Influence of N-Acylation on the Stability of Double-Stranded Polydeoxynucleotides

F. Newton Hayes,* Elizabeth Hansbury, Victoria E. Mitchell, and Terence J. Scallen

Contribution from the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544, and the Department of Biochemistry, School of Medicine, The University of New Mexico, Albuquerque, New Mexico 87106. *Received August 12, 1970*

Abstract: Thermal hyperchromism of polydeoxynucleotide mixtures has been studied relative to degree and type of purine N-acylation. Thermally labile acyl groups confused interpretation of melting curves until a technique of fast heating and rapid cooling gave needed reproducibility. In a series with $ac^6A \cdot T$ hydrogen bonding, a linear relationship of T_m to degree of acetylation was found. Relative stabilities of hydrogen-bonded association proved to be $A \cdot T > ac^6A \cdot T > ib^6A \cdot T$ and $ac^6A \cdot T > ac^2G \cdot C$. Purine acetylation in polydeoxynucleotides has made possible novel and fundamental thermal hyperchromism studies as well as allowing enzymatic chain-lengthening reactions which would otherwise have been impossible due to aggregation and interfering hydrogen bonding.

It has been observed[1] during a study of synthetic polydeoxynucleotides that monoacetylation of the 6-amino group of adenine sharply decreased the midpoint (T_m) in the thermal hyperchromicity transition (melting curve) of the Watson and Crick[2] A·T double-hydrogen bonding (Figure 1). At the same time it was reported[1] that melting studies on the effect of monoacetylation of guanine, presumed to be on the 2-amino group, failed to establish existence of the acetylated G·C triple-hydrogen bonding (Figure 2), since no sharp melting transitions were observed. Subsequently, Lefler and Bollum[3] reported that they could obtain no evidence for definite complexes formed in physical mixtures of single-stranded polydeoxycytidylate (dC_n)[4] with poly-*N*-acetyldeoxyguanylate $d(ac^2G)_n$.

Now in a detailed corroboration of our early observations, melting curves along with associated ultraviolet absorption data have been obtained that describe various degrees of double-helix destabilization resulting from acylation of amino groups in purines of synthetic polydeoxynucleotides.

Experimental Section

Polydeoxynucleotide concentrations are expressed in phosphorus concentrations determined according to Petersen, *et al.*[5] Ultraviolet absorption measurements were carried out on a Unicam SP-800 spectrophotometer equipped[6] for plotting of melting curves. Each T_m value was determined as the temperature of maximum slope, and Δ_{max} is the maximum slope expressed as the increase in *A* units per degree; both of these parameters were evaluated from calculated differential melting curves.[6]

The solvents used were 0.1 *M* Na^+, pH 7.0 (0.08 *M* NaCl–0.01 *M* Na_2HPO_4, pH 7.0), 0.5 *M* Na^+, pH 7.8 (0.48 *M* NaCl–0.01 *M* Na_2HPO_4, pH 7.8), 1.0 *M* Na^+, pH 7.0 (0.98 *M* NaCl–0.01 *M* Na_2HPO_4, pH 7.0), and 1.0 *M* Na^+, pH 8.1 (0.98 *M* NaCl–0.01 *M* Na_2HPO_4, pH 8.1).

Oligodeoxynucleotides dT_6, dT_{15}, and dC_4 were prepared by chemical polymerization according to Khorana and Vizsolyi.[7] Using dT_6 and dC_4 as initiators with appropriate deoxynucleoside 5′-triphosphates and calf thymus terminal deoxynucleotidyltransferase,[8] the polydeoxynucleotides $dT_{\overline{47}}$, $dC_{\overline{87}}$, $d(T_6\text{-}A_{\overline{50}})$, $d(T_6\text{-}A_{\overline{65}})$, and $d(T_6\text{-}ac^2G_{\overline{68}})$ were synthesized and characterized.[9] Chemical acylation[10] of $d(T_6\text{-}A_{\overline{50}})$ and $d(T_6\text{-}A_{\overline{65}})$ gave $d(T_6\text{-}ac^6A_{\overline{50}})$, $d(T_6\text{-}ac^6A_{\overline{65}})$, and $d(T_6\text{-}ib^6A_{\overline{65}})$. Ammonolysis at 26° of $d(T_6\text{-}ac^6A_{\overline{65}})$ (66 μM in 1.3 *M* aqueous NH_3) reached 50% deacetylation in 13 hr. The degree of acetylation was established by comparing uv absorption λ_{max} values with those obtained from a set of standard mixtures of nonacetylated with totally acetylated polymers. Complete acetylation was judged by finding no change in properties on further acetylation.

