

The Hydrogen Bonding of Cytosine with Guanine: Calorimetric and $^1\text{H-NMR}$ Analysis of the Molecular Interactions of Nucleic Acid Bases

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Synopsis

The enthalpy of hydrogen-bond formation between guanine (G) and cytosine (C) in *o*-dichlorobenzene and in chloroform at 25°C has been determined by direct calorimetric measurement. We derivatized 2'-deoxyguanosine and 2'-deoxycytidine at the 5'- and 3'-hydroxyls with triisopropylsilyl groups; these groups increase the solubility of the nucleic acid bases in nonaqueous solvents. Such derivatization also prevents the ribose hydroxyls from forming hydrogen bonds. Consequently, hydrogen-bond formation in our system is primarily between the bases, and to a lesser extent, between base and solvent, and can be measured directly with calorimetry. To obtain the data on base-pair formation, we first took into account the contributions from self-association of each base, and where possible, have determined the ΔH of self-association. From isoperibolic titration calorimetry, our measured ΔH of C_2 formation in chloroform is -1.7 kcal/mol of C. Our measured ΔH of C:G base-pair formation in *o*-dichlorobenzene is -6.65 ± 0.32 kcal/mol. Since *o*-dichlorobenzene does not form hydrogen bonds, the ΔH of C:G base-pair formation in this solvent represents the ΔH of the hydrogen-bonding interaction of C with G in a nonassociating solvent. In contrast, our measured ΔH of C:G base-pair formation in chloroform is -5.77 ± 0.20 kcal/mol; thus, the absolute value of the enthalpy of hydrogen bonding in the C:G base pair is greater in *o*-dichlorobenzene than in chloroform. Since chloroform is a solvent known to form hydrogen bonds, the decrease in enthalpic contribution to C:G base pairing in chloroform is due to the formation of hydrogen bonds between the bases and the solvent. The ΔH of hydrogen bonding of G with C reported here differs from previous indirect estimates: Our measurements indicate the ΔH is 50% less in magnitude than the ΔH based on spectroscopic measurements of the extent of interaction. We have also observed that the enthalpy of hydrogen bonding of C with G in chloroform is greater when G is in excess than when C is in excess. This increased heat is due to the formation of C:G_{*n*>1} complexes that we have observed using $^1\text{H-nmr}$. Although C:G₂ structures have previously been observed in triple-stranded polymeric nucleic acids, higher order structures have not been observed between C and G monomers in nonaqueous solvents until now. By using monomers as a model system to investigate hydrogen-bonding interactions in DNA and RNA, we have obtained the following results: A direct measurement of the ΔH of hydrogen bonding in the C:G complex in two nonaqueous solvents, and the first observation of C:G_{*n*>1} complexes between monomers. These results reinforce the importance of hydrogen bonding in the stabilization of various nucleic acid secondary and tertiary structures.

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INTRODUCTION

Selective hydrogen bonds formed between complementary nucleic acid bases have two important biological roles. First, selective hydrogen bonding determines the fidelity of replication and transcription processes, and second, hydrogen bonds contribute to stabilization of nucleic acid secondary and tertiary structures. However, the thermodynamic parameters of hydrogen bonding between the bases have been difficult to determine experimentally. One experimental approach to measuring the thermodynamic parameters of hydrogen bonding involves the decomposition of the energy that stabilizes double-stranded DNA into its two major components: stacking interactions (which occur on the monomer level in aqueous solution) and hydrogen-bonding interactions (which occur on the monomer level in nonaqueous solution). In this paper we describe an approach that permits calorimetric measurement of the ΔH of hydrogen bonding of the C : G base pair in solution.

With spectroscopic¹⁻⁶ and crystallographic^{7,8} techniques, it has been shown that monomeric guanine and cytosine in 1 : 1 mixtures can form a planar dimer stabilized by three hydrogen bonds. This dimer (Fig. 1), formed by monomers in nonaqueous solution but not in aqueous solution, lacks propeller twist but otherwise appears identical to the Watson-Crick base pair formed by guanine and cytosine in DNA.

The limited solubilities of cytosine and guanine (and the corresponding nucleosides) in nonpolar solvents have been an impediment to extensive physical-chemical investigations into hydrogen bonding between the two bases. Substitution of alkyl, trityl, or acyl groups at the glycosidic nitrogens of the bases or at the ribose hydroxyls of the nucleosides^{2,3,5} resulted in only moderate increases in solubility in suitable solvents. For this reason the thermodynamic parameters of hydrogen bonding of guanine with cytosine have only been indirectly estimated from spectroscopic data.⁴⁻⁶ Accurate thermodynamic parameters of hydrogen bonding of guanine with cytosine in nonaqueous solvents have not been determined by calorimetric measurement.

