(1977a) *J. Biol. Chem. 252,* 4007.
Weber, L. A., Hickey, E. D., Nuss, D. L., & Baglioni, C. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74,* 3254.
Zan-Kowalczewka, M., Bretner, M., Sierakowska, H., Szezesna, E., Filipowicz, W., & Shatkin, A. J. (1977) *Nucleic Acids Res. 4,* 3065.

Role of Hypermodified Bases in Transfer RNA. Solution Properties of Dinucleoside Monophosphates†

Mark Terrill Watts*,‡ and Ignacio Tinoco, Jr.

ABSTRACT: The hypermodified dinucleoside monophosphates, uridylyl(3′–5′)-*N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine (Upt^{6}A), adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)adenosine (Api^{6}A), adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)-2-methylthioadenosine (Apms^{2}i^{6}A), and adenylyl(3′–5′)-1,N^6-ethenoadenosine (ApεA, a synthetic model for adenylyl(3′–5′)wybutosine, ApyW), which represent the most common sequences found as the third letter of the anticodon triplet and its adjacent 3′ neighbor, have been isolated. Their solution properties have been investigated using ultraviolet absorption, circular dichroism (CD), and high resolution proton magnetic resonance. The properties of these molecules have been compared with those of their unmodified counterparts, uridylyl(3′–5′)adenosine (UpA) and adenylyl(3′–5′)-adenosine (ApA). These properties measured as a function of temperature have been analyzed employing a two-state intramolecular stacking model. All of the properties show that the stacking of Upt^{6}A is stabilized relative to UpA, while Api^{6}A, Apms^{2}i^{6}A, and ApεA are slightly destabilized relative to ApA. Thus, Upt^{6}A, Api^{6}A, Apms^{2}i^{6}A, and ApεA have comparable stacking equilibria, indicating that the modifications remove the large difference in stacking between UpA and ApA. Furthermore, cytidylyl(3′–5′)adenosine (CpA), which is the most common unmodified sequence in this particular anticodon region, exhibits a stability similar to those of the hypermodified dinucleoside phosphates. Hypermodification therefore seems to keep the flexibility of this crucial part of the tRNA constant. It is proposed that this may result in a more smoothly regulated translation step. Also, it is proposed that the enhanced stacking of Upt^{6}A relative to UpA prevents the incorrect wobble base pairing of this U residue in the tRNA during translation.

We have attempted in this investigation to define and understand on a molecular level the functions of hypermodified bases in tRNAs. That these bases perform some function during protein synthesis has been supported by experiments with polymer directed polypeptide synthesis, and binding assays using ribosomes and synthetic polymer messages (Gefter & Russell, 1969; Odom et al., 1974; Kitchingman et al., 1976; Miller et al., 1976). tRNAs modified or deficient in their normal amount of hypermodification were generally found to be less efficient in messenger binding and translation. In most of these studies, the aminoacylations of the tRNAs which were changed in their content of hypermodification were affected little if any by the changes. Binding studies with tRNAs and complementary trimers, or with two tRNAs having complementary anticodon triplets have also shown a stabilization of the binding when hypermodified bases are present (Högenauer et al., 1972; Grosjean et al., 1976). Thus, with only a few exceptions (Litwack & Peterkofsky, 1971; Kimball & Söll, 1974), the presence of the hypermodified bases has been demonstrated to affect in vitro polypeptide synthesis. This effect, at least in part, appears to be due to a more efficient binding of the tRNA to the ribosome–mRNA complex.

Aside from these effects upon translation, some authors have suggested that the role of t^{6}A[1] is to prevent wobble on the 3′ side of the anticodon triplet (Ghosh et al., 1967; Takeishi et al., 1968; Dube et al., 1968; Stewart et al., 1971; Jukes, 1973; Elkins & Keller, 1974). Also, on the basis of crystal structures of the hypermodified bases, several workers have proposed that the role of hypermodification is to prevent the formation of a fourth base pair between the tRNA and mRNA (Bugg & Thewalt, 1972; Parthasarathy et al., 1974a,b).

Except for the hypothesis of the prevention of a fourth base pair, it is not known how these possible functions are related to the molecular properties of the hypermodified bases. Therefore, we have chosen to approach this problem through the study of the smallest unit of a hypermodified tRNA which still exhibits the characteristics of a polynucleotide—the dinucleoside monophosphate. In this study, the solution spectral properties of Upt^{6}A, Api^{6}A, Apms^{2}i^{6}A, ApεA, and their

† From the Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, Berkeley, California 94720. *Received November 18, 1977.* This work was supported by National Institutes of Health Grant GM10840 and by the Division of Biomedical and Environmental Research of the United States Department of Energy. Presented in preliminary form at the 61st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 1977, Abstract No. 2234.

‡ This work is taken from a dissertation submitted to the Graduate Division, University of California at Berkeley, in partial fulfillment of the requirement for the Ph.D. degree, June 1977. Present address: The Proctor and Gamble Co., Miami Valley Laboratories, P. O. Box 39175, Cincinnati, Ohio 45247.

[1] Abbreviations used: t^{6}A, *N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine; i^{6}A, N^6-(Δ^2-isopentenyl)adenosine; ms^{2}i^{6}A, N^6-(Δ^2-isopentenyl)-2-methylthioadenosine; εA, 1-N^6-ethenoadenosine; yW, wybutosine (Kasai et al., 1976); pt^{6}A, 5′-monophosphate of t^{6}A; Upt^{6}A, uridylyl(3′–5′)-*N*-[9-(β-D-ribofuranosyl)purin-6-ylcarbamoyl]-threonine; Api^{6}A, adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)adenosine; Apms^{2}i^{6}A, adenylyl(3′–5′)-N^6-(Δ^2-isopentenyl)-2-methylthioadenosine; ApεA, adenylyl(3′–5′)-1,N^6-ethenoadenosine; ApyW, adenylyl(3′–5′)-wybutosine.

0006-2960/78/0417-2455$01.00/0

component monomers have been measured and compared with those of the unmodified counterparts. Because of its additional adduct ring, ϵA was selected as a simple model for the yW type bases.

Experimental Procedure

pt^{6}A and pUpt^{6}A were obtained from snake venom phosphodiesterase digests of crude tRNA (Cunningham & Gray, 1974). The crude tRNA was mixed bakers' yeast tRNA which was deficient in tRNA^{Phe} and tRNA^{fMet} (Freier & Tinoco, 1975). Authentic samples kindly supplied by Dr. Michael Gray (Dalhousie University) were used as chromatographic markers and then pooled with the molecules isolated here. Upt^{6}A was obtained by treating partially purified pUpt^{6}A with bacterial alkaline phosphatase (Borer, 1972). pt^{6}A and Upt^{6}A were both purified and desalted chromatographically using DEAE A-25 Sephadex, LH-20 Sephadex, Bio-Gel P-2, and Chelex-100 resins (Watts, 1977), and paper chromatography (Cunningham & Gray, 1974). pt^{6}A was left as the triethylammonium salt, while the final Upt^{6}A product was the pure sodium salt.

