terms of co-operative hydrogen bonding. Four types of the sets of two hydrogen bonds were classified, and it was clarified that while the hydrogen bond sets were consisted exclusively of N-H····O bond in the negative cases of interaction, N-H····N bond was common to the hydrogen bond sets of the systems which showed positive results. The cause of negative results was ascribed to the O-H····O bond formed between keto group of a base and surrounding methanol molecules, which might interfere with pairing of base moieties of nucleosides.

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The doubly stranded helical model proposed by Watson and Crick,[1)] though slight reformation has been made upon it,[2)] has corroborative evidences through X-ray diffraction studies and chemical analysis of DNA, and is generally accepted for the secondary structure of DNA *in vivo*. One of the essential requisites for the composition of this model is the formation of specific hydrogen bonds between complementary purine and pyrimidine base moieties which stand opposite to each other on each strand of the double helix. Neverthless, any attempts to demonstrate the specific

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1) J.D. Watson, F.H.C. Crick : Nature, **171**, 737 (1953); *Idem* : *Ibid.*, **171**, 964 (1953); F.H.C. Crick, J.D. Watson : Proc. Roy. Soc., London A, **223**, 80 (1954).

2) R. Langridge, H.R. Wilson, C. Hooper, M.H.F. Wilkins, L.D. Hamilton : J. Mol. Biol., **2**, 19 (1960), and other papers.

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base pairings in solution at the level of unit monomers, *i.e.*, that of nucleobases, nucleosides or mononucleotides, have not been achieved until a recent date, although X-ray crystallographic³⁾ and infrared⁴⁾ evidences of monomer-monomer interactions in solid state have been accumulated by making use of mixed crystals of nucleosides or N-alkylated nucleobases.

Levedahl, *et al.*⁵⁾ compared rotatory dispersion of individual nucleosides with the mixture of nucleosides in aqueous solution, but found no detectable interaction between deoxyadenosine and deoxythymidine, or deoxyguanosine and deoxycytidine. Miles'⁶⁾ infrared studies with the mixture of sodium salts of uridylic and adenylic acid in heavy water did not point out any interaction of those mononucleotides. Ts'o, *et al.*⁷⁾ studied interaction and association of bases and derivatives in aqueous solutions of relatively high concentration ($>0.1M$). The association did not proceed stoichiometrically, and the vertical stacking interactions, in opposition to the horizontal hydrogen bonding interactions, had been proposed for the mode of association, whether it is concerned with self-association of molecules or with interactions between two molecular species. Hamlin, *et al.*⁸⁾ found that a series of new absorption bands appears in the infrared spectrum when dilute deuterochloroform solutions of 9-ethyladenine and 1-cyclohexyluracil are mixed together, which is ascribable to the formation of hydrogen-bonded complex of 1:1 molar ratio.

Such are the works hitherto done,*5 and the aims of the present study is to obtain any informations about stoichiometry and specificity of hydrogen bonding interactions of the unit monomers of nucleic acids in solution by another means of approach.

As referred to above, the bases are stacked with each other in aqueous media when concentration of the solution is high and no interaction is detectable in dilute solution. Since it was considered to be probable that base pairing in dilute aqueous solution is interfered by hydrogen bonding between individual interactant and surrounding water molecules, the authors used methanol as the solvent for interactants because of its low dielectric constant ($\varepsilon=32.63$, 25°) compared with water. Nucleosides were chosen as interactants because participation of extra imino group of nucleobase or the ionic phosphate group of nucleotide was considered to make things intricate. The authors adopted conductance measurement for the detection of interaction of nucleosides because of unsuitability of usual ultraviolet or infrared spectroscopic techniques.

Results and Discussion*6

Table I shows specific conductance values of mixed methanol solutions of nucleosides. In the mixed systems of Cyd-Guo, Urd-Ado, Cyd-Ado, and dCyd-dGuo, specific

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*5 After the completion of this work, we were aware of the reports of L. Katz and S. Penman (J. Mol. Biol., **15**, 220 (1966)) and of C. Helene (Biochem. Biophys. Res. Commun., **22**, 237 (1966)). The formers observed interactions of nucleosides in dimethyl sulfoxide and in dimethyl sulfoxide-benzene by means of proton magnetic resonance spectroscopy, and the latter observed interactions of nucleosides or derivatives in frozen aqueous mixtures by emission spectroscopy.

