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Nuclear Magnetic Resonance Study of the Interactions of Guanosine and Cytidine in Dimethyl Sulfoxide

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Abstract: Guanosine forms hydrogen-bonded dimers in DMSO. The equilibrium constant for dimer formation is 0.18 l./mol at 32°, and there is no evidence of higher order aggregates. The structure of the dimer is probably similar to the hydrogen bonding thought to occur in guanylic acid gels but it is not possible from our results to assign a unique hydrogen-bonding scheme. Cytidine and 1-methylguanosine show little or no evidence of dimer formation in DMSO. When both guanosine and cytidine are present in DMSO a Watson-Crick hydrogen-bonded dimer results. The equilibrium constant for dimer formation is 3.7 ± 0.6 at 32°, and the enthalpy of formation is about −6 kcal/mol. There is no evidence of base stacking or the formation of larger hydrogen-bonded complexes in DMSO. Thus this solvent is an excellent system in which to study the effects of base pairing on the formation of specific complexes between mono- or oligonucleotides.

A basic feature of the macromolecular structure of nucleic acids is the occurrence of guanine–cytosine and adenine–thymine (or uracil) base pairs.[1] It would be of considerable interest to know just how much the interaction between two hydrogen-bonded bases contributes to the stability of double strand DNA or RNA helices.[2] One would also like to understand whether the specificity involved in base-pair formation is a result of the structures of the individual monomers themselves, or is mostly due to the constraints of forming monomers into an ordered helix. This is of special importance when we consider the possible types of interactions which might arise in a transfer RNA or other nucleic acid that is less than 100% double helix. It is not surprising that there have been a large number of studies on the interactions between nucleic acid bases or nucleosides.[3-14] These represent the simplest systems which may yield information about the specificity of base–base interactions.

Mononucleosides can be expected to interact by either base stacking or hydrogen bonding. There is a great deal of evidence that vertical stacking interactions occur between mononucleosides in aqueous solution. Ts'o and his collaborators have pioneered efforts to determine the extent of these interactions by studying the nuclear magnetic resonance (nmr) of the non-exchangeable base protons.[3-7] It would also be of interest to determine the enthalpy and free energy of hydrogen bonding between bases in aqueous solution. Here considerable competition from the solvent is expected. In fact, DeVoe and Tinoco have suggested that the enthalpy for hydrogen bonding may be positive in aqueous solution.[2] Little evidence exists for the formation of specific hydrogen-bonded complexes between mononucleosides in water. That a small amount of base pairing may occur is suggested by the chromatographic studies of Tuppy and Kuchler.[14] Unfortunately it is not easy to study hydrogen bonding in water by nmr because of the rapid exchange of the amino protons with the solvent. When mixtures of complementary mononucleosides or even dinucleotides or trinucleotides are examined by optical techniques at the low concentrations necessary for the measurements, usually no complex formation is apparent. At very high concentrations ApApA:UpUpU and GpCpC:GpGpC will form complexes but these are 1:2 instead of the 1:1 pairing found in DNA and some viral RNA's.[15,16]

To be able to study the properties of specific hydrogen-bonded complexes one must find solvents in which these complexes are easily formed. Kyogoku, *et al.*, have shown that the equilibrium constant for the formation of hydrogen bonds between adenine and uracil derivatives or guanosine and cytidine derivatives in chloroform solution at room temperature is greater than 100 l./mol.[10] The enthalpy of formation of the A:U pair is −6.2 kcal/mol. Dimethyl sulfoxide (DMSO) is a very strong proton acceptor and we would expect the degree of hydrogen bonding in this solvent to be far more similar to interactions in water than in chloroform. Previous nmr studies have shown that hydrogen bonding occurs between guanosine and cytidine in DMSO and that there is no base stacking in this solvent.[8] However, only qualitative statements about the existence of a complex could be made. We have extended the studies in DMSO to

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(13) J. Pitha, R. N. Jones, and J. Pithova, *Can. J. Chem.*, **44**, 1044 (1966).
(14) H. Tuppy and B. Kuchler, *Biochim. Biophys. Acta*, **80**, 669 (1964).

(15) G. Felsenfeld and H. T. Miles, *Ann. Rev. Biochem.*, **36**, 407 (1967).
(16) S. R. Jaskunas, C. R. Cantor, and I. Tinoco, Jr., manuscript in preparation.

obtain the interaction enthalpy, association constant, and identification of the structure of the complex. Katz and Penman observed that the chemical shift of the guanosine amino protons was linearly proportional to the mole fraction of cytidine at constant total nucleoside concentration.[8] We show below that this linear dependence should occur for the formation of 1:1 complex if the formation constant is small. The slope of the line is equal to the product of the equilibrium constant and the chemical shift of the amino protons in the 1:1 complex. We have obtained data at a large number of additional concentrations in order to determine both the equilibrium constant and the chemical shift of the complex.

In addition to GC pairing we have also observed the hydrogen-bonded association between two guanosines or two cytidines. The former interaction represents an unusual type of base pairing which is thought to stabilize aggregates of guanylic acid, oligoguanylates, and poly G. Our results permit several of the possible structures for the guanosine dimer to be excluded but they cannot clearly distinguish between the two remaining base-pairing schemes. These studies are part of a long-term effort to compile a library of nmr data on simple model compounds which will be useful in interpreting spectra of synthetic and natural polynucleotides.

Theory

Nmr can be used to study the chemical equilibrium in any reaction in which the chemical shift of a magnetic nucleus in the product differs from the chemical shift of the same nucleus in the reactant.[17] If there is a rapid equilibrium between products and reactants, then we observe only the weighted average chemical shift in the nmr. Hydrogen bonding probably provides the best example of the use of nmr in this situation because the chemical shift of a hydrogen-bonded proton normally moves downfield over 1.5 ppm from a non-hydrogen bonded proton and these shifts can readily be measured to ±0.005 ppm. Huggins, et al., first described the use of nmr to study hydrogen bonding.[18] We will briefly describe the theory as it applies to the calculations performed below.