Api^{6}A, Apms^{2}i^{6}A, and ApϵA were synthesized from N^6,-$O^{2'}$,$O^{5'}$-triacetyl 3'-AMP and the respective hypermodified nucleosides (Follman, 1967). The i^{6}A nucleoside was obtained from Sigma. ms^{2}i^{6}A was the gracious gift of Dr. Roy Morris (Oregon State University). ϵA was synthesized by Dr. Che-Hung Lee of this laboratory (Secrist et al., 1972). Api^{6}A and Apms^{2}i^{6}A were purified by DEAE-Sephadex A-25 and Sephadex LH-20 chromatography (Watts, 1977). ApϵA was also column chromatographed in DEAE-Sephadex A-25, and paper chromatographed in 95% EtOH/1 M ammonium acetate (70% v/v) (Borer, 1972). These dimers were left as the triethylammonium salts.

pi^{6}A and pms^{2}i^{6}A were obtained from snake venom phosphodiesterase digests of Api^{6}A and Apms^{2}i^{6}A, respectively (Watts, 1977). They were purified chromatographically as described above for the dimers. pϵA was obtained from Sigma.

The identities of Upt^{6}A, Api^{6}A, Apms^{2}i^{6}A, and ApϵA were verified by chromatography with markers of the NaOH, snake venom phosphodiesterase, and bovine spleen phosphodiesterase digests of the dimers. The characteristic UV and NMR spectra of the monomers and dimers also aided in their identification.

All unmodified counterparts (e.g., ApA) were obtained commercially. UV absorption spectra were obtained on Cary spectrophotometers (Models 14, 15, and 118c). Absorbance–temperature profiles (0–80 °C) for monomers and dimers were measured using a Beckman DU with a Gilford multiple sample absorption unit and a Neslab variable temperature bath system. CD spectra were measured with a Cary spectropolarimeter (Model 60) equipped with a circular dichroism assembly (Model 6001). CD data were directly encoded to a PDP8/e minicomputer, where they were smoothed and corrected for baseline deviations (Blum et al., 1972). Variable temperatures (0–80 °C) were obtained with the help of a frigistor heating and cooling system which was controlled by a Hallikainen Thermotrol (Allen et al., 1972). Checks for evaporation after the heating of the absorption and CD variable temperature experiments were always made after the cell had reequilibrated to room temperature. Those few experiments which gave a different absorbance before and after the experiment were discarded. Corrections for solvent expansion were routinely made for the UV absorption and CD variable temperature data.

Solvents for absorption and CD measurements were generally either water (pH ~ 5.5–7) or more frequently 2 mM sodium phosphate buffer (pH ~7; 1.2×10^{-3} M Na_2HPO_4, 0.8×10^{-3} M NaH_2PO_4).

Extinction coefficients and percent hypochromicities for the dinucleoside monophosphates were determined by degrading the dimers to their component monomers with NaOH (Borer, 1972; Watts, 1977). Using the reported values for the extinction coefficients of the monomers pA, pU, and pC (P-L Biochemicals, 1973), i^{6}A (Grimm & Leonard, 1967), ms^{2}i^{6}A (Hecht et al., 1969), ϵA (Secrist et al., 1972), and t^{6}A (Hall, 1971), the extinction coefficients and hypochromicities of the dimers can be calculated. t^{6}A is, however, converted completely to A upon the NaOH treatment (Chheda et al., 1969; Watts, 1977). Thus, the extinction of A was used for t^{6}A after the treatment with NaOH. ϵA was likewise degraded by NaOH. However, after ~36 h of treatment with NaOH an isosbestic point at 260.5 nm was observed which was independent of time. An extinction coefficient equal to 3.8×10^3 was determined for the resulting mixture of two products at that time. From 36 h and longer this is the value of the extinction for the unknown mixture of products formed from ϵA at 260.5 nm. As with the other dimers, ApϵA was treated with NaOH for 48 h.

High resolution NMR spectra were obtained on a Bruker HSX 360 MHz spectrometer located at the Stanford Magnetic Resonance Laboratory. Spectra were obtained at various temperatures (0–75 °C) in the FT mode. Pulse amplitude, duration, and delay were kept at levels such as not to cause appreciable saturation. Samples were prepared under dry nitrogen in 100.0% D_2O at concentrations of 5 mM or less, where intermolecular effects were negligible (Ts'o et al., 1967; Chan & Nelson, 1969; Watts, 1977). Sodium trimethylsilylpropionate (TSP) was added as an internal standard. Nucleoside monophosphates were prepared with pD = 5.5 (pH_{meter} 5.1), whereas dinucleoside monophosphates and nucleosides were prepared with pD = 7.0 (pH_{meter} 6.6) (Ts'o et al., 1967; Izatt et al., 1971; Watts, 1977). Chemical shifts from TSP (δ_{TSP}) are judged accurate within ±0.005 ppm, while coupling constants (J) are in error by ±0.2 Hz. Most of the monomers' protons have been assigned previously (Ts'o et al., 1967; Schweizer et al., 1969; Secrist et al., 1972). In the case where there was question concerning the proton assignments, comparisons between nucleoside and nucleotide, between modified and unmodified monomers, or between different pDs were performed (Schweizer et al., 1960; Danyluk & Hruska, 1968). Peak assignments of the dimers' protons were made by comparing their high temperature spectra with the characterized monomers' spectra. Also of use in the dimer proton assignments were the characterized spectra of the unmodified dimers (Ts'o et al., 1967).

Results

Table I presents the extinction coefficients (per mole of dimer) for the first absorption maximum of the dinucleoside monophosphates at 25 °C. Also tabulated are the percent hypochromicities (% h) (Bloomfield et al., 1974) at that wavelength at 25 °C. Percent h's were determined at every 5 °C from 0 to 80 °C and are tabulated elsewhere (Watts, 1977). Figure 5 illustrates some representative temperature profiles. No effects upon the extinction coefficients or the % h temperature profiles were observed upon the addition of dilute buffer (the 2 mM sodium phosphate buffer described above), sodium chloride (up to 100 mM), $MgCl_2$ (10 mM), EDTA, or combinations thereof.

Figures 1 and 2 illustrate the CD spectra at room temperature of the dimers studied ($\Delta\epsilon$ per base residue). In obtaining

TABLE I: Extinction Coefficients (per Mole of Dimer) and Percent Hypochromicities (% h) of the Dinucleoside Monophosphates at 25 °C.[a]

Dimer	λ_{max} (nm)	$\epsilon \times 10^{-3}$ at λ_{max}	% h at λ_{max}
ApA	258	27.1	11.7 ± 0.7
Api^{6}A	262.5	29.5	10.8 ± 0.8
Apms^{2}i^{6}A	263	24.4	7.7 ± 1.0
ApϵA	260	17.6	13.1 ± 1.0
UpA	258	24.4	3.4 ± 1.3
Upt^{6}A	268	30.4	11.2 ± 1.3
CpA	261	21.2	7.1 ± 0.9

[a] ϵ's are in error by ±0.2 to 0.3 × 10^3 L/(M cm) (calculated as the standard deviation from the mean of 5–10 measurements). Error in % h includes propagated error in monomer and dimer ϵ's. % h = $(1 - \epsilon_{dimer}/\Sigma\epsilon_{monomers}) \times 100$.