*6 Symbols for monomeric units of nucleic acids used in this section are as follows: A, C, G, I, T, and U stand for adenine, cytosine, guanine, hypoxanthine, thymine, and uracil, respectively; Ado, Cyd, Guo, Ino, and Urd for ribonucleosides of A, C, G, I, and U, respectively; dAdo, dCyd, dGuo, and dThd for deoxyribonucleosides of A, C, G, and T, respectively.

3) K. Hoogsteen: Acta Cryst., **12**, 822 (1955), and many papers.
4) Y. Kyogoku, W. Tsuboi, T. Shimanouchi, I. Watanabe: Nature, **189**, 120 (1961).
5) B.H. Levedahl, T.W. James: Biochem. Biophys. Acta, **26**, 89 (1957).
6) H.T. Miles: *Ibid.*, **30**, 324 (1958).
7) P.O.P. Ts'o, I.S. Melvin, A.C. Olson: J. Am. Chem. Soc., **85**, 1289 (1963); P.O.P. Ts'o, S.I. Chan: *Ibid.*, **86**, 4176 (1964); S.I. Chan, M.P. Schweizer, P.O.P. Ts'o, G.K. Helmkamp: *Ibid.*, **86**, 4182 (1964); M.P. Schweizer, S.I. Chan, P.O.P. Ts'o: *Ibid.*, **87**, 5241 (1965).
8) M. Hamlin, Jr., R.C. Lord, A. Rich: Science, **148**, 1735 (1965).

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conductance gave minimum values at the equimolar points. And as shown in Fig. 1, the conductance-concentration curve of mixed systems of dThd-dAdo is inflected at the equimolar point. However, neither minimum nor inflection point was obtained from the data of mixed solutions of Urd and Guo, dThd and Guo, and Urd and Ino. In the case of three component system of Urd-Cyd-Guo, where uridine was added to equimolar mixture of cytidine and guanosine, specific conductance gave the minimum value at the equimolar ratio of these three nucleosides.

Table I. Specific Conductance of Mixed Methanol Solutions of Nucleosides of Various Molar Ratios

Component nucleosides	Temp. (±0.01°C)	Concentration ($10^{-4}M$) (A)	(B)	(C)	Molar ratio (A):(B):(C)	k (10^{-7}ohm^{-1}cm^{-1})
Cyd (A) Guo (B)	0.5	2.5	12.5	—	1 : 5 —	8.3
	0.5	5.0	10.0	—	1 : 2 —	7.2
	0.5	7.5	7.5	—	1 : 1 —	6.8
	0.5	10.0	5.0	—	2 : 1 —	8.8
	0.5	12.5	2.5	—	5 : 1 —	9.7
Cyd (A) Ado (B)	0	4.8	0	—	— — —	8.0
	0	4.8	1.6	—	3 : 1 —	7.8
	0	4.8	2.4	—	2 : 1 —	7.1
	0	4.8	4.8	—	1 : 1 —	6.8
	0	4.8	9.6	—	1 : 2 —	8.4
dCyd (A) dGuo (B)	−16	6.0	2.0	—	3 : 1 —	2.4
	−16	6.0	3.0	—	2 : 1 —	2.1
	−16	6.0	6.0	—	1 : 1 —	2.0
	−16	6.0	12.0	—	1 : 2 —	2.3
	−16	6.0	18.0	—	1 : 3 —	2.9
Urd (A) Guo (B)	0	2.0	6.0	—	1 : 3 —	1.8
	0	3.0	6.0	—	1 : 2 —	2.0
	0	6.0	6.0	—	1 : 1 —	2.3
	0	12.0	6.0	—	2 : 1 —	3.4
dThd (A) Guo (B)	0	2.0	6.0	—	1 : 3 —	2.5
	0	3.0	6.0	—	1 : 2 —	2.5
	0	6.0	6.0	—	1 : 1 —	2.6
	0	12.0	6.0	—	2 : 1 —	2.7
	0	18.0	6.0	—	3 : 1 —	2.9
Urd (A) Ino (B)	0	2.0	6.0	—	1 : 3 —	2.3
	0	3.0	6.0	—	1 : 2 —	2.4
	0	6.0	6.0	—	1 : 1 —	2.5
	0	12.0	6.0	—	2 : 1 —	2.8
	0	18.0	6.0	—	3 : 1 —	3.1
Guo (A) Cyd (B) Urd (C)	0.5	5.0	5.0	1.0	5 : 5 : 1	11.1
	0.5	5.0	5.0	2.5	2 : 2 : 1	10.4
	0.5	5.0	5.0	5.0	1 : 1 : 1	8.4
	0.5	5.0	5.0	10.0	1 : 1 : 2	8.8
	0.5	5.0	5.0	15.0	1 : 1 : 3	9.7
	0.5	5.0	5.0	20.0	1 : 1 : 4	11.0

Thus the stoichiometry of the interaction of nucleosides in methanol is as shown in Table II.