If we assume that only a 1:1 complex, GC, forms, then the equilibrium constant for the reaction $G + C \rightleftarrows GC$ is given by

$$K = \frac{x}{(G_0 - x)(C_0 - x)} \tag{1}$$

where x is the concentration of the complex and G_0 and C_0 are the initial concentrations of guanosine and cytidine, in moles/liter. The averaged chemical shift of the guanosine protons is given by

$$\delta_{obsd} = \frac{G_0 - x}{G_0}\delta_G + \frac{x}{G_0}\delta_{GC} \tag{2}$$

where δ_G is the chemical shift of pure guanosine and δ_{GC} the chemical shift of the guanosine protons in the complex. To simplify the equations, we choose our reference for an equation of the above form to be the chemical shift of the pure species. Then

(17) J. W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Nuclear Magnetic Resonance Spectroscopy," Vol. 1, Pergamon Press, Ltd., London, 1965, Chapter 9.

(18) C. M. Huggins, G. C. Pimentel, and J. N. Schoolery, *J. Chem. Phys.*, 23, 1244 (1955).

$$\Delta_{obsd} = \frac{x}{G_0}\Delta_{GC} \tag{3}$$

where

$$\Delta_{obsd} = \delta_{obsd} - \delta_G$$

and

$$\Delta_{GC} = \delta_{GC} - \delta_G$$

We wish to determine K from measurements of Δ_{obsd} as a function of concentration. The standard technique is to prepare solutions which are very dilute in one component. If dilute guanosine solutions are prepared, then $x < G_0 \ll C_0$. Equation 1 reduces to $x = KC_0[G_0 - x]$. Solving for x and substituting into eq 3 gives

$$\Delta_{obsd} = \frac{KC_0}{1 + KC_0}\Delta_{GC} \tag{4}$$

If Δ_{obsd} is determined at two different cytidine concentrations, then eq 4 can be written as two equations in the two unknowns, K and Δ_{GC}, for two sets of data. This method gives an equilibrium constant subject to considerable experimental error for two reasons. The guanosine N_1H resonance is very broad, and the NH_2 resonance overlaps the cytidine NH_2 in solutions containing a much larger cytidine concentration than guanosine concentration.

To obtain a second set of results, solutions dilute in cytidine were prepared. Then $x < C_0 \ll G_0$ and eq 1 gives $x = KG_0[C_0 - x]$. The analysis is exactly the same as the derivation of eq 4 and yields

$$\Delta_{obsd} = \frac{KC_0}{1 + KG_0}\Delta_{GC} \tag{5}$$

A plot of Δ_{obsd} against C_0 gives a straight line with slope

$$S_{obsd} = \frac{K}{1 + KG_0}\Delta_{GC} \tag{6}$$

This time, solutions with two different initial guanosine concentrations must be studied as a function of cytidine concentration in order to obtain two equations in K and Δ. Unfortunately this second method also gives an equilibrium constant subject to considerable error because both guanosine and cytidine self-associate in solution.

The equilibrium constant for the association reaction $G + G \rightleftarrows G_2$ is given below. The concentration of complex is y. The chemical shifts due to association
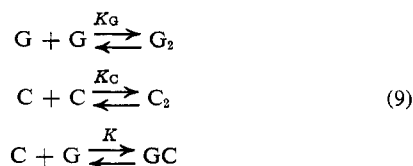
$$K_G = y/(G_0 - 2y)^2 \tag{7}$$

are much less than the shifts observed in mixtures of guanosine and cytidine. The assumption that K_G is small is valid independent of concentration. Thus $y \sim K_G(G_0)^2$ and

$$\Delta_{obsd} = K_G G_0 \Delta_{GC} \tag{8}$$

An additional factor of 2 is inserted in eq 8 if a cyclic dimer is assumed in which there are twice as many protons observed in the complex as in the monomer. A plot of Δ_{obsd} against G_0 gives a straight line under these conditions. If an equilibrium involving monomers and tetramers is postulated, then $K_G \sim y/(G_0)^4$ and $\Delta_{obsd} = 4K_G(G_0)^3\Delta_G$. In this case Δ_{obsd} is not linearly

proportional to the guanosine concentration. The experimental results show that only dimers are formed in DMSO solution (*vide infra*), but it is not possible to obtain both K_G and Δ_{GG} from the experimental data. We assume that $\Delta_{GG} = \Delta_{GC} = \Delta_{CC}$ in the following, and discuss this assumption further below.

In mixtures of guanosine and cytidine the simultaneous equilibria which occur are given by

$$G + G \underset{}{\overset{K_G}{\rightleftharpoons}} G_2$$

$$C + C \underset{}{\overset{K_C}{\rightleftharpoons}} C_2 \qquad (9)$$

$$C + G \underset{}{\overset{K}{\rightleftharpoons}} GC$$

The equations to be solved are

$$K_G = \frac{y}{(G_0 - x - 2y)^2}$$

$$K_C = \frac{z}{(C_0 - x - 2z)^2}$$

$$K = \frac{x}{(G_0 - x - 2y)(C_0 - x - 2z)} \qquad (10)$$

where x, y, and z are the concentrations of guanosine-cytidine dimer, guanosine–guanosine dimer, and cytidine–cytidine dimer, respectively. A simple iteration procedure was used to analyze the data. First, a trial value for the chemical shift of one of the protons of the complex, Δ_{GC}, is guessed. Then K_G and K_C are obtained from eq 8. A trial value for K is then selected. For each solution studied the concentration of the three dimers is determined by a simple iteration procedure using the exact equations (10) for the three equilibria. The chemical shift of each solution is calculated from

$$\Delta_{obsd} = \frac{x + y}{G_0} \Delta_{GC} \qquad (11)$$

The equilibrium constants K and K_G are then systematically changed until the best least-squares fit is obtained between the calculated and observed chemical shifts, Δ_{obsd}. A different value for Δ_{GC} is then guessed and the entire iteration procedure repeated. This procedure determines the best values of Δ_{GC} and K and also allows a reasonable determination of the errors in these values. Allowing K_G to vary greatly improves the least-squares fit of the data, but changes Δ_{GC} and K by <10%. This adjustment is equivalent to allowing Δ_{GG} to differ from Δ_{GC}. Using trial parameters determined from eq 4 and 6 reduced the time required for the calculation to less than 10 sec on the CDC 6400 computer.