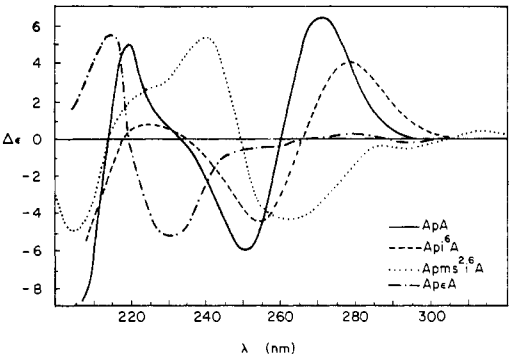

FIGURE 1: CD of dinucleoside monophosphates (per residue). ApA (—), Api^{6}A (- - -), Apms^{2}i^{6}A (· · ·), ApϵA (— · —), at pH 7 and 25 °C, for all except ApϵA which is at 20 °C. Monomer CDs have been subtracted.

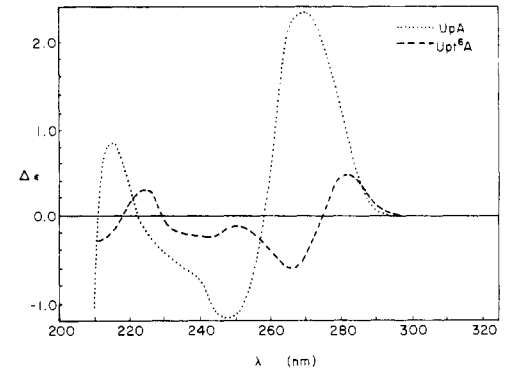

FIGURE 2: CD of dinucleoside monophosphates (per residue). UpA (· · ·), Upt^{6}A (- - -) at pH 7 and 0 °C. Monomer contributions have been subtracted. t^{6}A monomer has a large CD band ($\Delta\epsilon$ = 4) at ~210 nm (Watts, 1977).

these curves, the sums of the component monomers' CD have been subtracted. The values of the monomer CD contributions at the wavelengths of the dimers' maxima or minima ranged from $|\Delta\epsilon|$ = 1 to 2 (or approximately 15 to 25% of the dimers total CD at low temperatures). Figure 3 presents a typical CD "melting" experiment. The values for the $\Delta\epsilon$'s (with monomer contributions subtracted) at the wavelength of the first peak or trough, and at every 10 °C from 0 to 80 °C are tabulated elsewhere (Watts, 1977). As with the absorption, no effects upon the CD spectra were observed from variations in the salt conditions.

NMR dimerization changes (Ts'o et al., 1967) were determined at several temperatures between 0 and 75 °C (usually 0, 10, 20, 25, 37, 45, 60, and 75 °C). The dimerization shifts ($\Delta\delta_{TSP}$) of the base protons, the 1′-sugar protons, and most of the hypermodified side chains' protons were measured. Figures 4a–c present some of the more important dimerization shift temperature profiles.

The dimerization changes in the 1′-sugar proton coupling constants ($\Delta J_{1'2'}$) were also determined as a function of temperature. Figure 4d presents some representative $\Delta J_{1'2'}$ temperature profiles. All of the dimerization changes have been tabulated elsewhere (Watts, 1977).

These dimer properties (%h, $\Delta\epsilon$, $\Delta\delta_{TSP}$, $\Delta J_{1'2'}$) have been analyzed using a two-state stacking model, the validity of which will be discussed in the following section. Thus, the measured property of a dimer at any temperature is:

$$P(T) = XP_S + (1 - X)P_U \quad (1)$$

where P_U is the temperature independent property of the unstacked state, U, and P_S is the temperature independent property of the stacked state, S, and X is the fraction of the stacked state, S. It is known that the properties of dimers closely approach the sum of the properties of their component monomers when the stacking is sufficiently reduced (Bloomfield et al., 1974). This was also observed in the present study. Upon the addition of ethanol ApA and Api^{6}A exhibited a 97% and a 93% loss of their hypochromicity, respectively (Watts, 1977). Therefore, since the monomers' properties have been subtracted from all of the dimers' measured properties, we have taken P_U to be equal to zero. Using this assumption, the U ⇌ S equilibrium constant is:

$$K(T) = \frac{P(T)}{P_S - P(T)} \quad (2)$$

P_S is unfortunately very difficult, if not impossible to obtain experimentally (Davis & Tinoco, 1968). We have further assumed, then, that the $\Delta H°$ for the stacking equilibrium is temperature independent (Davis & Tinoco, 1968; Topal, 1974, Topal & Warshaw, 1976). The criteria of fit of the experimental data, $P(T)$, to the two-state model was thus to choose the value of P_S which when substituted into eq 2 resulted in a set of $K(T)$ which formed the best straight line in a van't Hoff plot (Topal, 1974). The "best" line was determined by minimizing the average deviation from the least-squares line of each of the ordinate (ln K) values, divided by the total ordinate range (the value chosen for P_S affected the size of the ordinate). Care was always taken to check that there was only one minimum as a function of the P_S chosen. Some of the data (the CD data for UpA and Upt^{6}A, and several of the NMR dimerization changes) did not yield minima and were not analyzed by this method.

Figure 5 illustrates some examples of two-state model fits of %h temperature profiles. Table II summarizes the results obtained from the two-state analysis of the UV absorption data. Table III presents the results from the analysis of the CD data, while Table IV summarizes the results of the fitting of some of the proton's dimerization changes.

An additional two-state model analysis procedure was used for the $J_{1'2'}$ coupling constants (Lee et al., 1976; Lee & Tinoco 1977). In this model, we have taken as our fully stacked state the stacked conformation found in double-stranded stacked helices. The geometry found for the sugar residues in this conformation (3′-endo) requires that the dihedral angle H1′–C–C–H2′ be 90°; this leads to the value for the stacked state's coupling P_S = 0.0 (Lee et al., 1976). Once again, the

TABLE II: Thermodynamic Results for the Two-State Model from Absorption Data.[a]

Dimer	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/(deg mol))	$K_{37°C}$	P_S (% h_S)
ApA	−7 (−5, −9)	−20 (−17, −27)	1.4 (0.9, 1.8)	18 (15, 21)
Api^{6}A	−3 (−2, −5)	−11 (−9, −15)	0.9 (0.4, 1.8)	20 (15, 37)
Apms^{2}i^{6}A	−6 (−4, −9)	−18 (−10, −27)	2.9 (1.1, 4.5)	10 (7, 13)
ApϵA	−4 (−3, −6)	−14 (−12, −20)	0.9 (0.5, 1.3)	24 (18, 36)
UpA	−2 (−2, −6)	−8 (−8, −17)	0.3 (0.1, 2.8)	13 (4, 25)
Upt^{6}A	−3 (−1, −5)	−8 (−6, −16)	1.4 (1.0, 3.9)	18 (12, 37)
CpA	−6 (−4, −10)	−19 (−14, −30)	0.9 (0.6, 1.9)	12 (9, 19)

[a] The parameters listed are for the reaction U → S. P_S is the best value for the % h of the stacked state. Listed in parentheses are the likely lower and upper limits for each value. These error limits include the errors in the % h (25 °C) measurement and the errors from the temperature variation in the absorption (Watts, 1977). They do not relate to the accuracy of the two-state model in describing the dimers' stacking.