To conduct physical-chemical experiments with the nucleic acid bases, we have substituted the ribose hydroxyls of guanosine and cytidine with the highly lipophilic triisopropylsilyl group. This modification renders the

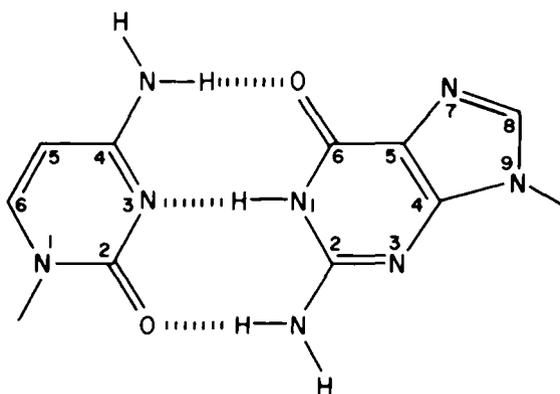


Fig. 1. The C : G Watson-Crick base pair.

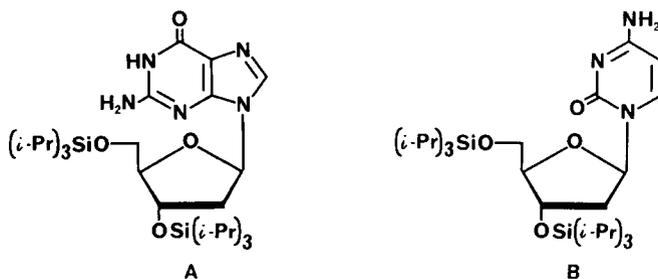


Fig. 2. The two nucleoside derivatives used in this study: (A) 2'-deoxy-3',5'-ditriisopropylsilylguanosine; (B) 2'-deoxy-3',5'-ditriisopropylsilylcytidine.

nucleosides soluble in certain nonaqueous solvents of relatively low dielectric constant at reasonable concentrations. For example, 2'-deoxy-3',5'-ditriisopropylsilylguanosine [Fig. 2(A)] and 2'-deoxy-3',5'-ditriisopropylsilylcytidine [Fig. 2(B)] are soluble at 20°C in chloroform at concentrations of 0.5*M* and in *o*-dichlorobenzene at concentrations of 0.04*M*. These nucleoside derivatives have provided a system amenable to calorimetric determination of the ΔH of hydrogen bonding of the C : G pair in two solvents.

In this report, we describe the use of isoperibolic titration calorimetry to determine the enthalpy of formation of the C : G base pair. In addition, the enthalpy of dimerization of C has been measured in one solvent. These studies have been conducted with ditriisopropylsilylated nucleosides in nonaqueous solution at 25°C. The purpose of the triisopropylsilyl groups is to obtain suitable solubility of the nucleic acid bases and to prevent the ribose hydroxyls from forming hydrogen bonds. Therefore, 2'-deoxy-3',5'-ditriisopropylsilylguanosine [Fig. 2(A)] will be referred to as guanine (G) and 2'-deoxy-3',5'-ditriisopropylsilylcytidine [Fig. 2(B)] will be referred to as cytosine (C) for the purposes of this article.

MATERIALS AND METHODS

2'-Deoxy-3',5'-ditriisopropylsilylguanosine and 2'-deoxy-3',5'-ditriisopropylsilylcytidine were prepared by a modification of published procedures.⁹ The two ditriisopropylsilylated nucleosides were obtained in yields of greater than 90% by allowing the reactions to proceed for 24 h at room temperature. In comparison, a 20-min reaction time produces the monotriisopropylsilylated adducts.⁹ The reaction products were purified by flash chromatography and recrystallized from methylene chloride/hexane. The products were pure as determined by elemental analysis, high-performance liquid chromatography, thin-layer chromatography, and ¹H-nmr. *o*-Dichlorobenzene (Aldrich, gold label) was used without further purification. Chloroform, washed several times with H₂SO₄, several times with water, and predried over CaCl₂, was heated under reflux for 24 h under positive argon pressure over P₂O₅.¹⁰ The chloroform required for a given experiment was distilled immediately prior to use. Deuteriochloroform (99.8% enriched) was distilled over P₂O₅ and stored in the dark under argon.