Although the present method gives us stoichiometry of interaction, it cannot afford, in its nature, to give us any direct informations about the binding sites of the molecules. However, it may be possible to obtain some informations about the mode of interaction by compairing the experimental results with chemical structure of the interactants.

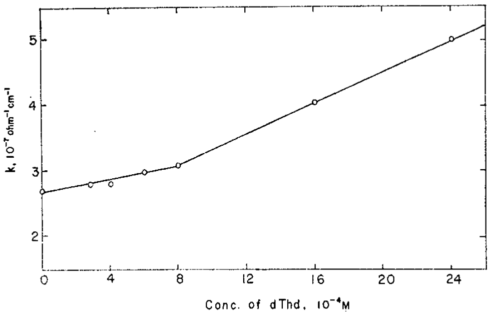

Fig. 1. Specific Conductance of Mixed Methanol Solutions of dAdo (8×10^{-4} *M*) and dThd at 0°

Table II. Stoichiometry of Detected Interactions between Nucleosides

Nucleoside pair	Molar ratio
Urd-Ado	1 : 1
Gyd-Ado	1 : 1
Cyd-Guo	1 : 1
dThd-dAdo	1 : 1
dCyd-dGuo	1 : 1
Guo-Cyd-Urd	1 : 1 : 1

The relations between specific conductance and concentration, and also equivalent conductance and square root of molar concentration of nucleosides are shown in Fig. 2 and Fig. 3, respectively, which indicate that nucleosides behave as weak electrolyte in methanol. There may exist equilibrium among molecules, ions, and intermoleculary associated species. It is, however, hardly conceivable that the observed decrease in the specific conductance value of the mixtures of nucleosides was resulted from mere acid-base interactions, because interactions have been detected not only with mixtures of two nucleosides which can afford to make ionic pairs, but also with the mixture of cytidine and adenosine, both of which have only base moieties of proton-accepting properties. Table I shows a remarkable fact that in all the cases of positive results of interaction, at least one of the pair carries 6-amino group in the base moiety with it, while the negative results of interaction are limited to systems whereof both interactants are 6-keto derivatives. 6-Amino base and 6-keto base have necessarily the partial structures $NH_2\text{-}\overset{6}{\underset{|}{C}}{=}\overset{1}{N}\text{-}$ and $O{=}\overset{6}{\underset{|}{C}}\text{-}\overset{1}{N}H\text{-}$, respectively. Thereupon, it seems that specificity of interaction and the whole experimental results are expected to be elucidated rather in terms of specific hydrogen bonding interactions.

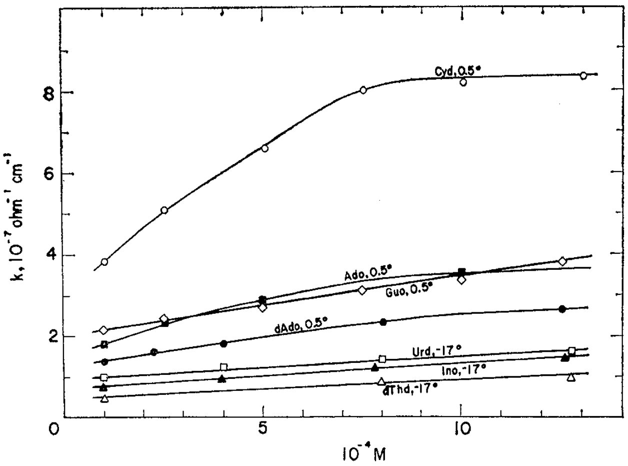

Fig. 2. Relation between Specific Conductance and Concentration of Methanol Solutions of Nucleosides

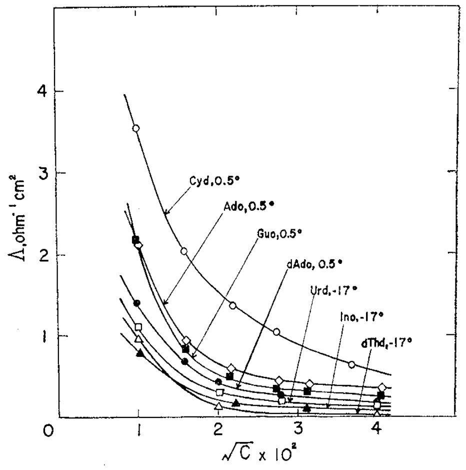

Fig. 3. Relation between Equivalent Conductance and Square Root of Molar Concentration of Methanol Solutions of Nucleosides