TABLE III: Thermodynamic Results for the Two-State Model from CD Data.[a]

Dimer	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/(deg mol))	$K_{37°C}$	P_S ($\Delta\epsilon_S$)
ApA	−6.5 (−6, −7.5)	−23 (−21, −25)	0.4 (0.3, 0.6)	17 (14, 20)
Api^{6}A	−4.5 (−4, −5.5)	−18 (−17, −20)	0.2 (0.1, 0.5)	17 (10, 30)
Apms^{2}i^{6}A	−5 (−3, −9)	−16 (−9, −25)	2.4 (0.7, 4.0)	−7 (−6, −11)
ApϵA	−6 (−5, −7)	−22 (−20, −25)	0.4 (0.2, 0.5)	−14 (−11, −21)

[a] $\Delta\epsilon$, adjusted for monomer contributions, vs. T. Wavelengths (nm) used are: ApA (271), Api^{6}A (279), Apms^{2}i^{6}A (264), ApϵA (231). See footnote to Table II for explanation of parameters and values in parentheses.

TABLE IV: Thermodynamic Results for the Two-State Model from NMR Data.[a]

Dimer	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/(deg mol))	$K_{37°C}$	P_S
Apa				
H2Ap	−3 ± 2	−13 ± 5	0.3 (0.1, 1.0)	0.9 ± 0.8
H8pA	−6 ± 2	−20 ± 6	1.4 (0.6, 2.2)	0.3 ± 0.2
H1′Ap	−2	−9	0.3	1.0
$\Delta J_{1'2'}$Ap	−3 ± 2	−13 ± 5	0.5 (0.2, 1.6)	8 ± 8
Api^{6}A				
H2Ap	−3 ± 2	−12 ± 2	0.4 (0.1, 1.0)	0.7 ± 0.8
H2pi^{6}A	−4	−13	0.7	0.3
H8pi^{6}A	−3 ± 2	−11 ± 3	0.9 (0.1, 1.8)	0.4 ± 0.8
UpA				
H1′Up	−4	−13	0.7	0.5
Upt^{6}A				
H1′Up	−4	−14	0.9	0.4
H6Up	−3	−8	2.5	0.2

[a] Dimerization changes. For a proton listing (e.g., H2Ap), the results are from the $\Delta\delta_{TSP}$ data. For a coupling constant listing (e.g., $\Delta J_{1'2'}$Ap), the results are from the $\Delta J_{1'2'}$ data. See footnote to Table II for explanation of parameters and values in parentheses.

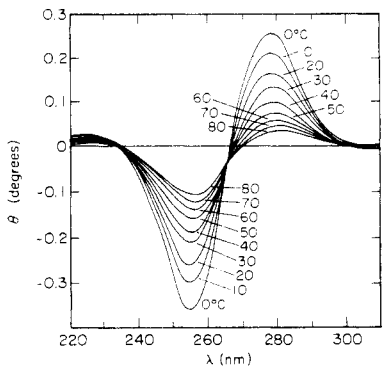

FIGURE 3: CD melting experiment of Api^{6}A at pH 7, 1-cm pathlength, $A_{max} \cong 1$. The monomer CDs have not been subtracted.

unstacked state's properties are taken as equal to the monomers' properties. Then,

$$A(T) = XP_S + (1 - X)B(T) \quad (3)$$

where $A(T)$ is the observed splitting in the dimer, $B(T)$ is the temperature dependent splitting of the monomer, and X is the fraction of the stacked state, S. Combining eq 3 with the expression for the equilibrium constant ($K(T) = [S]/[U]$), and using our assumption that $P_S = 0$, we arrive at:

$$K(T) = (B(T) - A(T))/A(T) \quad (4)$$

This method differs from that in eq 2 only in that the value of P_S was deduced by assumptions rather than fitting procedures based on a temperature independent $\Delta H°$. Using eq 4, which relates only *measured* properties with the equilibrium constant,