Results

Spectral Measurements. Mixing curves were run and analyzed according to Riley, *et al.*,[11] with incremental addition of $dT_{\overline{47}}$ to $d(T_6\text{-}ac^6A_{\overline{65}})$ in 1.0 *M* Na^+, pH 7.0 at 21°, of $dT_{\overline{47}}$ to $d(T_6\text{-}ib^6A_{\overline{65}})$ in the same solvent at 8°, and of $d(T_6\text{-}ac^2G_{\overline{68}})$ to $dC_{\overline{87}}$ in 0.5 *M* Na^+, pH 7.8 at 8°. Plots of $\epsilon(P)$ against the composition of the mixture gave typical intersecting lines whose intercepts within ±15% all indicated the existence of 1:1 complexes.

A mixture of $d(T_6\text{-}ac^6A_{\overline{50}})$ (39.0 μM in $d(ac^6A)$ units) and dT_{15} (39.3 μM) in 1.0 *M* Na^+, pH 7.0 (mixture I), was spectrophotometrically analyzed at 0 and 40° as shown in Figure 3. For the 0° curve 1, λ_{max} = 263.5 nm, $A_{250}/A_{260} = 0.78$, $A_{280}/A_{260} = 0.67$; for the 40° curve 2, λ_{max} = 268 nm, $A_{250}/A_{260} = 0.74$, $A_{280}/A_{260} = 0.78$; the maximum hyperchromicity ratio (2/1) = 1.47 at 276 nm. A mixture of $d(T_6\text{-}A_{\overline{50}})$ (40.8 μM in dA units) and dT_{15} (39.3 μM) in 1.0 *M* Na^+, pH 7.0 (mix-

* Address correspondence to this author at Los Alamos Scientific Laboratory.

(1) F. N. Hayes, E. Hansbury, V. E. Mitchell, R. L. Ratliff, and D. L. Williams, *Eur. J. Biochem.*, **6**, 485 (1968).

(2) J. D. Watson and F. H. C. Crick, *Nature (London)*, **171**, 737 (1953).

(3) C. F. Lefler and F. J. Bollum, *J. Biol. Chem.*, **244**, 594 (1969).

(4) Abbreviations used are those of the IUPAC-IUB Combined Commission on Biochemical Nomenclature, *Biochemistry*, **9**, 4022 (1970). The substituent designations ac and ib refer to acetyl and isobutyryl, respectively. Overlining of a subscript means that the number is the average of a distribution.

(5) D. F. Petersen, L. B. Cole, E. H. Lilly, and V. E. Mitchell, Los Alamos Scientific Laboratory Report No. LAMS-2780, 1962, p 300.

(6) F. N. Hayes, E. H. Lilly, R. L. Ratliff, D. A. Smith, and D. L. Williams, *Biopolymers*, **9**, 1105 (1970).

(7) H. G. Khorana and J. P. Vizsolyi, *J. Amer. Chem. Soc.*, **83**, 675 (1961).

(8) M. Yoneda and F. J. Bollum, *J. Biol. Chem.*, **240**, 3385 (1965).

(9) F. N. Hayes, V. E. Mitchell, R. L. Ratliff, A. W. Schwartz, and D. L. Williams, *Biochemistry*, **5**, 3625 (1966).

(10) E. Hansbury, V. N. Kerr, V. E. Mitchell, R. L. Ratliff, D. A. Smith, D. L. Williams, and F. N. Hayes, *Biochim. Biophys. Acta*, **199**, 322 (1970).

(11) M. Riley, B. Maling, and M. J. Chamberlain, *J. Mol. Biol.*, **20**, 359 (1966).