Experimental Section

Approximate amounts of guanosine and cytidine were put into volumetric flasks and stored under vacuum overnight to remove adsorbed water. Dimethyl sulfoxide (DMSO) was dried over Linde No. 5A Molecular Sieves. Concentrations were determined from the uv absorbance in neutral buffer using extinction coefficients at λ_{max} of 9010 and 13,650 for cytidine and guanosine, respectively.[19] The extinction coefficient of 1-methylguanosine was determined to be approximately 7700 at 259 mμ. This value may be somewhat low, but it cannot significantly affect any of our

results. Some data was taken in DMSO, but more accurate chemical shifts were obtained in DMSO-d_6. The deuterated solvent was used for most of the measurements. Approximately 1% by volume of DMSO was added to the DMSO-d_6 solutions for a reference lock signal.

All nmr spectra were taken on a Varian HA-100 spectrometer at 100 MHz operating in the frequency sweep mode. Chemical shifts were determined from the internal DMSO lock signal using the Hewlett-Packard No. 5512A counter to determine the difference between the lock frequency and observation frequency to ±0.1 Hz. The molar concentrations were corrected for the change in volume of DMSO as a function of temperature. It was assumed that the volume change was independent of solute concentration.

Circular dichroism measurements were made using the Cary Model 6001 attachment to the Cary 60 spectropolarimeter. A demountable Beckman microcell with a 0.012-mm Teflon spacer was used for measurements in DMSO. Standard 22-mm diameter short-path length cylindrical cells were employed for solutions in $CHCl_3$.

Results and Discussion

Parallel stacking of rings and hydrogen-bonded configurations are expected in solutions of nucleic acid bases. Stacking leads to upfield shifts of 0.5 to 2.0 ppm of all the protons which are oriented above or below the aromatic ring of a second molecule.[20,21] Hydrogen bonding results in the formation of planar base pairs. The ring protons are deshielded 0.15 to 0.30 ppm due to the presence of ring currents in the same plane. The amino and NH protons are further deshielded another 2 to 5 ppm in the hydrogen bond. Ts'o and his coworkers observe substantial upfield shifts of the ring protons of bases in aqueous solution.[4,6] We observe small downfield shifts of the ring protons of guanosine and cytidine in DMSO and large downfield shifts of the amino protons. Since only the average shift from the proton in all possible environments is observed, it is only possible to conclude that stacking is more important than hydrogen bonding in aqueous solutions whereas hydrogen bonding is dominant in DMSO. The excellent agreement between the observed chemical shifts and those calculated from the theory outlined above, which assumes only dimers form, indicates that stacking is not significant in DMSO solutions. The proton spectra of guanosine and cytidine have been studied and the chemical shifts assigned by the Jardetskys.[21] We have studied the concentration dependence of all the amino and ring protons in guanosine, 1-methylguanosine, and cytidine except for the cytidine C_5H which overlaps the $C_{1'}$ proton of the sugar. The chemical shifts are given in Table I.

1-Methylguanosine. The amino protons of 1-methylguanosine move *upfield* as the concentration is increased. If hydrogen bonding occurred between 1-methylguanosines we would expect a downfield shift. On the other hand, DMSO is a strong proton acceptor and we would expect a hydrogen bond between the amino protons and the sulfoxide group. Upon increasing the methylguanosine concentration and decreasing the DMSO concentration the extent of hydrogen bonding to solvent should decrease and the amino resonance should move upfield as observed. Shifts of a similar magnitude and in the same direction have been observed for solutions of chloroform in acetone.[22] (In our solutions the concentration of DMSO

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(21) C. D. Jardetsky and O. Jardetsky, *J. Am. Chem. Soc.*, 82, 222 (1960).
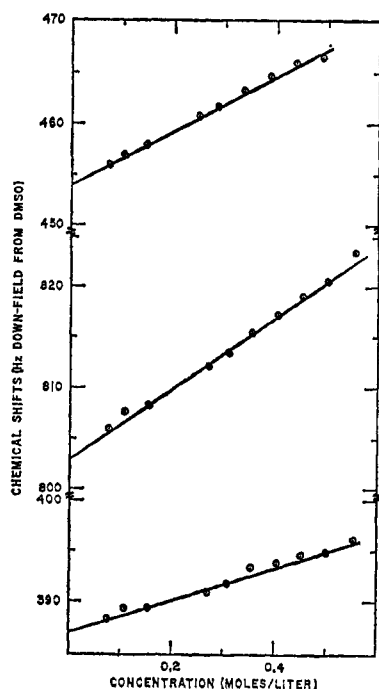(22) I. D. Kuntz and M. D. Johnston, Jr., *ibid.*, 89, 6008 (1967).

Figure 1. Concentration dependence of several proton chemical shifts in solutions of either guanosine or cytidine in DMSO. Shifts are expressed in hertz downfield from DMSO at 100 MHz: upper line, cytidine $-NH_2$; middle line, guanosine $-N_1H$; lower line, guanosine $-NH_2$.