For calorimetric and nmr experiments, nucleosides were dried at 25°C, 0.4 torr for 24 h before use. Weighed samples were then placed in the appropriate

vessel, either a dewar (reaction vessel) or a 10-mL volumetric flask, and redried for 24 h at 25°C, 0.4 torr. Dry solvent was then injected through a septum into the volumetric flask. Solvent was added directly under argon to the dewar employed in the calorimetric experiments.

Calorimetric experiments were conducted using an isoperibolic titration calorimeter (Tronac Model 450). A detailed description of the operation of the Tronac 450 has been published elsewhere.^{11,12} The syringe for the Tronac 450 was assembled, fitted with a needle, and dried at 25°C 0.4 torr for 24 h. The nucleoside solution in the volumetric flask was withdrawn directly into the syringe.

The syringe (containing 2 mL of relatively concentrated nucleoside solution) and the dewar (containing 35 mL of either relatively dilute nucleoside solution or pure solvent) were equilibrated for a minimum of 45 min prior to the first injection. The volume delivered per injection was on the order of 0.2 mL. Calibration runs were performed before and after each injection, and measurements were made under moisture-free argon. To obtain each ΔH of interaction or dilution, 4 to 6 measurements were averaged or fitted with a linear least-squares program. Each experiment was repeated at least twice on different days with different stock solutions. The dilution experiments were performed by injecting the same solutions used for the base-pairing experiments into pure solvent. The performance of the calorimeter was checked periodically by measurement of the ΔH of protonation of an aqueous solution of Tris hydroxymethyl aminomethane with a standard aqueous hydrochloric acid solution.¹²

¹H-nmr was performed at 300 MHz on a Varian XL-300. Each spectrum of the continuous variation series was obtained with 16,000 double precision (32-bit) data points over a sweep width of 4,500 Hz. The nondeuterated chloroform contaminant in the deuteriochloroform was used as a reference. The probe temperature was maintained at $22 \pm 1^\circ\text{C}$.

RESULTS

The heats of interaction and dilution of various concentrations of C and G in *o*-dichlorobenzene and in chloroform at 25°C have been investigated with isoperibolic titration calorimetry. The choice of solvents has been dictated by the solubility of the nucleosides, in particular, by the general lack of solubility of G and its derivatives in nonaqueous solvents. (For a discussion of the solubility properties of G and its derivatives, see Ref. 13.)

We investigated the solubility properties of G derivatives in order to find nucleosides and solvents suitable for physical-chemical studies of the hydrogen-bonding interactions of nucleic acid bases. The substitution of triisopropylsilyl groups at the hydroxyl positions of nucleosides resulted in a particularly useful class of compounds. This method is synthetically trivial and yields stable, easily purified compounds with high solubility in solvents such as chloroform. We have observed the following: (1) 2'-deoxy-3',5'-ditriisopropylsilylguanosine has reasonable solubility ($> 0.40M$) in nonaqueous solvents that possess hydrogen-bond proton donor sites (chloroform, methylene chloride, or methanol); (2) 2'-deoxy-3',5'-ditriisopropylsilylguanosine has low solubility ($< 0.05M$) in nonaqueous solvents that do not possess hydrogen-bond

proton donor sites (hexane, carbon tetrachloride, toluene, ethyl acetate, dimethylsulfoxide, or *o*-dichlorobenzene); (3) addition of trace amounts of water to solvents that do not possess hydrogen-bond proton donor sites increases the solubility of 2'-deoxy-3',5'-ditriisopropylsilylguanosine; (4) substitution of both hydroxyls of 2'-deoxyguanosine with triisopropylsilyl groups decreases the solubility in dimethylsulfoxide from $> 0.50M$ to $< 0.050M$.

The solubilization of G in nonaqueous solvents is enhanced by hydrogen bonding in solution. However, interaction with hydrogen-bond proton donors exerts a far more pronounced effect than interaction with hydrogen-bond proton acceptors. The hydrogen-bond proton donor can be solvent, impurities (water), or unprotected ribose hydroxyl groups presumably via intermolecular interaction. This suggests that certain aspects of previous experiments with unprotected nucleosides in nonhydrogen-bond-donating solvents such as dimethylsulfoxide^{1,4} could be reinterpreted in terms of intermolecular base : ribose hydrogen-bonding interactions.