Donohue, *et al.*,[9] from pure mathematical point of view, found that a total of twenty-nine base pairings, each of them being stabilized by two hydrogen bonds, are

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[9] J. Donohue: Proc. Nat. Acad. Sci., **42**, 60 (1956); J. Donohue, K. N. Trueblood: J. Mol. Biol., **2**, 363 (1960).

sterically feasible among the combinations of any two of the normal four bases of DNA.

The varieties of hydrogen bonds which appear in the Donohue's schemes are limited to either N-H···N or N-H···O, and the set of two hydrogen bonds which join two bases X and Y together, after all, falls under one of the four types shown in Fig. 4, which will be called types a, b, c and d as indicated. For instance, hydrogen bond sets of two A-C pair schemes given by Donohue, *et al.*[9)] may be expressed as shown in Fig. 5, and the both may be counted as type a. The hydrogen bond set of A-T pair in Watson-Crick model corresponds to type b.

Now let us scrutinize the experimental results from the point of view mentioned above. In Table III the base pairs studied are arranged in comparison with the corresponding sets of hydrogen bonds which have been found to be feasible by Donohue, *et al.*

$$\mathrm{X}\left\{\begin{array}{c}\mathrm{N{-}H\cdots N}\\ \mathrm{N\cdots H{-}N}\end{array}\right\}\mathrm{Y} \qquad \mathrm{X}\left\{\begin{array}{c}\mathrm{N{-}H\cdots O}\\ \mathrm{N\cdots H{-}N}\end{array}\right\}\mathrm{Y}$$

Type a Type b

$$\mathrm{X}\left\{\begin{array}{c}\mathrm{N{-}H\cdots O}\\ \mathrm{N{-}H\cdots N}\end{array}\right\}\mathrm{Y} \qquad \mathrm{X}\left\{\begin{array}{c}\mathrm{N{-}H\cdots O}\\ \mathrm{O\cdots H{-}N}\end{array}\right\}\mathrm{Y}$$

Type c Type d

Fig. 4. Four Types of Hydrogen Bond Sets

For description see text.

$$\mathrm{Ade}\left\{\begin{array}{c}\mathrm{(6)\,N{-}H\cdots N\,(1)}\\ \mathrm{(1)\,N\cdots H{-}N\,(6)}\end{array}\right\}\mathrm{Cyt} \qquad \mathrm{Ade}\left\{\begin{array}{c}\mathrm{(6)\,N{-}H\cdots N\,(1)}\\ \mathrm{(7)\,N\cdots H{-}N\,(6)}\end{array}\right\}\mathrm{Cyt}$$

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Fig. 5. Feasible Sets of Hydrogen Bonds between Ade and Cyt

Figures in the parentheses indicate the position of the electronegative atoms in the molecule.

Table III. Comparison of Base Pairs with the Hydrogen Bond Sets

Base pair		Groups at position-6		Types of H-bond set[a)]	Interaction
(A)	(B)	(A)	(B)		
Ade	Cyt	NH_2	NH_2	a, a[b)]	+
Ade	Ura	NH_2	C=O	b, b, b, b	+
Ade	Thy	NH_2	C=O	b, b, b, b	+
Cyt	Gua	NH_2	C=O	b, c, c	+
Gua	Thy	C=O	C=O	d, d	−
Hypoxanthine	Ura	C=O	C=O	d, d	−

a) See Fig. 4.
b) Two a's indicate that there are two different forms of interactions as shown in Fig. 5, for example.