Figure 2. Some of the possible base-paired structures for the dimers G_2, C_2, or GC.[23] Structures I–IV are the four plausible G_2 dimers. Structure V is the Watson–Crick G–C pair. Structure VI is one possible C_2 pair between nonprotonated bases. R is ribose.

only changes from 13 to 15 M; the concentrations of acetone ranged from 0 to 15 M because cyclohexane could be used as an inert solvent in the experiments of Kuntz and Johnston.) The small downfield shift of the ring proton in 1-methylguanosine is probably at-

Table I. Chemical Shifts of the Low-Field Proton Peaks in Guanosine, 1-Methylguanosine, and Cytidine at 32° [a]

Concn, M	NH_2	NH	C_8H	Concn, M	NH_2	C_6H[b]
Guanosine				Cytidine		
0.556	395.9	823.4	541.9	0.493	466.6	526.5
0.503	394.9	820.6	541.6	0.441	465.9	526.6
0.453	394.5	819.0	541.1	0.388	464.7	526.1
0.405	393.7	817.2	540.9	0.338	463.2	525.8
0.355	393.3	815.5	540.3	0.287	461.7	525.5
0.309	391.8	813.5	539.9	0.250	460.8	525.6
0.270	391.0	812.2	539.7	0.149	458.0	525.3
0.154	389.3	808.3	538.7	0.104	456.9	525.2
0.109	389.2	807.6	538.7	0.074	455.9	525.1
0.077	388.2	806.0	538.3	1-Methylguanosine[c]		
0.023	387.8	804.1	537.9			C_8H
0.013	387.2	803.7	538.0	0.316	525.7	684.4
0.007	387.3	804.0	538.0	0.198	528.0	683.7
				0.108	528.5	683.2
				0.068	529.2	682.6

[a] Values are in hertz downfield from DMSO at 100 MHz. Concentrations are ±2% and chemical shifts ±0.3 Hz. [b] Upfield peak of doublet, $J = 7.4$ Hz. [c] The chemical shift of the methyl group of 1-methylguanosine is 149.0 Hz downfield from DMSO and is concentration independent.

tributable to an anisotropic solute–solvent complex formation in which there is a preferred orientation of DMSO relative to the aromatic rings.[22] If the downfield shifts were due to the formation of planar base
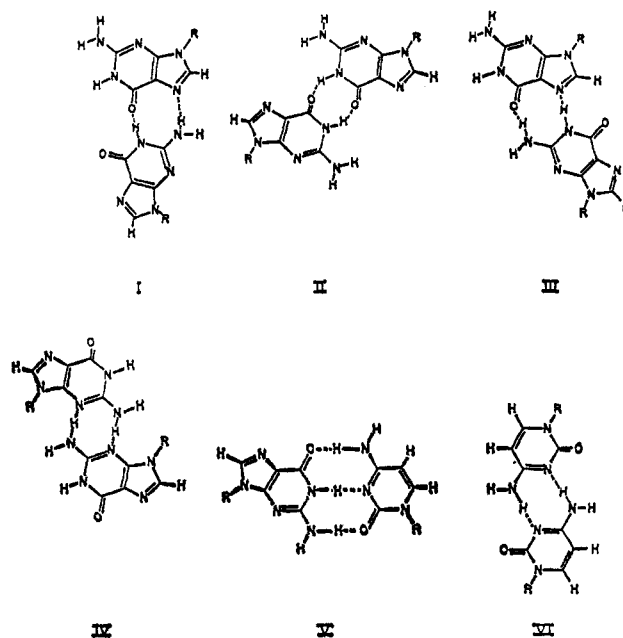
pairs then we would observe much larger downfield shifts of the amino protons.

Guanosine. In solutions of guanosine there are *downfield* shifts of the amino protons with increasing concentrations. These are due to hydrogen bonding between guanosine to form dimers or larger aggregates. These shifts are small compared to those observed in mixtures of guanosine and cytidine. This suggests that only a small fraction of the molecules are engaged in hydrogen bonding. The excellent linearity of the amino and NH shifts as a function of concentration (see Figure 1) shows that only dimers form. If we assume that $\Delta_{CG} = \Delta_{GG}$ (*vide infra*) then the equilibrium constant for guanosine dimer formation is 0.18 l./mol. The change in the N_1H chemical shift is almost twice that of the NH_2 chemical shift and indicates either (a) a number of different hydrogen-bonded structures form such that on the average an NH proton is involved in a bond as often as an NH_2 proton or (b) a specific dimer forms requiring one of the NH_2 protons and the NH proton simultaneously. We observe the average chemical shift of both of the amino protons because of rapid exchange between the protons in the three possible environments—NH_2 protons in a free guanosine and NH_2 protons in the dimer in which one of the protons is forming a hydrogen bond and the other one is free and unshifted. Donahue has proposed two models of guanosine dimers which support explanation b above.[23] Structures I and III are both dimers in which one NH_2 and the N_1H engage in hydrogen bonding. These are shown in Figure 2 along with two other possible dimers: structure II consists of two NH hydrogen bonds and no amino hydrogen bonds and structure IV contains two amino hydrogen bonds and no NH bonding. Experimental evidence exists for guanosine hydrogen-bonded pairs with structure IV in crystals of guanosine hydro-

(23) J. Donahue, *Proc. Natl. Acad. Sci. U. S.*, **42**, 60 (1956).