Self-Association of Cytosine

C (derivatized at the N¹ for solubility purposes) forms a hydrogen-bonded self-associate in nonaqueous solvents.⁴⁻⁶ The likely structure of the hydrogen-bonded cytosine dimer is shown in Fig. 3.

According to LeChatelier's principle, infinite dilution is expected to disrupt self-association. By extrapolating the heat of dilution of three different concentrations to infinite dilution, we have obtained the ΔH of dilution of the three concentrations of C in chloroform. It is assumed that all heat effects upon dilution are due to dissociation of hydrogen-bonded complexes. As shown in Table I, the ΔH of dilution of C in chloroform decreases with decreasing total concentration of C, indicating that the extent of self-association decreases with decreasing concentration (consistent with LeChatelier's principle). The ΔH of dilution of C appears to approach a limiting value of 1.7 kcal/mol of C at the higher end of our concentration range in chloroform. This is consistent with ¹H-nmr chemical-shift data (to be published elsewhere) indicating nearly 100% self-association of C when [C] is greater than 0.150M at 25°C in deuteriochloroform. Assuming, therefore, 100% self-association of C at the higher end of our concentration range, the negative of the high

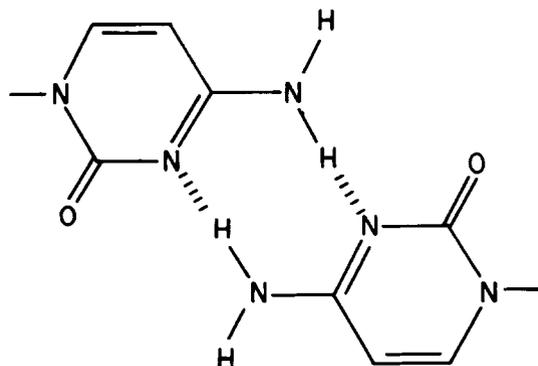


Fig. 3. The cytosine dimer.

TABLE I
 ΔH of Dilution^a of Cytosine (or C)

[C] (<i>M</i>)	ΔH (kcal/mol of C)	Solvent
0.383	1.70 ± 0.03	Chloroform
0.268	1.58 ± 0.06	Chloroform
0.0566	0.78 ± 0.10	Chloroform
0.0415	5.05 ± 0.74	<i>o</i> -Dichlorobenzene
0.0344	4.41 ± 0.68	<i>o</i> -Dichlorobenzene

^aAt 25°C as determined by isoperibolic titration calorimetry.

concentration limit of ΔH of dilution is equal to the ΔH of self-association. Thus, the ΔH of self-association of C in chloroform is -1.70 kcal/mol of C.

The limited solubility of C in *o*-dichlorobenzene precludes a study of the concentration dependence of the heat of dilution of C in this solvent. As shown in Table I, the ΔH of dilution of relatively low concentrations (0.0344–0.0415*M*) of C in *o*-dichlorobenzene is considerably higher than the ΔH of dilution of higher concentrations of C in chloroform. This is convincing evidence that the ΔH of self-association of C is lower in *o*-dichlorobenzene than in chloroform. By assuming that the extent of dimerization in *o*-dichlorobenzene is 100% or less at 0.0415*M*, we conclude that the upper limit of the ΔH of self-association of C in *o*-dichlorobenzene is -5.1 kcal/mol of C. Thus, it appears that the self-associates of C have higher enthalpy of formation in chloroform than in *o*-dichlorobenzene.

Self-Association of Guanine

G, like C, is capable of forming hydrogen-bonded self-associates in nonaqueous solvents.⁴⁻⁶ However, in contrast to the behavior of C, hydrogen-bonded G_n complexes with $n \geq 2$ exist in deuteriochloroform. ¹H-nmr chemical shifts of G (to be published elsewhere) do not vary with concentration in deuteriochloroform in a manner expected for simple dimer formation. Chemical shifts do not approach limiting values as concentration is increased, even in a relatively high concentration range ($> 0.20M$). Our observations with ¹H-nmr are consistent with those of previous investigators,⁵ who concluded that, even at low concentrations in chloroform (deuteriochloroform was used as a solvent in the ir experiments of Ref. 5), the self-association behavior of G derivatives cannot be described by simple dimerization.

The ΔH of dilution of G in chloroform varies with concentration (as shown in Table II), and appears to approach a limiting value of approximately 1.5 kcal/mol of G. However, there is insufficient experimental evidence to ascertain what self-associative process produces this heat. Fortunately, no assumptions regarding the extent or stoichiometry of self-association are required for calorimetric determination of the enthalpy of hydrogen bonding of C with G (see below).