It is remarkable from the table that while sets of hydrogen bonds in the negative cases of interaction are limited to type d, those of positive cases of interaction fall under type a, b, or c, but not d. Type d set differs from others in that it is consisted exclusively of N-H···O bonds, while N-H···N bond is common to other types of set. This is closely related to the fact that if both bases of a pair are 6-keto compounds the resulting set of bonds cannot be other than type d, but if at least one of a pair is

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6-amino compound the resulting set of bonds cannot be type d. So that it may be reasonable to attribute the cause of difference of the experimental results to the differences in the combination of two hydrogen bonds. And it is quite within the range of possibilities that the negative results of interaction might have come from interference by the O-H···O hydrogen bonds formed between keto group of a base and surrounding methanol molecules, which is predominant over N-H···O bond between two bases.*[7]

Thus the whole experimental results are explainable consistently in terms of hydrogen bonding. That is to say, the observed decrease of specific conductance could be taken as indication of formation of complexes stabilized by two co-operative hydrogen bonds between the component nucleosides concerned. The decrease of specific conductance may have resulted from suppression of molecular dissociation caused by intermolecular association, which brings about a shift of equilibrium to the direction of diminution of ionic species, and the minimum or the inflection values may be regarded as indication of maximum interactions at these points.

In connection with the results mentioned above, Fig. 2 gives us a noteworthy fact that while specific conductance-concentration curve of guanosine, uridine, thymidine, and inosine are linear, those of cytidine, adenosine, and deoxyadenosine, whose base moieties have amino group at the position-6, are not linear. The unlinearity may possibly come from association of identical bases through combination of the proton-electron pair codes[10] in a manner expressible with type a hydrogen-bond set. Pairing of identical bases have been postulated by Stent[11] for the identical RNA duplex which could serve as a template for the directed synthesis of fresh DNA.

It is noticeable that the base pairings proposed for the duplication of DNA and RNA, *i.e.*, A-T, A-U, and G-C pairs, have not been ruled out from the positive results of interaction. Other types of base pairings than Watson-Crick's, such as A-C and U-C-G, could well be detected under the present experimental conditions. A-C pair has been proposed by Donohue, *et al.*[9] as one of the twenty-nine base pairs, and Zubay's[12] cyclic scheme which was built for the elucidation of directed synthesis of RNA on template DNA may be presented for the configuration of the observed equimolar triplet of U-C-G.

The experimental arrangements shown here may provide a new way of approach to the nature of specific interaction of nucleic acid bases, and further researches in this direction are under persuit with the hope of obtaining more detailed informations.

Experimental

Materials and Solutions

Methanol——Commercial methanol of special grade was used with further purification by distillation; specific conductance, 0.8×10^{-7} ohm^{-1}cm^{-1} (20°); content of water, 0.03% by Karl Fischer's method.

Nucleosides——Ribonucleosides were obtained from Tokyo Kasei Co., and deoxyribonucleosides from N.B.C.; all of them were recrystallized and dried *in vacuo*.

Stock Solutions——Purine nucleosides or pyrimidine nucleosides were dissolved in a small amount of methanol in volumetric flasks at room temperature without shaking or stirring. After these were dissolved completely, the solutions were diluted by addition of methanol to the final concentration of either 2×10^{-3} *M* (purine nucleosides) or 10^{-2} *M* (pyrimidine nucleosides).

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*[7] Order of strength of hydrogen bonds may be estimated provisionally as follows: O-H···O>O-H···N>N-H···O>N-H···N (cf. G.C. Pimental, A.L. McClellan: "The Hydrogen Bond," pp. 285～293 (1960), W.H. Freeman and Co., San Francisco).

10) P. Löwdin: Biopolymers Symposia, No. 1, "Quantum Aspects of Polypeptides and Polynucleotides, pp. 163 (1964), Interscience Publishers, Easton, Penn.

11) G. Stent: Adv. Virus Res., **5**, 95 (1958).

12) G. Zubay: Nature, **182**, 1290 (1958).

Preparation of Mixed Solutions by Continuous Variation Method——In the preparation of mixed solutions of various molar ratios of guanosine and cytidine, total concentration of nucleoside was kept at 1.5×10^{-3} M.

Preparation of Mixed Solutions by Molar Ratio Method——In the preparation of mixed solutions of nucleosides other than those of guanosine and cytidine, purine nucleosides were kept at a fixed concentration between 5×10^{-4} and 8×10^{-4} M, and concentration of pyrimidine nucleosides was changed.

Measurements of Conductance

Conductance apparatus of the type CMID and electrodes of the type CG-2001-PL supplied by Towa Dempa Co. were used in these experiments. Platinum-black was left off from thin platinum plate electrodes. Haake Ultra-thermostat equipped with a refrigerator was used as a bath. After the electrodes were dipped into a cell filled with a definite amount of methanol solution of nucleoside, the cell was sealed with a stopper and stored under slight nitrogen bubbles for about 20 min. in the bath. Conductance was measured when equilibrium of temperature was reached.

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