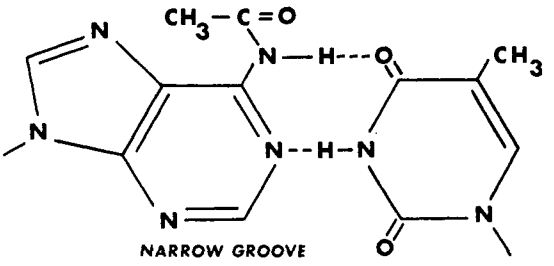


Figure 1. A drawing of the ac⁶A·T association.

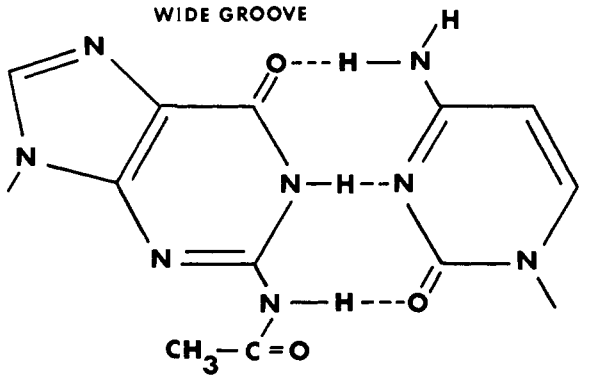


Figure 2. A drawing of the ac²G·C association.

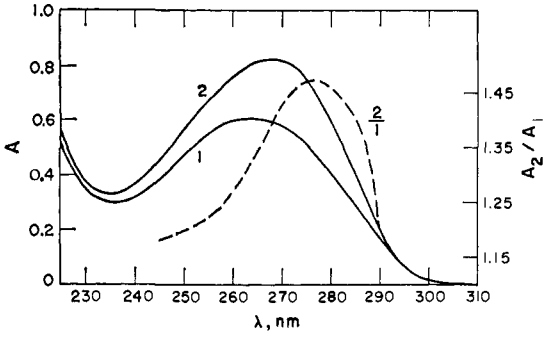


Figure 3. Ultraviolet spectra of d(T₆-ac⁶A$\bar{50}$) plus dT₁₅ (mixture I).

ture II) was spectrophotometrically analyzed at 10° (1) and at 65° (2) as shown in Figure 4. For the 10° curve, λ_{max} = 257 nm, A_{250}/A_{260} = 0.88, A_{280}/A_{260} = 0.55; for the 65° curve, λ_{max} = 258.5 nm, A_{250}/A_{260} = 0.82, A_{280}/A_{260} = 0.45; the maximum hyperchromicity ratio (2/1) = 1.50 at 266 nm. A mixture of d(T₆-ac²-G$\bar{68}$) [36.7 μM in d(ac²G) units] and dC$\bar{87}$ (39.7 μM) in 0.5 M Na⁺, pH 7.8 (mixture III), was spectrophotometrically analyzed at 1° (1) and at 70° (2) as shown in Figure 5. For the 1° curve, λ_{max} = 259 nm, A_{250}/A_{260} = 0.94, A_{280}/A_{260} = 0.61; for the 70° curve, λ_{max} = 257.5 nm, A_{250}/A_{260} = 0.96, A_{280}/A_{260} = 0.80; The maximum hyperchromicity ratio (2/1) = 1.55 at 280 nm.

Melting Curves. Differential melting curves are displayed in Figure 6, where curve 1 run at 276 nm is for mixture I, curve 2 at 266 nm is for mixture II, and curve 3 at 270 nm is for a 1:1 mixture of I and II. The T_m values are: 1, 19.3°; 2, 52.2°; and 3, 17.7 and 52.5° with Δ_{max} values for 1, 0.020, and for 2, 0.018. Curves 1 and 2 are exactly reproduced on cooling and remelting; the lower melting portion of curve 3 is significantly flatter on cooling.