As shown in Table II, the ΔH of dilution of a relatively low concentration of G (0.0367*M*) in *o*-dichlorobenzene is approximately the same as the ΔH of

TABLE II
 ΔH of Dilution^a of Guanine (or G)

[G] (M)	ΔH (kcal/mol of G)	Solvent
0.242	1.40 ± 0.20	Chloroform
0.196	1.45 ± 0.15	Chloroform
0.0516	0.61 ± 0.13	Chloroform
0.0367	1.21 ± 0.08	<i>o</i> -Dichlorobenzene

^aAt 25°C as determined by isoperibolic titration calorimetry.

dilution of a much higher concentration of G (0.242M) in chloroform. The ΔH of dilution of the relatively low concentration of G (0.0367M) in *o*-dichlorobenzene is twice the ΔH of dilution of a slightly higher concentration of G (0.0516M) in chloroform. Thus, it appears that the self-associates of G have higher enthalpy of formation in chloroform than in *o*-dichlorobenzene. However, the effect of solvent appears much less pronounced on the self-association of G than on the self-association of C.

The C : G Base Pair

Upon combining G and C in nonaqueous solvents, the two bases form a Watson-Crick-type base pair¹⁻⁶ as shown in Fig. 1. The ΔH of C : G base-pair formation from the "free" bases (i.e., nonassociated bases) cannot be obtained simply by measuring the heat of mixing C and G. Prior to mixing, the bases are not free but are each involved in self-association. To determine the ΔH of C : G base-pair formation, the heats obtained upon mixing the two bases (shown in Table III) must be corrected for the heat associated with disruption of the self-associates upon base-pair formation. For determination of the ΔH of C : G base-pair formation from free bases, the correction term for self-association has been obtained from the ΔH of dilution of the appropriate concentrations of each base (shown in Tables I and II).

The use of the ΔH of dilution of C as one of the appropriate correction terms for self-association can be understood by the following: At a given concentration of C, a certain fraction (X , which is dependent on concentra-

TABLE III
 ΔH of Injection^a of Guanine into Excess Cytosine

[G] ^b (M)	[C] ^c (M)	ΔH (kcal/mol)	Solvent
0.242	0.058	-3.42 ± 0.03	Chloroform
0.196	0.051	-3.70 ± 0.02	Chloroform
0.0367	0.037	-0.72 ± 0.16	<i>o</i> -Dichlorobenzene

^aAt 25°C as determined by isoperibolic titration calorimetry.

^bConcentration in the syringe.

^cConcentration in the 35-mL reaction vessel.

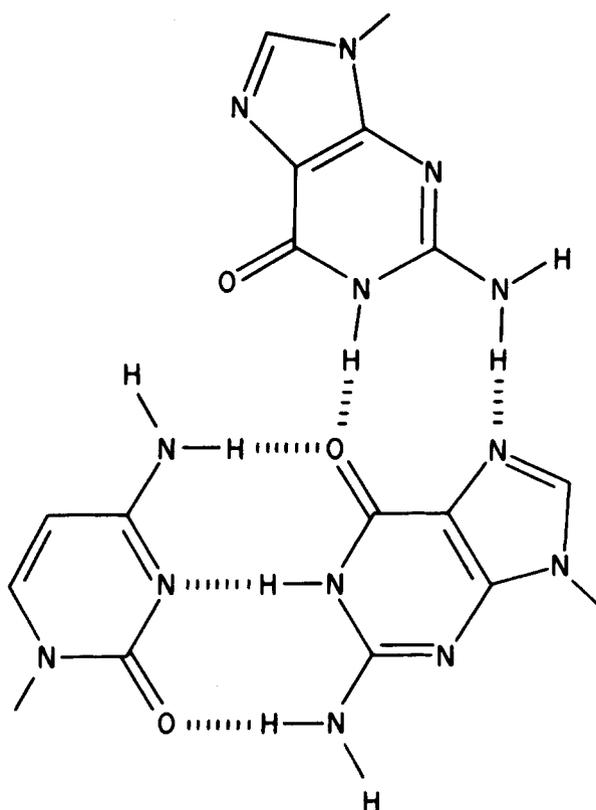


Fig. 4. A C:(G)₂ hydrogen-bonded triplet. (Note that other conformations and stoichiometries are consistent with the data shown here.)

tion) of C in the reaction vessel will be involved in dimers. If $[C] \gg [G]$ then X will not change during the course of the reaction. The same fraction X of the C that reacts with G will be converted from dimers to monomers during the reaction with G. The ΔH associated with the process of conversion of the fraction X of dimerized C to monomer C is the ΔH of dilution of the given concentration of C.