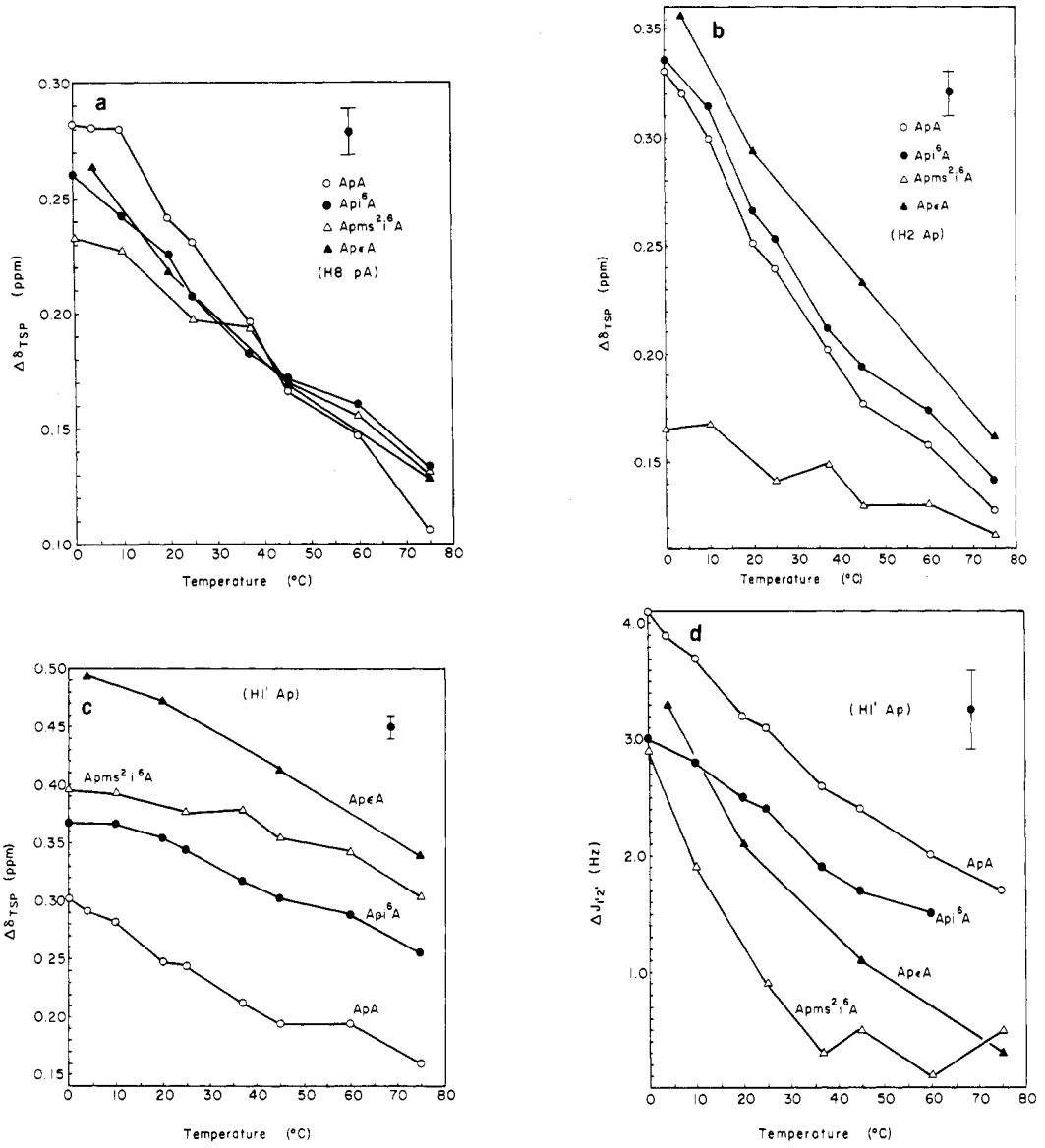

FIGURE 4: Dimerization changes of dinucleoside monophosphates. ApA (○), Api⁶A (●), Apms²i⁶A (△), ApεA (▲) at 5 mM concentrations. Solid lines are present only as visual aids. Errors are ca. ±0.01 ppm for the chemical shift dimerization changes and ca. ±0.3 Hz for the coupling constant dimerization changes. (a) Dimerization shifts of the H8 proton of the -pA residue. (b) Dimerization shifts of the H2 proton of the Ap- residue. (c) Dimerization shifts of the H1′ proton of the Ap- residue. (d) Dimerization changes of the coupling constant of the H1′ proton of the Ap- residue.

we can obtain a K at every temperature. Then, from a least-squares line of ln K vs. $1/T$, we can arrive at a van't Hoff $\Delta H°$.

Figure 6 presents such a plot using the data for the Ap- residue of ApA and Api⁶A. Table V summarizes the 3′-endo method results for the dimers studied. The $J_{1'2'}$ values at high temperatures for the Up- residue of UpA and Upt⁶A did not appear to approach the monomer's value, and thus these data were not analyzed by this model (Lee & Tinoco, 1977).

Discussion

The properties of dinucleoside monophosphates at one temperature have often been used to compare their stacking interactions (Bloomfield et al., 1974; Ts'o, 1974b). The problem with this simple approach is that we are not certain that a given amount of stacking will yield the same values for the measured properties for dimers containing different monomers. For the hypermodified bases studied here, which have very different electronic transition properties and different shielding ring currents than those of adenosine, this uncertainty is especially evident. We have therefore chosen to monitor the properties of the dimers as a function of the position of the stacking equilibria. Since the equilibria are very temperature dependent, it is possible to extract relative thermodynamic parameters of the stacking interactions of a model which describes the equilibrium. The simplest model is a two-state model. There is a large body of literature dealing with the two-state model and its use in describing dimer stacking equilibria (Bloomfield et al., 1974; Ts'o, 1974b).

ApA Derivatives. The use of the model in this study indicates that hypermodification of ApA changes its stacking ability (Tables II–V). Since stacking is believed to be caused mainly by attractive forces, it has often been discussed in terms of its ΔH (Ts'o, 1974a). The $\Delta H°$ values obtained in this study for ApA are in agreement with literature values using corresponding techniques (Watts, 1977). With few exceptions the hypermodified counterparts of ApA have been found here to have less favorable stacking enthalpies than ApA. For instance, the $\Delta H°$ values from absorption and CD studies of ApA are -7 and -6.5 kcal mol^{-1}, respectively. The corresponding

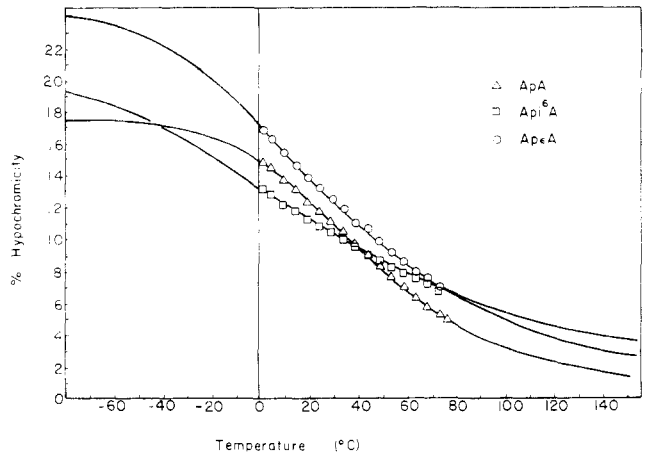

FIGURE 5: Percent h vs. T data for ApA (△, 258 nm), Api^{6}A (□, 262.5 nm), and ApεA (○, 260 nm) at pH 7. Smooth curves are the calculated fits for the two-state model.

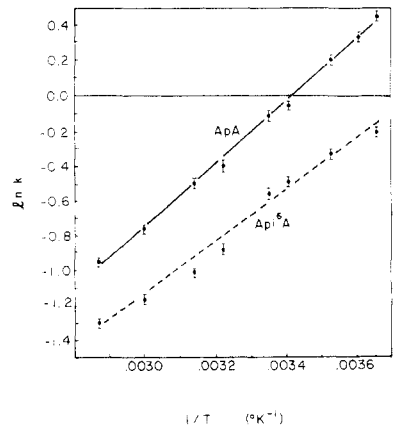

FIGURE 6: van't Hoff plots of two-state ln Ks derived from the 3′-endo approach to the coupling constants, $J_{1'2'}$. The lines are the least-squares lines for ApA (Ap residue, ——), and Api^{6}A (Ap residue, - - -).

values for Api^{6}A are -3 and -4.5 kcal mol^{-1}. This is in contrast to the effect of methylation of the base moieties of nucleosides upon their intermolecular associations (Ts'o, 1974a). There the ΔH increases with methylation. That the free energies of the hypermodified dimers are not widely different from ApA's is a result of a more favorable entropic contribution in each case. Unfortunately, the experimental error alone (not including the uncertainty due to the choice of the two-state model) in almost all cases allows an overlap of the values of $\Delta H°$ and $\Delta S°$ for ApA and its hypermodified dimers. Also, although the mentioned trends are the same from one technique to the next, the absolute values of the $\Delta H°$'s and $\Delta S°$'s for a given dimer are in general different for the absorption, CD, or NMR results.