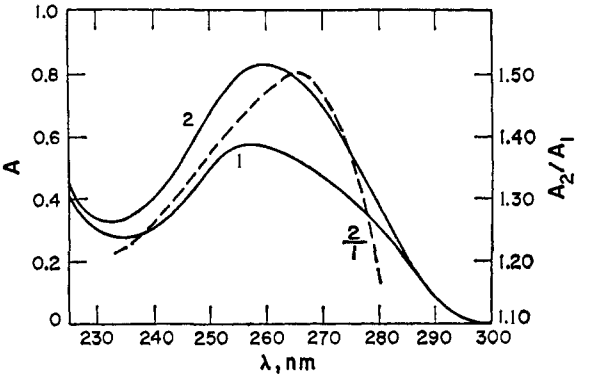


Figure 4. Ultraviolet spectra of d(T₆-A$\bar{50}$) plus dT₁₅ (mixture II).

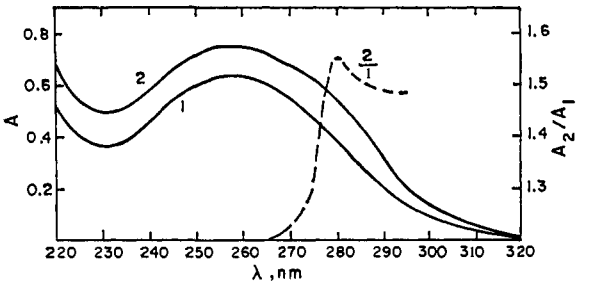


Figure 5. Ultraviolet spectra of d(T₆-ac²G$\bar{68}$) plus dC$\bar{87}$ (mixture III).

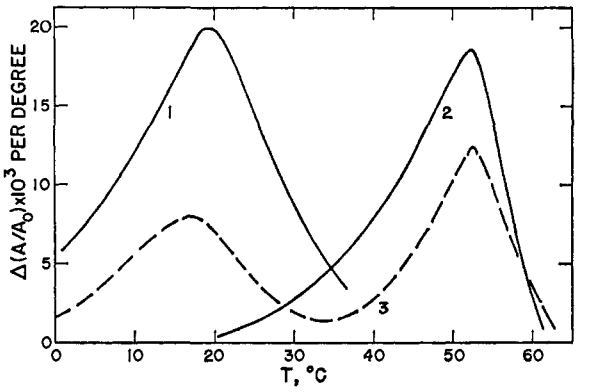


Figure 6. Differential melting curves of mixtures I and II.

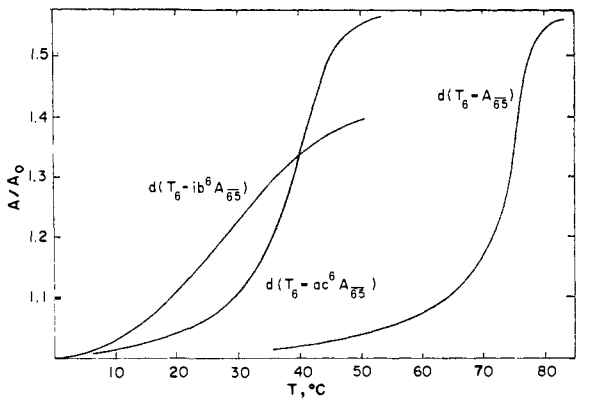


Figure 7. Integral melting curves of the indicated polynucleotides in mixture with dT$\bar{47}$.

In Figure 7 are shown integral melting curves for mixtures of the indicated polydeoxynucleotides with dT$\bar{47}$; in each case the solvent was 1.0 M Na⁺, pH 7.0,

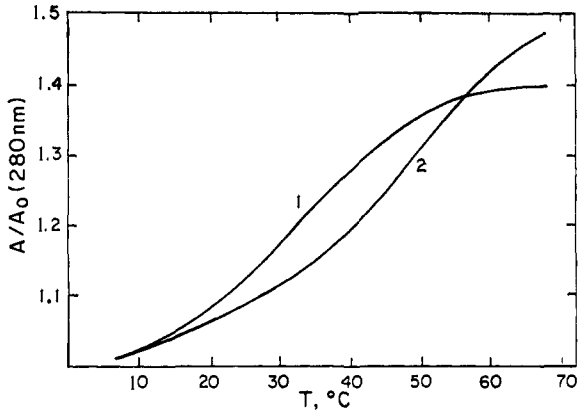


Figure 8. Successive integral melting curves of mixture III: (1) slow and (2) fast.

and the concentration of hydrogen-bonding nucleotide units was 40 μM for both components. Characteristics of these curves are: d(T_6-$A_{\bar{6}5}$), T_m = 75.0°, Δ_{max} = 0.076; d(T_6-ac$^6A_{\bar{6}5}$), T_m = 41.7°, Δ_{max} = 0.037; and d(T_6-ib$^6A_{\bar{6}5}$), T_m = 28.1°, Δ_{max} = 0.012.