Due to a complementary geometric arrangement of hydrogen-bond donors and acceptors, C and G have the potential to form hydrogen bonds without complete disruption of the G_n species to produce a C : G_{n+1} complex (possible structure shown in Fig. 4). As shown below, this occurs to a significant extent in the presence of excess G ($[G] > [C]$). In the alternative case, when $[C] > [G]$, formation of C : G hydrogen-bonded species is favored over the C : G_2 . When mixing C and G for determining the ΔH of formation of the C : G base pair, we have utilized experimental conditions that ensure that $[C] \gg [G]$ in the reaction vessel. Under these conditions, the correction term obtained from the dilution of G is applicable for calculating the ΔH of formation of the C : G base pair from free bases.

The ΔH of formation of the C : G dimer has been determined by the summation of three terms: (1) the ΔH of injection of G into C, (2) the $-\Delta H$ of dilution of the appropriate concentration of C, and (3) the $-\Delta H$ of dilution of

the appropriate concentration of G:

$$\Delta H_{CG} = \Delta H_{\text{injection G into C}} - \Delta H_{\text{dilution of G}} - \Delta H_{\text{dilution of C}}$$

These calculations involve only the assumption that self-associates are disrupted upon base-pair formation. No assumptions are made regarding the extent or stoichiometry of self-association prior to mixing. Conditions employed when determining the first term ensure that $[C] \gg [G]$ in the reaction vessel, such that C:G base-pair formation is favored over C:G₂ complex formation. In conclusion, the ΔH of formation of the C:G hydrogen-bonded base pair from free bases has been determined to be -6.65 ± 0.32 kcal/mol in *o*-dichlorobenzene and -5.77 ± 0.15 kcal/mol in chloroform.

Erroneous experimental values of ΔH of base-pair formation would arise if the extent of C:G base pairing is not close to 100% in the reaction vessel. This possibility is inconsistent with two independent observations: (1) the magnitude of the ΔH of base-pair formation from free bases does not increase with increasing concentration and; (2) ¹H-nmr data [for example, see Fig 5(A) below] show that, in deuteriochloroform, at concentrations typical of those occurring in the reaction vessel in the calorimetric experiments ($[C] \cong 0.05M$, $[G] \cong 0.005M$), the chemical shifts of G do not vary with change in mole fraction. The absence of change of chemical shift with mole fraction indicates that nearly 100% of G is involved in Watson-Crick type base pairing at these concentrations.

Higher Order Associates (C : G_{*n*>1})

The $-\Delta H$ of hydrogen bonding of C with G is greater in the case when G is in excess than in the case when C is in excess (data not shown). This apparent discrepancy results from the formation of higher order associates of G with C (possible structure shown in Fig. 4).

We have employed ¹H-nmr to investigate the nature of the hydrogen-bonding interactions between C and G. To determine stoichiometry (stoichiometries) of interaction(s), a ¹H-nmr continuous variation experiment^{14,15} was conducted. The total concentration ($[C] + [G]$) was held constant at 0.050M in deuteriochloroform while mole fractions of the two components were varied. In the mole fraction range where $[C] > [G]$, chemical shifts of the H¹ resonance of G and the H⁵ resonance of C show a linear relationship with mole fraction [Fig. 5(A, C)] (the amino protons of C are exchange broadened and unobservable in this mole fraction range). The linear relationship between chemical shift and mole fraction in this mole fraction range indicates that C_{*n*}:G complexes with $n > 1$ do not form in solution. The sharp discontinuity at $[C] = [G]$ is evidence for formation of a 1:1 complex (the C:G base pair). In the mole fraction range where $[G] > [C]$, chemical shifts of the H¹ resonance of G, the hydrogen-bonded amino resonance of C, and the H⁵ resonance of C show a nonlinear change with mole fraction (Fig. 5) (in this mole fraction range the two amino protons of C are observable as separate resonances). The nonlinear relationship between chemical shift and mole fraction in the mole fraction range where $[G] > [C]$ indicates clearly that, in addition to the C:G base pair, at least one other type of C:G_{*n*} complex can exist in solution. One