Because of the difficulty in interpreting changes in the separate contributions to the free energies, the remainder of the discussion of the dynamic stacking abilities will be centered about the equilibrium constants. This is also advantageous because the experimental errors in the K's are smaller than those in the $\Delta H°$'s and $\Delta S°$'s. Still, however, it is readily apparent that the K's for a given dimer are different from technique to technique. Even within the realm of one technique, NMR, there is a variance of K's for a given dimer depending upon the particular dimerization change monitored. It is possible that in some of these cases the discrepancies can be explained by experimental error, but it is believed that the differences are the result of the choice of the two-state model. Different equilibria may be measured by the different techniques. This is also suspected because of the trends in the K's from technique to technique. The K's from absorption tend to be generally higher than those found by CD or NMR (3′-endo approach).

TABLE V: Thermodynamic Results for the Two-State Model from the 3′-Endo Approach to the NMR Data.[a]

Dimer	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/(deg mol))	$K_{37°C}$ (±0.08)
3′ Residues			
ApA (Ap)	−3.5	−12	0.71
Api^{6}A (Ap)	−3.0	−11	0.45
Apms^{2}i^{6}A (Ap)	−7.5	−29	0.10
ApεA (Ap)	−7.5	−27	0.22
5′ residues			
ApA (pA)	−5.5	−20	0.38
Api^{6}A (pi^{6}A)	−2.9	−12	0.30
Apms^{2}i^{6}A (pms^{2}i^{6}A)	−2.2	−10	0.24
ApεA (pεA)	−3.9	−15	0.31
UpA (pA)	−4.4	−18	0.17
Upt^{6}A (pt^{6}A)	−2.3	−11	0.22

[a] Parameters are for the reaction U → S. Errors in Ks are from propagation of errors in the coupling constants of the monomers and dimers (Watts, 1977). They do not include the uncertainty due to the choice of the model.

Nevertheless, with only a few exceptions, certain trends are evident from technique to technique in the K's for ApA, Api^{6}A, Apms^{2}i^{6}A, and ApεA. It appears from the K's in Tables II, III, and V that these hypermodifications slightly decrease the stacking ability relative to ApA. The notable exceptions to this are the rather abnormally high K's found for Apms^{2}i^{6}A with absorption (2.9) and CD (2.4). The experimental errors found with the K's for Apms^{2}i^{6}A were generally significantly larger than for the other dimers (ca. ±1.7 for both the absorption and the CD). This occurs because the properties monitored for Apms^{2}i^{6}A generally showed small changes with temperature relative to the changes observed for the other dimers. Also, the P_S values for this dimer with CD and absorption were very low relative to the other dimers' P_S's (Watts, 1977). If one simply picks values of P_S close to those found with the other dimers (18–24 for % h_S, 14–17 for $\Delta\epsilon_S$), then values for the K's obtained with both the absorption and the CD are less than that for ApA. These values of P_S still yield reasonable straight lines for the ln K vs. $1/T$ plots. Certainly the 3′-endo results of Apms^{2}i^{6}A are consistent with this trend.

Because of the large variation between the K's for the various protons of a given dimer, it is impossible to judge whether the dimerization changes agree with this trend (Table IV; Watts, 1977). Also, large fitting errors relative to the absorption or CD were observed with almost all protons. Likewise, for a given proton (even those with the better fitting errors) the ± values for the K's are large. Even if one compares corresponding protons from ApA to Api^{6}A, no consistent trend is observed. All this stems from the fact that the changes from the extremes of the temperature range were generally no more than ~0.15 ppm and often only 0.1 ppm or less. Those protons listed in Table IV exhibited the largest changes with temperature, but still exhibit large differences for the same dimer. Thus, the dimerization changes unfortunately cannot be taken as either supporting or conflicting evidence for the trend mentioned above.

UpA Derivatives. Another trend to be noticed in Tables II,

IV, and V is the fact that, with all the techniques used, Upt^{6}A appears to be stabilized relative to UpA. The absorption yields a large difference in the K's (1.4 vs. 0.3) while the NMR shows a smaller but consistent difference (0.22 vs. 0.17, 3′-endo method). The H1′Up dimerization change also yields the same trend but is unreliable considering the discussion above.

The results taken together indicate that i^{6}A, ms^{2}i^{6}A, and ϵA slightly destabilize the stacking of ApA, while t^{6}A stabilizes the stacking of UpA. The fact that these trends appear in all of the techniques used adds weight to their validity.

Two-State Model. The basic argument against the use of the two-state model for these systems is that different thermodynamic parameters are obtained for the different properties monitored. It is thus somewhat surprising that isosbestic points are observed in the CD melting experiments (Figure 3). These isosbestic points were observed for all of the dimers studied by CD. This type of observation has been studied for the absorption and CD of ApA and CpC (Powell et al., 1972). These authors conclude that, for optical data, thermodynamic parameters can be obtained from a two-state model. They state that if there are more than two states, the intermediates between the stacked and unstacked forms do not have recognizable identities in terms of spectroscopic properties. It is possible that there exists a set of stacked states which exhibit indistinguishable absorptions. Likewise there may be a set of states with similar CD spectra. That these two sets of states might not overlap would be conceivable when one considers the different geometry dependences of the absorption and CD (Bloomfield et al., 1974; Davis, 1967). The absorption tends to be less geometry dependent and depends more on simply the distance between the bases than does the CD. Perhaps this is why the CD appears to "melt" out with increasing temperature before the absorption does ($K_{abs} > K_{CD}$ in general). NMR, with its probes on various portions of the dimer, may be more sensitive to the nature of the stacked states (Ts'o, 1974b; Lee & Tinoco, 1977). This could explain the difference between results from different protons. Each proton may "melt" away in a different fashion.

It is clear then that there exist more than two states for the dimer stacking. It is not clear whether the use of a two-state model is a valid means of obtaining accurate thermodynamic parameters from temperature data.

The other major assumption used in fitting the data to the two-state model is that $\Delta H°$ must be independent of temperature. Figure 6 presents data which supports this. The ln K's from the 3′-endo method (which places no restrictions upon $\Delta H°$) plotted vs. $1/T$ give very reasonable straight lines.

The 3′-endo method is also a two-state model, and is a specific probe on only one portion of the dimer. From the above discussion, the results from the 3′-endo method probably do not reflect how the dimer melts—only the sugar region. However, if we are only to compare that region from dimer to dimer (and not from property to property), then the trends obtained in the parameters may very well describe the trends in the "overall" stacking of the different dimers. It is of interest to note that the parameters from the 3′-endo analysis of the Ap- residue of ApA agree quite well with the fit parameters from the dimerization change $\Delta J_{1'2'}$Ap (Tables IV and V).

Although there are difficulties in using the two-state model to analyze the data, the consistency from technique to technique of the trends observed here suggests that it is a worthwhile model to use in obtaining relative thermodynamic parameters. One should not expect accurate absolute stacking parameters, nor agreement in values from technique to technique. The model should be useful in attempting to learn of differences in stacking abilities.