A series containing partially deacetylated samples derived from d(T_6-ac$^6A_{\bar{5}0}$) by limited ammonolysis was analyzed for absorbance λ_{max} and then mixed with $dT_{\bar{4}7}$ (both at 36 μM) in 0.1 M Na^+, pH 7.0; melting curves were then run. Each λ_{max} was converted to its per cent acetylation value; the resulting relationship between T_m and per cent acetylation was found to be linear between the limits: 15.6°, 100%; 59.0°, 0%.

In Figure 8 are shown two successive melting curves of mixture III. A fresh mixture was allowed to stand at 21° for 15 hr to promote annealing and then inserted into the thermostated spectrophotometer also at 21°. After slow cooling (about 1.5 hr) to 0°, a melting curve at normal rate was run up to 70° (150-min duration, Figure 8, curve 1). Then the sample was quick-cooled in ice to 21°, at which it remained for 15 hr. Subsequently, after slow cooling to 0°, the second melting curve was run. This time by inactivating the normal, slow, and programmed rate-of-heating control and setting the thermostatic control directly to 80°, the same temperature span was traversed in 20 min (Figure 8, curve 2). The characteristics of these curves are: 1, T_m = 32.5°, Δ_{max} = 0.010; 2, T_m = 50.5°, Δ_{max} = 0.013. Another set of melting curves with fresh mixture III was run at the fast (20 min) rate for both curves. In this case the T_m values were 32.5° followed by 37.0°. A variant of mixture III was prepared using the same solutes and concentrations but 1.0 M Na^+, pH 8.1, as solvent. A fast melting curve gave T_m = 34.2° and Δ_{max} = 0.009. Then after holding the melted sample at 70° for 90 min and quick-chilling, another fast melting gave T_m = 70.2° and Δ_{max} = 0.015.

The Acetylation Site on Guanine. Ralph, *et al.*,[12] considered it improbable for the acetyl group in acetylated dGMP to be on either N^1 or O^6 because of the demonstrability of an ionization around pH 10–11. They tentatively assigned the acetyl group to N^2, while allowing the possibility that it is on the 3 position nitrogen. Studies using nmr were carried out to establish more clearly the acetylation site.

(12) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *J. Amer. Chem. Soc.*, 85, 1983 (1963).

In order to achieve high solubility in dimethyl-d_6 sulfoxide, acetylated deoxyguanosine 5′-phosphate[1] (181 μmol in 1.8 ml of hexamethylphosphoric triamide) was dephosphorylated by adding 10 mg of *Escherichia coli* alkaline phosphatase and water to a final volume of 6 ml, which was incubated at 37° for 1 hr. Gel filtration chromatography on Sephadex G-10 (3.1 × 90 cm, H_2O) gave as the fourth of five peaks, eluting at an A_{max} volume of 616 ml, 134 μmol of acetylated deoxyguanosine. Thin-layer chromatography R_f values are given in Table I.

Table I. Thin-Layer Chromatography R_f Values

Compd	R_f[a]
Deoxyguanosine	0.68
Guanine	0.46
dGMP	0.31
d(ac^{2}G)MP	0.48
N^2-Acetyldeoxyguanosine	0.79

[a] Solvent: 1.0 M NH_4Ac (pH 7.5)–95% ethanol, 1:1 v/v; Merck cellulose F tlc plates.