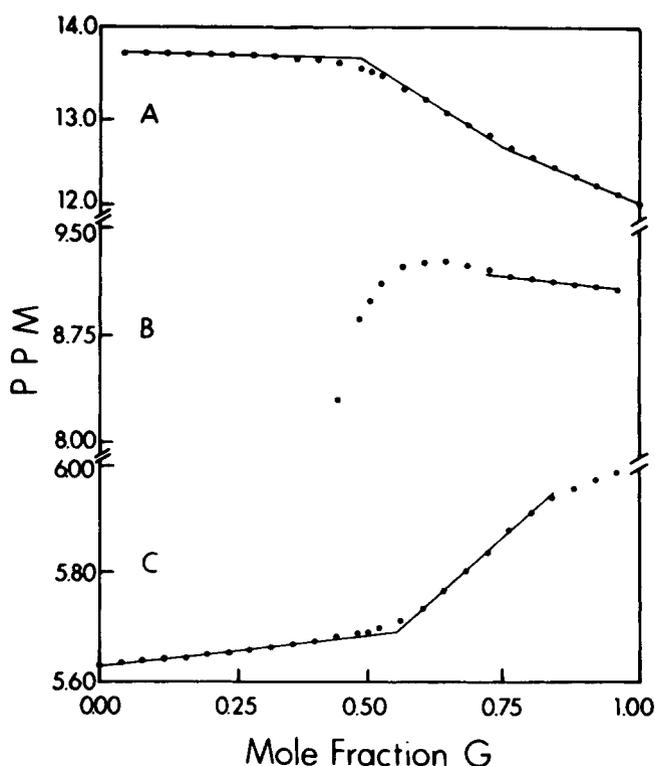


Fig. 5. ^1H -nmr chemical shifts from the continuous variation of G and C. The total nucleoside concentration is $0.050M$ in deuteriochloroform at 22°C for each data point. (A) The H^1 resonance of G. (B) The hydrogen-bonded amino resonance of C. (C) The H^5 resonance of C.

likely $\text{C} : \text{G}_{n>1}$ complex is the $\text{C} : \text{G}_2$ structure shown in Fig. 4. This conformation is consistent with nuclear Overhauser effects observed between the amino protons of G and the H-8 proton of G in 1 : 2 mixtures of C and G in solution (to be published elsewhere).

The formation of higher order complexes reported here between monomers is an indication of behavior on the monomer level similar to that previously observed between bases in polymeric ribonucleic acids where poly rC and poly rG exhibit 1 : 2 stoichiometry of interaction.¹⁶

DISCUSSION

We have determined the ΔH of formation of the C : G Watson-Crick-type base pair (shown in Fig. 1) in *o*-dichlorobenzene (-6.65 ± 0.32 kcal/mol) and in chloroform (-5.77 ± 0.20 kcal/mol). The ΔH of formation of the C_2 dimer in chloroform is -1.7 kcal/mol of C and the upper limit of the ΔH of formation of the C_2 dimer in *o*-dichlorobenzene is -5.1 kcal/mol of C. The enthalpies of formation reported here are directly obtained thermodynamic parameters of nucleic acid hydrogen-bonding interactions.

Previous estimates of the enthalpies of hydrogen bonding of nucleic acid bases in chloroform obtained from spectroscopic data⁵ are not in good agreement with the values reported here. The ΔH of formation of the C : G base

pair in chloroform was indirectly estimated with ir spectroscopy to be twofold greater in magnitude than we have observed calorimetrically. Considering the greater inherent accuracy of direct calorimetric measurements as compared to indirect spectroscopic estimates of heats of hydrogen bonding, the values reported here should be considerably more accurate. Further, in the particular ir spectroscopic study cited here, a linear relationship between $\log K$ and $1/T$ was not obtained due to the high degree of experimental uncertainty in the extent of association of the two bases at each temperature. Therefore, only a crude estimation of the ΔH of hydrogen bonding of G with C could be obtained. The result of such spectroscopic studies is qualitative thermodynamic data, whereas calorimetric measurements of the type described here provide accurate, quantitative data.