Table II. Chemical Shifts in Mixtures of Guanosine and Cytidine at 32° [a]

Guanosine concn, M	Cytidine concn, M	Guanosine NH$_2$ shift[b]	Guanosine NH$_2$ line width	Guanosine NH shift[b]	Guanosine NH line width	Guanosine C$_8$H shift[c]	Cytidine NH$_2$ shift[b]	Cytidine C$_6$H shift[c]
0.458	0.0489	15.1 ± 0.3	5.2	34.1 ± 0.3	7.0	541.6	52.1 ± 2.0	539.9
0.458	0.0245	11.1 ± 0.3	5.1	24.7 ± 0.3	5.4	541.3	53.5 ± 4.0	...
0.381	0.0195	9.4 ± 0.3	5.0	21.0 ± 0.3	5.0	540.5	50.6 ± 3.0	538.7
0.381	0.0390	13.4 ± 0.3	5.0	29.2 ± 0.4	6.0	540.8	48.6 ± 3.0	538.6
0.381	0.0782	20.7 ± 0.3	5.6	44.6 ± 0.4	9.5	541.2	47.6 ± 2.0	538.6
0.255	0.245	48.9 ± 0.3	5.5	103.8 ± 0.6	15.5	541.4	39.2 ± 2.0	534.6
0.255	0.0782	21.9 ± 0.3	5.0	46.7 ± 0.6	9.8	540.2	41.0 ± 2.0	535.6
0.127	0.0195	8.0 ± 0.3	4.6	17.6 ± 0.3	4.8	538.8	33.6 ± 2.0	532.4
0.127	0.0390	14.2 ± 0.3	6.5	30.9 ± 0.5	8.3	539.1	32.5 ± 1.0	532.3
0.127	0.0782	25.0 ± 0.3	4.5	53.8 ± 0.3	10.4	539.5	31.3 ± 0.6	531.8
0.127	0.122	34.5 ± 0.3	4.8	73.3 ± 0.6	9.9	539.7	29.3 ± 0.6	531.0
0.0636	0.0098	4.7 ± 0.5	4.0	10.4 ± 0.5	4.3	538.3	21.4 ± 1.0	529.3
0.0636	0.0390	15.0 ± 0.3	4.0	32.4 ± 0.6	7.8	538.4	20.5 ± 0.6	528.9
0.103	0.110	33.2 ± 0.3	5.0	70.7 ± 0.6	12.0	539.6	27.1 ± 0.5	530.5
0.0554	0.0725	25.5 ± 0.3	4.8	54.6 ± 0.6	11.4	539.0	17.9 ± 0.3	528.5
0.0860	0.173	46.7 ± 0.3	5.5	98.7 ± 1.5	19.0	540.6	23.2 ± 0.3	529.8
0.0458	0.146	43.4 ± 0.3	5.3	93.0 ± 1.5	14.8	539.8	15.3 ± 0.3	527.5
0.0094	0.390			159.0 ± 3.0	15.0		13.2 ± 0.3	
0.0094	0.146			98.0 ± 3.0	15.0		14.9 ± 0.3	

[a] Concentrations are ±2%. [b] Δ_{obsd} in Hz downfield from the peak at infinite dilution, at 100 MHz. δ_G = 387.1 Hz downfield from DMSO for the guanosine NH$_2$, 803.0 for the NH, and 453.9 for the cytidine NH$_2$. [c] Chemical shifts downfield from DMSO at 100 MHz. The up-field peak of the cytidine doublet is given.

chloride,[24] and with structure I in gels of 3′- and 5′-guanylic acid.[25] Nash and Bradley have calculated the self-interaction energy of guanosine in vacuo. Their results predict that structure II is most stable; structures I and III are less stable than II, but much more stable than IV.[26]

One possible explanation of our nmr results is that all four structures of Donahue are formed to approximately the same extent in DMSO. 1-Methylguanosine lacks an NH proton and thus can only form structure IV. The upfield shift of the NH$_2$ proton with increasing concentration strongly suggests that hydrogen-bonded complexes do not form in solutions of 1-methylguanosine. This makes structure IV unlikely for guanosine as well. Since structure IV is eliminated as a major contributor to the G$_2$ dimer, structure II can be neglected as well since it is only by having an identical amount of II and IV that the chemical shifts of guanosine NH and NH$_2$ could be kept in the observed ratio of 2:1. We are left with a choice between structures I and III. If good enough data were available these could be distinguished on the basis of the breadth of the resonances since the proton hydrogen bonded to a nitrogen acceptor would experience additional quadrupolar broadening. Our data on this point thus far are inconclusive. It is interesting that guanosine appears to form dimers in DMSO while large aggregates are formed in water. This suggests that a large amount of the free energy of formation of the aggregates must come from base stacking. The C$_8$ proton shifts in solutions of guanosine are in the same direction and of the same magnitude as the C$_8$ proton shifts in methylguanosine. Since guanosine dimers form, but 1-methylguanosine dimers do not, base pairing must have little effect on the resonance of the C$_8$ proton. This conclusion is consistent with the observation of only small shifts in ring protons upon formation of guanosine–cytidine dimers.

Cytidine. Pure cytidine also associates in solution. It seems reasonable that cytidine also dimerizes, but in a form (structure VI of Figure 2) in which one of the amino protons from both monomers is hydrogen bonded. The extra factor of 2 must be inserted in eq 8 to obtain the equilibrium constant for dimer formation. The result is 0.10 l./mol.

Mixtures of Guanosine and Cytidine. The downfield chemical shifts here are an order of magnitude greater than those observed from self-association. Consequently the effect of self-association on the determination of the equilibrium constant is only a small perturbation. The observed chemical shifts for mixtures of guanosine and cytidine in DMSO-d_6 solution are given in Table II.

The large shifts of the amino protons are typical of those expected from hydrogen bonding. The equilibrium constant and chemical shift of the dimer were calculated as outlined above using the guanosine data. From the amino peaks, K = 3.6 and Δ_{GC}(NH$_2$) = 137 Hz. From the chemical shifts of the N$_1$H peaks, K = 4.0 and Δ_{GC}(N$_1$H) = 276 Hz. If the equilibrium constant is changed by ±0.6, the least-squares fit is 50% worse. The average difference between the calculated and observed peaks is 0.6 Hz for the N$_1$H data and 0.3 Hz for the amino data, reflecting the accuracy with which the chemical shifts could be determined.