Static Properties. It would be of interest to note whether a comparison of the properties of the dimers at one temperature would lead to conclusions regarding the stacking abilities similar to those obtained by the dynamic analyses. Unfortunately, the static properties are complicated by the fact that the monomers' properties themselves can influence the properties of the dimers in ways not directly related with stacking. For instance, the % h is affected by the strength of the monomer absorption transitions (Bloomfield et al., 1974). Additional complications arise with static CD data. The sensitive geometry dependence could (e.g., in the case of tilted bases) obscure large stacking as measured by the % h or $\Delta\delta$. Also, cancellation of positive and negative CD bands can occur.

Likewise, dimerization shifts are greatly influenced by the ring currents of the mononucleotides, making direct comparisons between dimers containing modified and unmodified bases difficult. Figures 4b and 4c illustrate the ambiguities one would encounter in interpreting shifts in terms of stacking abilities. Even if one compares the dimerization shifts of the -pA residue in which the shielding base is the same (either Ap- or Up-), the inferences from stacking stabilities are not clear. Thus, it might appear that the base-base interaction of ApA is not largely affected by hypermodification (Figure 4a). Upt^{6}A on the other hand appears to have greater base-base interaction than UpA, as judged by the large difference (0.14 ppm) in the dimerization shifts of the H8pA protons of the molecules. Unfortunately, with the presence of the large side chains on these dimers, it is not known that the aromatic base-base interaction is the only factor affecting their stacking stabilities.

We therefore conclude that the static data alone cannot be considered as supporting or conflicting evidence for the stacking trends observed. Certainly, the bulk of the static data is consistent with the trends (for a more detailed discussion, see Watts, 1977), but it is not surprising that notable exceptions exist (see Figures 2 and 4c). Finally, it is important to reemphasize that the static properties reflect conformations and indicate only through inference the stabilities of the conformations. Thus, they will prove more useful in discussing conformations rather than stacking abilities, especially if one considers more than one stacked state (Watts, 1977; Lee & Tinoco, 1977).

Some of the static properties reported here are at variance with previously reported data. Our dimerization shifts for Api^{6}A relative to ApA are in disagreement with those reported elsewhere (Schweizer et al., 1971). These workers generally found smaller dimerization shifts for Api^{6}A than ApA. We have found Api^{6}A to have very similar if not larger dimerization shifts than ApA. We suspect the differences to be concentration related, as these workers employed 20 mM solutions, where concentration dependent shifts are known to occur (Schweizer et al., 1971; Ts'o et al., 1967; Chan & Nelson, 1969). Likewise, the hypochromicity of Api^{6}A is less than that of ApA. Studies on models of these two dimers yield the opposite trend (Leonard et al., 1969). Thus, trimethylene bridged base moieties do not appear to be good models for dinucleoside phosphates in this regard.

Our data regarding salt binding to the dimers demonstrated no effect upon the absorption from the addition of salt or Mg^{2+}. These results for Api^{6}A were unexpected on the basis of some studies with poly(i^{6}A) (Thedford & Straus, 1972). These authors attributed Mg^{2+} induced changes in the optical melts of poly(i^{6}A) to specific interaction of the ion with the base moiety. Also Upt^{6}A does not appear to bind any cations that affect the absorption or CD. That salt binding to the -COO^- might change the absorption at all was supported by the large

absorption changes observed when the $-COO^-$ group was protonated. (In fact, it was observed during the spectrophotometric titration of pt^6A that the absorption change due to the $-COO^-$ protonation was greater than that for the N1 protonation.) On the basis of these observations, the proposed Mg^{2+} binding to t^6A in *E. coli* $tRNA^{11c}$ (Miller et al., 1976) may be in question.

Finally, the dimerization changes for the side chain protons of the dimers Api^6A and $Apms^2i^6A$ are not those which would be expected from a situation in which the chains are folded between the bases, as proposed by other workers (Schweizer et al., 1971). The shifts are large, but for Api^6A the order of shifting is $-CH_2- > =CH- > -CH_3$. Thus, the further the proton is from the attachment to the base, the less its shift. This is not to say there is no interaction of the chain with the neighboring base, for even the methyl groups show significant shifts (~0.15 ppm, as compared with the base protons ~0.2–0.3 ppm). Furthermore, the two methyl groups are now very much more inequivalent than in the monomer (a difference of about 0.03 ppm). The same is found for $Apms^2i^6A$, $=CH-$ being shifted more than the inequivalent $-CH_3$'s of the isopentenyl chain. (In contrast to the side chain protons of these ApA type dimers, the $-CH_3$ protons of Upt^6A are completely unaffected by the presence of the neighboring U.)

Conclusion

We conclude on the basis of our findings with the two-state model that i^6A, ms^2i^6A and ϵA slightly destabilize ApA, while t^6A stabilizes UpA. Thus the large difference in stacking ability between UpA and ApA may be removed by hypermodification. If we assume that these dimer stacking stabilities are an indication of the flexibilities allowed in the linkage adjacent to the anticodon triplet, then tRNAs containing these hypermodifications will have similar flexibilities at this crucial point in the anticodon loop. With this in mind, we examined the relative stacking ability of CpA, the next most prevalent dimer found in this position in tRNAs (Watts, 1977). The results of the dynamic analysis (Table II) indicate that the stacking ability of CpA is between those of ApA and UpA. In fact, it is close in stability to those of Api^6A and $Ap\epsilon A$ found by absorption. Thus, approximately 40 of the 70 sequenced tRNAs contain dimers in the region adjacent to the first letter of the anticodon triplet which exhibit very similar stacking abilities (if indeed $Ap\epsilon A$ is a reasonable model for ApyW type dimers). It may very well be that the small modifications of A, G, and I (m^2A, m^6A, m^1G, m^1I), which occur adjacent to the 3′ side of the anticodon triplet in the bulk of the remaining 30 tRNAs, confer on the dimers containing them stacking abilities similar to the other 40.

We propose then that a role of the hypermodified bases is to maintain a constant flexibility in this region of the tRNA. In light of the large effects of unbonded bases adjacent to the double-stranded regions (Grosjean et al., 1976; Martin et al., 1971; Yoon et al., 1976), a constant flexibility of this point in the tRNA could aid in making the anticodon–codon interaction proceed with the same efficiency or rate in all tRNAs. This could be of help in the regulation of protein synthesis by allowing a smoother progression of codon–anticodon interactions.[2] In addition, the stronger stacking of Upt^6A relative to UpA may serve another role in the tRNA. There is always a pyrimidine (and usually a U) next to the 5′ side of the anticodon triplet. UpX type dimers are known to exhibit very little stacking relative to dimers not containing U (Warshaw & Tinoco, 1966). Thus in all tRNAs there probably exists a very flexible linkage adjacent to the 5′ side of the anticodon triplet which favors wobble (Fuller & Hodgson, 1967). However, on the 3′ side of the anticodon triplet, the only UpX dimer found is Upt^6A, which exhibits less flexibility than UpA. Hence on the basis of this work, the t^6A modification of A may reduce the flexibility and thus prevent wobble on the 3′ side of the anticodon.