The influence of solution environment on the ΔH of formation of nucleic acid hydrogen-bonding interactions has been explored. The three species investigated (C : G, C₂, and G_n) have higher enthalpies of formation in chloroform than in *o*-dichlorobenzene. Since hydrogen-bonding interactions generally have a large electrostatic component, the dielectric constants of *o*-dichlorobenzene ($\epsilon = 9.93$) and chloroform ($\epsilon = 4.81$) would lead us to predict the opposite, i.e., that the hydrogen-bonded associates have higher enthalpies of formation in *o*-dichlorobenzene than in chloroform. It appears that the hydrogen-bonding ability of chloroform¹⁷ must play a considerable role in destabilizing base-base hydrogen-bonding interactions. Hydrogen-bond interactions between chloroform and 1-methyl cytosine have been reported¹⁸ and such interactions would be expected to destabilize base-base hydrogen-bond interactions as observed here with calorimetry. Since *o*-dichlorobenzene is not known to form hydrogen bonds, the ΔH of C : G base-pair formation in this solvent represents a measure of the ΔH of the hydrogen-bonding interaction of C with G in a nonassociating solvent.

Chloroform has a greater effect on the hydrogen-bonding interactions of C than on hydrogen-bonding interactions of G. As compared with *o*-dichlorobenzene, chloroform causes an increase in the enthalpy of formation, which follows the order C₂ > C : G > G_n. This implies that specific hydrogen bonds can form between chloroform (or any other hydrogen-bonding solvent) and the nucleic acid bases (also see Ref. 18). Hence, certain assumptions used in the calculation of hydrogen-bonding energies, e.g., that the entropy of formation in chloroform of a specific nucleic acid dimer is the same as the entropy of formation of a different dimer,^{5,19} are of questionable validity. Similarly, the assumption that the entropy of formation of a base pair in carbon tetrachloride is roughly the same as in chloroform²⁰ is also of questionable validity.

The ΔH of formation of the C : G Watson-Crick-type base pair in *o*-dichlorobenzene provides a basis for comparison with the experimental *in vacuo* ΔH of formation²¹ (for a discussion of the accuracy of the experimental *in vacuo* data, see Ref. 22). When corrected with the macroscopic dielectric parameter of *o*-dichlorobenzene, the ΔH of hydrogen bonding of the C : G base pair measured *in vacuo* (-21.0 kcal/mol), would be expected to increase to -2.1 kcal/mol. The experimentally determined ΔH in *o*-dichlorobenzene reported here (-6.65 kcal/mol) is evidence that contributions other than electrostatic (i.e., dipole-dipole, polarization, dispersion, or charge transfer) provide significant stabilization energy to the C : G base pair in solution. Note that the ΔH

of hydrogen bonding in chloroform is coincidentally similar to the dielectrically corrected *in vacuo* value. However, this is a result of solute-solvent hydrogen bonding, which destabilizes the C:G base pair in chloroform. The data reported here should provide a basis for the quantitative comparison of experimental with theoretical^{19,20} enthalpies of hydrogen bonding of nucleic acid bases.

The previous spectroscopic estimate of the ΔH of formation of the adenine:uracil base pair in chloroform (-6.2 kcal/mol; Ref. 5) must be considered questionable in light of the discrepancy between the calorimetrically determined enthalpies of formation reported here (for the C:G base pair and the two self-associates) and previous spectroscopic estimates of these values.⁵ Calorimetric measurement of the heat of hydrogen bonding of adenine (A) with uracil (U) monomers for determination of the ΔH of formation of the A:U Watson-Crick base pair has not been attempted. The multiplicity of dimeric species formed by monomeric A and U in nonaqueous solution (Hoogsteen, reverse Hoogsteen, Watson-Crick and reverse Watson-Crick)^{23,24} makes direct measurement of the ΔH of formation of any one of these dimers difficult.

We have observed that C and G monomers can form hydrogen-bonded structures other than the Watson-Crick-type C:G base pair. Specifically, C and G can combine to form a 1:2 hydrogen-bonded triplet. The hydrogen-bonding scheme of the C:G₂ triplet formed between monomers appears to resemble that previously observed between bases in polymeric ribonucleic acids where poly rC and poly rG form a triple helix in aqueous solution.¹⁶ The formation of C:G₂ triplets on the monomer level lends support to the utility of monomers in nonaqueous solvents as model systems for understanding hydrogen-bonding interactions in the hydrophobic interior of nucleic acid double and triple helices. Further, the similarity in the hydrogen-bonding schemes observed between monomers and between polymers underscores the importance of specific hydrogen bonding in the formation and stabilization of a variety of DNA and RNA secondary and tertiary structures.

This report describes the first application of a direct calorimetric technique for determining the enthalpies of important hydrogen-bonding interactions between nucleic acid monomers. We have accurately measured these enthalpies in nonaqueous solvents (previous estimates are inconsistent with our directly determined values), and the results should be of utility to investigators involved in both theoretical and experimental aspects of nucleic acid chemistry.

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