The chemical shift for the guanosine NH protons is almost exactly twice the shift for the NH$_2$ protons. As in the case of the guanosine association, this shows that only one of the NH$_2$ protons is hydrogen bonded. The structure of the guanosine–cytidine dimer is undoubtedly the well-known Watson–Crick structure with three hydrogen bonds (structure V of Figure 2).[27] Thus, we find that the chemical shift, Δ_{GC}, of the hydrogen-bonded proton is independent of whether the proton is in a N—H···N bond or a N—H···O=C bond. This

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enables us to assume that Δ_{GG} in the guanosine dimer is approximately the same as Δ_{GC} in the mixed dimer. This assumption affects the equilibrium constant for guanosine–cytidine formation by less than 10%, but it enables us to determine the extent of self-association given above.

In all spectra the guanosine peaks give symmetrical Lorentzian-like lines, even when the N_1H peaks are 20 Hz broad. The cytidine NH_2 peak, however, is asymmetric in most of the guanosine–cytidine mixtures. The chemical shifts of the cytidine amino protons in Table II refer to the shift at the maximum absorption of this asymmetric peak. Attempts to fit these apparent chemical shifts to the equations developed above were unsuccessful; for any given value of Δ_{GC} there were always several observed shifts which differed several hertz from the calculated chemical shifts for the best value of K. Since the asymmetry is a function of concentration it is not surprising that the apparent peak location is not a true representative of the extent of hydrogen bonding. An explanation for this asymmetry is provided by the results of Shoup, et al.[12,28] They studied 1-methylcytosine-7-^{15}N. The substitution of ^{15}N for ^{14}N on the cytosine amino group results in a sharp doublet for the amino protons, $J(NH) \cong 90$ Hz. The amino protons are a *pair* of doublets in solutions of this compound with 9-ethylguanine at $-10°$ in a $1:1$ mixture of DMSO and dimethylformamide. In the dimer, rotation of the amino group in cytosine is insufficiently fast to average the amino protons. One doublet of the amino protons is shifted about twice as far downfield as the other doublet with reference to the single doublet observed in pure cytosine at $-10°$. The simplest explanation of these spectra is that rotation of the amino group about the C–N bond is slower than the rate of formation of guanine–cytosine complexes. As a consequence there is a greater probability that the amino proton which engages in hydrogen bonding in the dimer (see Figure 2) will still be adjacent to the cytosine N and hydrogen bond the next time a dimer forms.

Downfield shifts of all the ring protons are also seen (Table II). The cytidine C_6 proton doublet shifts 12 Hz over the concentration range studied in Table II, or about 10% as much as the amino proton shifts. The shifts observed for the guanosine C_8 proton are about a factor of 4 less. The downfield shifts of the ring protons may possibly be explained by hydrogen bonding having an effect similar to protonation of the ring nitrogens.[29a] Additional deshielding due to the presence of a second ring in the same place may also contribute to the shifts. This effect is likely to be small since the six-membered ring of guanosine is not very aromatic[29b] and the five-membered ring is very far from the cytidine C_6 proton. The shifts could also be attributed to the combined anisotropies of all guanosine double bonds.[30] The shift of the C_8 proton in guanosine should be much less because it is further from a cytidine molecule which contains fewer anisotropic groups. The cytidine C_6H shifts are sufficiently large that solvent effects can be neglected. Equations 9 and 10 were used to calculate an equilibrium constant and the chemical shifts of the complex from the observed shifts of the ring protons. The result is $K = 3.5 \pm 0.9$ which agrees well with the equilibrium constant determined from the guanosine data and confirms the assumption that a $1:1$ dimer forms. The shift, $\Delta_{GC}(C_6)$, is 25 Hz.

The large downfield shifts observed at the amino and NH protons are due to a combination of hydrogen bonding and ring currents; it is not possible to separate these two effects in Δ_{GC} calculated for the amino protons. If the ring current contribution were appreciable then the NH chemical shifts would not be approximately twice the NH_2 shifts because the nonhydrogen bonding amino proton is still sufficiently close to the cytidine that it should also experience a downfield shift in the dimer.

Katz and Penman also studied mixtures of guanosine and cytidine in DMSO but all their reported results were at constant total nucleoside concentration equal to 0.5 M.[8] They observed that the chemical shift of the guanosine amino protons is linearly proportional to the cytidine concentration. If $K \lesssim 1$ and the concentrations are much less than 1 M, then eq 1 reduces to $x = KC_0G_0$ and eq 2 predicts that the observed guanosine chemical shift should be linearly proportional to the cytidine concentration. Our calculations show that even when $K = 3.5$ a straight line is predicted for cytidine concentrations up to 0.35 M, at which point the line should curve slightly. It is not necessary to postulate the existence of $2:1$ or $1:2$ complexes of guanosine and cytidine in DMSO to fit within experimental error all the available data on the change in chemical shifts with concentration. The only reasonable structure for a $1:1$ complex in which three hydrogen bonds form involving the cytidine and guanosine NH_2 and guanosine N_1–H is the Watson–Crick arrangement. This contrasts with adenine and uracil mixtures where at high concentrations a $1:2$ complex may form.