Nmr studies were conducted using 5–10% solutions of nucleoside in dimethyl-d_6 sulfoxide with tetramethylsilane as internal reference. An authentic sample of deoxyguanosine gave a spectrum in agreement with observations made by Gatlin and Davis[13] and by Goldschmidt, *et al.*[14] Specifically, deoxyguanosine showed a two-proton singlet at δ 6.45 (N^2-amino group) and a purine ring NH at 10.67. The spectrum of N-acetylated deoxyguanosine showed: (1) disappearance of the N^2-amino group singlet, (2) appearance of acetyl protons at δ 2.20, and (3) a broad two-proton peak at δ 11.8 containing the 1- and N^2-amide protons. These observations establish the acetylation site of deoxyguanosine at the N^2-amino group. No O-acetylation of the sugar hydroxyls was present, since the protons of the hydroxyl groups on 3′ and 5′ carbons were the same in both spectra.

Discussion

Inspection of molecular models of polydeoxynucleotides assembled as 15 base pairs in the B form of double-stranded DNA[2] shows that acetylation both of adenine and guanine (as in Figures 1 and 2) can occur without dislocation of neighboring atoms and with very minor interference of free rotation around the C–N amide bond. Isobutyryl groups on adenine can also be constructed, but the amide bond rotation is severely hindered. From steric considerations, acetylation should weaken association of the two strands somewhat, and isobutyrylation should lead to considerably greater weakening. Counteracting the steric effects, the increased acidity that acylation imparts to the amide hydrogen forming the hydrogen bond should strengthen that bond and, therefore, the whole double helix. However, in ac^{2}G, the acetyl group can provide an electronic destabilization of the charge polarization that can be postulated[15] for G·C and that cannot exist

(13) L. Gatlin and J. C. Davis, Jr., *ibid.*, 84, 4464 (1962).

(14) B. M. Goldschmidt, T. P. Blazej, and B. L. Van Duuren, *Tetrahedron Lett.*, 1583 (1968).

(15) The authors are grateful to referee II for suggesting that charge polarization contributes to the stability of the G·C pair and that the acetyl group would work in reverse to this polarization in the guanine

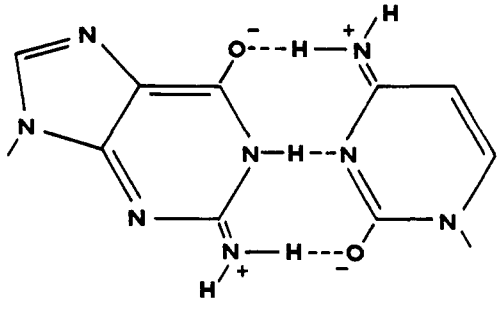
for A·T. Thermal hyperchromicity studies show that the net result of steric and electronic effects is that association between strands is weakened for all three cases of acylation that were studied.

In the case of $ac^6A \cdot T$ association (Figure 1), the drop in T_m is almost identical (32.9°, 33.3°) in 1.0 *M* Na^+ whether using dT_{15} or $dT_{\overline{47}}$ to form the double-stranded complex. Similar results could not be obtained for the $ib^6A \cdot T$ association because dT_{15} gave too low a T_m with $d(T_6\text{-}ib^6A_{\overline{65}})$ to be measured. From the $dT_{\overline{47}}$ experiment (Figure 7), the drop in T_m from $ac^6A \cdot T$ to $ib^6A \cdot T$ was 13.0°. It is suggested, therefore, from T_m data that replacing the free hydrogen on the 6-amino group of adenine in A·T with an acetyl group produces more weakening of the double helix than the further replacement of acetyl with isobutyryl.

Through the melting range of $d(T_6\text{-}ac^6A_{\overline{50}})$ with dT_{15} (Figure 6, curve 1) in 1.0 *M* Na^+, pH 7.0 (0–40°), there is very little, if any, loss of acetyl groups as judged by good reproducibility in recycling melting and cooling curves. However, in the mixed melting of Figure 6, curve 3, where the $d(T_6\text{-}ac^6A_{\overline{50}})$ was taken up to 70°, the cooling curve showed changes in the $ac^6A \cdot T$ portion, suggesting partial loss of acetyl groups. By use of controlled ammonolysis, a set of partially acetylated polymers was obtained that showed a linear change in T_m relative to per cent acetylation.