DMSO is a very strong proton acceptor and there is undoubtedly considerable hydrogen bonding between the monomer and the solvent. This causes substantial downfield shifts of the reference monomer. All the solutions we have studied contain DMSO in great excess, between 0.95 and (in the extrapolated limit) 1.00 mole fraction. The change in mole fraction of the DMSO is insufficient to determine the interaction between the solute and solvent. Porter and Brey have studied self-association of phenols in DMSO at concentrations up to 0.4 mole fraction phenol and find that the results can be interpreted only if it is assumed that the phenols are either hydrogen bonded to the DMSO or to each other; no free phenol OH protons are observed.[31] They observed no change in the equilibria from the addition of small amounts of water. We also found no change in the chemical shifts upon addition of up to 0.5 M water to either the guanosine or guanosine–cytidine solutions. Additional evidence that the monomers are hydrogen bonded to the solvent is provided by the upfield shift of the N_1H and amino protons as the temperature is increased (see Table III and ref 8). Hydrogen bonding in DMSO is exothermic and should decrease as the temperature increases. Larger chemical shifts for base–base inter-

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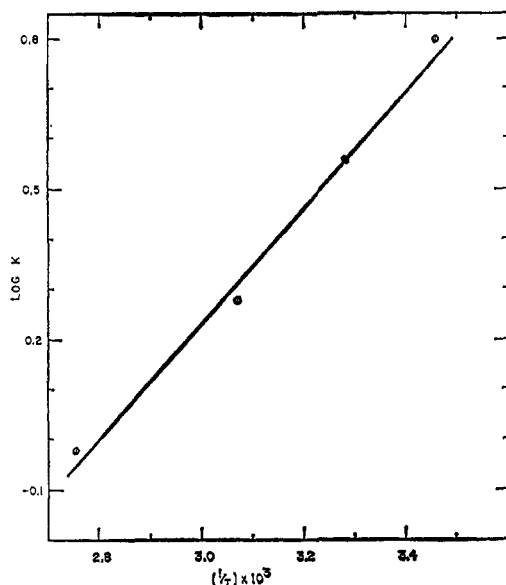(31) D. M. Porter and W. S. Brey, Jr., *J. Phys. Chem.*, **71**, 3779 (1967).

Figure 3. van't Hoff plot of the equilibrium constant for guanosine–cytidine hydrogen-bonded dimer formation in DMSO.

action are observed in chloroform. This is because the reference state in chloroform is at least partially a nonhydrogen-bonded monomer and because the equilibrium constant for dimer formation is larger.[8]

Table III. Chemical Shifts in Mixtures of Guanosine and Cytidine at 53 and 90°

Temp, °C	Guanosine concn, M	Cytidine concn, M	Guanosine[a] NH$_2$	Guanosine[b] C$_8$H	Cytidine[b] C$_6$H
53	0.438	0.0467	13.2	538.0	534.6
	0.365	0.0748	17.4	537.6	533.4
	0.250	0.240	34.2	536.5	528.4
	0.121	0.0750	14.3	534.8	526.0
	0.0436	0.139	31.8	536.6	525.0
90	0.424	0.0452	9.9	532.7	527.2
	0.353	0.0724	12.7	532.5	526.2
	0.242	0.232	25.0	531.4	522.8
	0.117	0.0726	9.3	529.9	520.7
	0.0422	0.134	20.3	531.1	520.1

[a] Δ_{obsd} in hertz downfield from the peak at infinite dilution, at 100 MHz. $\delta_G = 378.9$ Hz downfield from DMSO at 53°; 365.9 Hz at 90°. [b] Chemical shifts downfield from DMSO at 100 MHz. The upfield peak of the cytidine doublet is given.

At the probe temperature (32°) the guanosine NH$_2$ peak width is independent of guanosine–cytidine dimer formation whereas the guanosine N$_1$H proton line width varies between 5 and 20 Hz and is directly proportional to the per cent of dimer formation. This behavior may be attributed to the fact that only the N$_1$H proton forms a hydrogen bond to another nitrogen nucleus, which can cause additional broadening of the proton resonance. It is also possible that hydrogen bonding decreases the relaxation time of the guanosine N$_1$ nitrogen. This would also cause broadening of the resonance.

Variable-Temperature Studies. Experimental results at 53 and 90° for five solutions containing both guanosine and cytidine are given in Table III. The widths of the guanosine N$_1$H and cytidine amino peaks are proportional to the amount of guanosine–cytidine dimer

present. In solutions which are 0.25 M in each base these peaks are over 30 Hz in width at 50°. If the width of the peak is due to quadrupole relaxation of the nitrogen nucleus, then it is expected to increase with temperature because the relaxation time decreases. The guanosine NH$_2$ peak also broadens as the temperature is increased, but its width remains independent of dimer formation. It was only possible to observe the chemical shift of the guanosine amino group and cytidine C$_6$H at the higher temperatures in all five solutions. Spectra were also taken of the individual nucleosides at 53 and 90°. The precision of the data is reduced at these temperatures because the experimental chemical shifts are less, the resonance lines are broader, and small temperature variations cannot be excluded. If both the equilibrium constants and Δ_{GC} are allowed to vary, no one set of values fits the data better than alternative sets in the range $0.1 < K < 4.0$. However, it is possible to obtain reliable estimates of the equilibrium constant by the following considerations.

The chemical shift of the amino proton in the dimer, relative to DMSO, $\delta_{GC}(NH_2)$, should be independent of temperature whereas the chemical shift of the protons in the monomer, $\delta_G(NH_2)$, moves upfield as the temperature is increased because the extent of hydrogen bonding to the solvent decreases. Values of $\delta_G(NH_2)$ are given in footnote a of Table III; $\delta_{GC}(NH_2) = 524$ Hz at 32° from the calculations above. Using these values we obtain $\Delta_{GC}(NH_2) = 145$ Hz at 53° and $\Delta_{GC}(NH_2) = 158$ Hz at 90°. The equilibrium constant K can then be fit to the observed chemical shifts, Δ_{obsd}. We obtained $K = 1.9$ at 53° and $K = 0.95$ at 90°. Katz and Penman studied mixtures of guanosine and cytidine at 16°.[8] Since the experimental values of $\Delta_{GC}(NH_2)$ at 32, 53, and 90° are a linear function of temperature, the results can be extrapolated to 16°, to yield $\Delta_{GC}(NH_2) = 132$ Hz. Then the best value of K to fit the observed chemical shifts at 16° is 6.3.