The early investigations[1,3] on $ac^2G \cdot C$ (Figure 2) association that immediately followed those on $ac^6A \cdot T$ were confusing in their negativity, since monoacetylation could directly affect only one out of three instead of one out of two of the hydrogen bonds as in $ac^6A \cdot T$ (Figure 1). In $ac^2G \cdot C$, the acetyl on the 2-amino group occupies space in the more restricted narrow groove of the double helix, but model building showed that sufficient space was available. There was a slight possibility, however, that the acetyl group could be on the nitrogen at ring position 1, a location that would completely disrupt the G·C association. Studies with nmr on *N*-acetyldeoxyguanosine, derived by enzymic dephosphorylation from the 5′-phosphate,gave proof that the acetyl group was on the N^2 position.

ring. Polarization in the G·C pair is illustrated in one extreme form contributing to the resonance hybrid. A similar structure is not



possible with the A·T pair.

Melting studies on $ac^2G \cdot C$ were performed at pH values just alkaline enough to eliminate most internal helix formation of polydeoxycytidylates[16] without being so alkaline as to catalyze rapid hydrolytic reversion of $d(ac^2G)$ to dG units. This acetyl group has been shown[1] to be somewhat labile by radiotracer studies of acetyl-1-^{14}C retention starting with acetic anhydride and going through the series $d(ac^2G)MP$, $d(ac^2G)TP$, and $d(T_6\text{-}ac^2G_{\overline{56}})$; at the end of the preparative sequence only 75% of the dG units were acetylated. During the melting studies in 0.5 *M* Na^+, pH 7.8 (Figure 8), and in 1.0 *M* Na^+, pH 8.1, with maximum temperature of 70°, it was observed that melting curves were not reproducible and that a fast melting run produced less change in the shape of the curve during melting than a standard slow run. Loss of acetyl groups is thought to be occurring such that the resultant increase in G·C content gives higher T_m values. Therefore, the true T_m of $ac^2G \cdot C$ in 1.0 *M* Na^+, pH 8.1, must be less than the indicated 34.2°.

Furthermore, it is not strictly correct to compare the strength of $ac^2G \cdot C$ association with that of $ac^6A \cdot T$ by comparing T_m's of $d(T_6\text{-}ac^2G_{\overline{68}}) \cdot dC_{\overline{87}}$ (34.2°) and $d(T_6\text{-}ac^6A_{\overline{65}}) \cdot dT_{\overline{47}}$ (41.7°) because of the difference in polymer lengths. It has been shown that T_m varies with the continuous hydrogen bonding length in mixtures of polydeoxynucleotides at the same molarity.[6] As was found in the $ac^6A \cdot T$ and A·T melting cases, the use of $dT_{\overline{47}}$ gave higher T_m's than with dT_{15}. Each of these polydeoxythymidylates determines the continuous hydrogen bonding length of the mixtures in which they are used. In the $ac^2G \cdot C$ case with minimum length 68 compared with 47 for the $ac^6A \cdot T$ case, the former has an undue advantage in length; for comparison, its T_m is too high. Therefore, based on the two effects, both of which lower the $ac^2G \cdot C$ T_m of 34.2°, the comparison with the $ac^6A \cdot T$ T_m of 41.7° shows that the $ac^2G \cdot C$ association is weaker than that of $ac^6A \cdot T$. Further supporting this conclusion is the change in Δ_{max} from 0.037 to 0.009 going from $ac^6A \cdot T$ to $ac^2G \cdot C$, showing a great drop in cooperativity of melting. It was not possible to study the G·C association in a double-stranded product such as $d(T_6\text{–}G_{\overline{68}}) \cdot dC_{\overline{87}}$ formed by mixing the strands because of the tendency of dG_n to aggregate rather than to complex with dC_n.[3]

Acknowledgments. This work was performed under the auspices of the U. S. Atomic Energy Commission. T. J. S. acknowledges support from U. S. Public Health Service Grant No. 5R01-AM-10,628 and from Life Insurance Medical Research Fund Grant No. G-70-20. We are grateful to D. L. Williams and V. N. Kerr for some of the deoxynucleotides used in this study, to Dr. R. L. Ratliff for calf thymus terminal deoxynucleotidyltransferase, and to Mrs. E. H. Lilly for phosphorus analyses.

(16) R. B. Inman, *J. Mol. Biol.*, 9, 624 (1964).