A van't Hoff plot of the log of the equilibrium constant against T^{-1} is given in Figure 3. The enthalpy for dimer formation is -5.8 kcal/mol and the entropy is -16 eu. Since there are three hydrogen bonds, the enthalpy for each one is only -1.9, but it is important to note that this is the heat of formation of the complex from the solvated monomers. From the chemical shifts of the individual nucleosides as a function of concentration (see eq 8), we obtain slopes $K_G\Delta_{GG}(NH_2)$ equal to 15.9 ± 0.6 at 32°, 13.8 ± 1.5 at 53°, and 12.5 ± 1.5 at 90° for guanosine solutions. Assuming $\Delta_{GG} = \Delta_{GC}$, then $K_G = 0.116, 0.095$, and 0.085, respectively. The van't Hoff plot gives $-\Delta H = 1.0 \pm 1.0$ kcal/mol for self-association of guanosine. For cytidine solutions the slopes $2K_C\Delta_{CC}(NH_2)$ are 27.2 ± 0.5 at 32°, 23.2 ± 2 at 53°, and $14. \pm 3$ at 90°. Then $K_C = 0.10, 0.083$, and 0.053, respectively, and $-\Delta H = 1.7 \pm 1.5$ kcal/ mol. These results show that the enthalpy for self-association is less than that for guanosine–cytidine dimer formation. The large errors arise because the shifts observed are on the order of 5 Hz and the lines are 5 Hz broad. The use of a ^{14}N spin decoupler to remove the quadrupole broadening from the proton spectra would permit more accurate high-temperature data to be obtained.

Mixtures of 1-Methylguanosine and Cytidine. Several solutions of 1-methylguanosine and cytidine were

also prepared. From the small shifts of the cytidine amino protons we conclude that the equilibrium constant for complex formation is at least an order of magnitude less for methylguanosine–cytidine dimer formation than the value for guanosine–cytidine dimer formation. Substitution of a methyl group at the N_1 position eliminates one of the three hydrogen bonds in the GC dimer (see Figure 2), but steric hindrance from the methyl should prevent more than one hydrogen bond from forming. Thus it is not surprising that little or no hydrogen bonding is observed. Pochon and Michelson draw similar conclusions from their results on the interaction of poly 1-methyl-G with poly C.[32]

Optical Studies. An attempt was made to directly observe the formation of GC base pairs in DMSO by the use of circular dichroism (CD). It is known that the poly G–poly C double strand complex has an optical rotatory dispersion curve quite different from the sum of the rotatory dispersion of the two polymer strands measured separately.[33,34] There must be a corresponding large change in the circular dichroism. The CD of a solution which contains 0.045 M guanosine and cytidine is slightly different from the CD measured for guanosine and cytidine separately in DMSO. A very weak new positive CD band appears upon interaction which is centered at 285 mμ. At this concentration the equilibrium constant derived from the nmr results implies that 13% of the guanosine and cytidine should be hydrogen bonded. Solutions of 5'-trityl-2',3'-benzylidylguanosine and 5'-trityl-2',3'-benzylidylcytidine show a large change in CD when the substituted nucleosides are allowed to interact in CHCl3. Here the equilibrium constant for interaction is considerably larger. A new positive CD band appears which is centered at 290 mμ. Some of the CD change may result from interaction between the aromatic derivatives and the bases and further optical studies in these systems are in progress. However, the similarity of the CD bands due to interaction in DMSO and CHCl3 is reasonable evidence that we are observing similar hydrogen-bonded complexes in both cases.

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Conclusions

The high dielectric constant (45) and strong proton acceptor properties of DMSO make it much more similar to water than CHCl3. The extent of hydrogen bonding between bases is much less in DMSO than in CHCl3. Pitha, *et al.*, have determined an equilibrium constant of over 10^5 for hydrogen bonding between guanosine and cytosine derivatives in CHCl3 from infrared measurements.[18] We find an equilibrium constant of about 4 for the association of guanosine and cytidine in DMSO. In water the degree of association is so weak that direct observation of GC complexes has not yet been achieved.

Kyogoku, *et al.*, have observed an enthalpy change of -6.2 ± 0.6 kcal/mol associated with the formation of a complex between 9-ethyladenine and 1-cyclohexyluracil in CHCl3. If this enthalpy is attributed entirely to hydrogen bonding the result is -3.1 kcal/mol per hydrogen bond. Our results of -5.8 kcal/mol for GC pairing in DMSO in turn yield -1.9 kcal/mol per hydrogen bond. The moderately large negative enthalpy found in DMSO may indicate that the enthalpy of hydrogen bond formation between guanosine and cytidine is still negative in aqueous solution. The entropy change found for dimer formation between guanosine and cytidine in DMSO was -16 eu. This compares reasonably well with the value of -11 eu found by Kyogoku, *et al.*, for AU pairing in CHCl3.

The structure of the GC pair in DMSO is almost certainly the same as the base-pairing scheme known to occur in double strand DNA. Formation of G_2 and C_2 pairs was also observed in DMSO. These complexes are much weaker than GC. Some information about the structure of these complexes can be obtained from the nmr results, but it is not yet possible to select a unique base-pairing scheme for them.

Communications to the Editor

Biosynthesis of Aflatoxins

Sir:

The aflatoxins are a structurally and biologically remarkable group of metabolites produced by some *Aspergillus* species.[1,2] Since their origin in nature is not obvious from inspection of their structures, we

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(2) R. I. Mateles and G. N. Wogan, *Advan. Microbial. Phys.*, **1**, 25 (1967).

examined the biosynthesis of aflatoxin-B_1 with the aid of radioactive precursors. Administration[2] of methionine-methyl-^{14}C to *Aspergillus flavus* yielded radioactive aflatoxin-B_1 (1). Zeisel degradation gave methyl iodide containing 97.8% of the activity of the starting material.[3]

Aflatoxin-B_1 prepared from acetate-1-^{14}C[4] was de-

(3) We are indebted to Dr. S. Brechbühler for this determination.
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