[2] In fact, it has been determined that the stabilities of various anticodon–anticodon complexes (the interaction between two tRNAs) do not vary systematically with G·C composition, unlike all other known double helices (H. J. Grosjean, S. de Henau, and D. M. Crothers, personal communication).

Acknowledgments

We thank Dr. Roy Morris (Oregon State University) and Dr. Michael Gray (Dalhousie University) for supplying molecules and advice. We are also indebted to Dr. Woody Conover, Dr. Steven Patt, and the Stanford Magnetic Resonance Laboratory for their generous help in obtaining the NMR spectra. Finally, we thank Dr. Che-Hung Lee of this laboratory for many helpful discussions and for his help in preparing and running NMR samples.

References

Allen, F. S., Gray, D. M., Roberts, G. P., & Tinoco, Jr., I. (1972) *Biopolymers 11*, 853.

Bloomfield, V. A., Crothers, D. M., & Tinoco, Jr., I. (1974) *Physical Chemistry of Nucleic Acids*, p 83, Harper & Row, San Francisco, Calif.

Blum, A. D., Uhlenbeck, O. C., & Tinoco, Jr., I. (1972) *Biochemistry 11*, 3248.

Borer, P. N. (1972) Ph.D. Thesis, University of California, Berkeley.

Bugg, C. E., & Thewalt, U. (1972) *Biochem. Biophys. Res. Commun. 46*, 779.

Chan, S. I., & Nelson, J. H. (1969) *J. Am. Chem. Soc. 91*, 168.

Chheda, B. G., Hall, R. H., Magrath, D. I., Mozejko, J., Schweizer, M. P., Stasiuk, L., & Taylor, P. R. (1969) *Biochemistry 8*, 3278.

Cunningham, R. S., & Gray, M. W. (1974) *Biochemistry 13*, 543.

Danyluk, S. S., & Hruska, F. E. (1968) *Biochemistry 7*, 1038.

Davis, R. C. (1967) Ph.D. Thesis, University of California, Berkeley.

Davis, R. C., & Tinoco, Jr., I. (1968) *Biopolymers 6*, 223.

Dube, S. K., Marcker, K. A., Clark, B. F. C., & Cory, S. (1968) *Nature (London) 218*, 232.

Elkins, B. N., & Keller, E. B. (1974) *Biochemistry 13*, 4622.

Follman, H. (1967) *Tetrahedron Lett. 22*, 2113.

Freier, S. M., & Tinoco, Jr., I. (1975) *Biochemistry 14*, 3310.

Fuller, W., & Hodgson, A. (1967) *Nature (London) 215*, 817.

Gefter, M. L., & Russell, R. L. (1969) *J. Mol. Biol. 39*, 145.

Gosh, H. P., Söll, D., & Khorana, H. G. (1967) *J. Mol. Biol. 25*, 275.

Grimm, W. A. H., & Leonard, N. J. (1967) *Biochemistry 6*, 3625.

Grosjean, H., Söll, D. G., & Crothers, D. M. (1976) *J. Mol. Biol. 103*, 499.

Hall, R. H. (1971) *The Modified Nucleosides in Nucleic Acids*, p 109, Columbia University Press, New York, N.Y.

Hecht, S. M., Leonard, N. J., Burrows, W. J., Skoog, F., Armstrong, D. J., & Occolowitz, T. (1969) *Science 166*, 1272.

Högenauer, G., Turnowsky, F., & Unger, F. M. (1972) *Biochem. Biophys. Res. Commun. 46*, 2100.

Izatt, R. M., Christensen, J. J., & Rytting, J. H. (1971) *Chem. Rev. 71*, 439.

Jukes, T. H. (1973) *Nature (London) 246*, 22.

Kimball, M., & Söll, D. (1974) *Nucleic Acids Res. 1*, 1713.

Kitchingman, G. R., Webb, E., & Fournier, M. J. (1976) *Biochemistry 15*, 1848.

Lee, C.-H., & Tinoco, Jr., I. (1977) *Biochemistry 16*, 5403.

Lee, C.-H., Ezra, F. S., Kondo, N. S., Sarma, R. H., & Danyluk, S. S. (1976) *Biochemistry 15*, 3627.

Leonard, N. J., Iwamura, H., & Eisenger, J. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 352.

Litwack, M. D., & Peterkofsky, A. (1971) *Biochemistry 10*, 994.

Martin, F. H., Uhlenbeck, O. C., & Doty, P. (1971) *J. Mol. Biol. 57*, 201.

Miller, J. P., Hussian, Z., & Schweizer, M. P. (1976) *Nucleic Acids Res. 3*, 1185.

Odom, O. W., Hardesty, B., Wintermeyer, W., & Zachau, H. G. (1974) *Arch. Biochem. Biophys. 162*, 536.

Parthasarathy, R., Ohrt, J. M., & Chheda, G. B. (1974a) *Biochem. Biophys. Res. Commun. 57*, 649.

Parthasarathy, R., Ohrt, J. M., & Chheda, G. B. (1974b) *Biochem. Biophys. Res. Commun. 60*, 211.

P-L Biochemicals (1973) *Ultraviolet Absorption Spectra*, Circular OR-10, 7th printing.

Powell, J. T., Richards, E. G., & Gratzer, W. B. (1972) *Biopolymers 11*, 235.

Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., & Hollis, D. P. (1960) *J. Am. Chem. Soc. 90*, 1042.

Schweizer, M. P., Chheda, B. G., Baczynskyj, L., & Hall, R. H. (1969) *Biochemistry 8*, 3283.

Schweizer, M. P., Thedford, R., & Slama, J. (1971) *Biochim. Biophys. Acta 232*, 217.

Secrist, J. A., Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry 11*, 3499.

Stewart, H. J., Sherman, F., Shipman, N. A., & Jackson, M. (1971) *J. Biol. Chem. 246*, 7429.

Takeishi, K., Ukita, T., & Hishimura, S. (1968) *J. Biol. Chem. 243*, 5761.

Thedford, R., & Straus, D. B. (1972) *Biochem. Biophys. Res. Commun. 47*, 1237.

Topal, M. D. (1974) Ph.D. Thesis, New York University.

Topal, M. D., & Warshaw, M. M. (1976) *Biopolymers 15*, 1975.

Ts'o, P. O. P. (1974a) in *Basic Principles in Nucleic Acid Chemistry*, (Ts'o, P. O. P., Ed.) Vol. I, p 453, Academic Press, New York, N.Y.

Ts'o, P. O. P. (1974b) in *Basic Principles in Nucleic Acid Chemistry*, (Ts'o, P. O. P., Ed.) Vol. II, p 305, Academic Press, New York, N.Y.

Ts'o, P. O. P., Kondo, N. S., Schweizer, M. P., & Hollis, D. P. (1967) *Biochemistry 8*, 997.

Warshaw, M. M., & Tinoco, Jr., I. (1966) *J. Mol. Biol. 20*, 29.

Watts, M. T. (1977) Ph.D. Thesis, University of California, Berkeley.

Yoon, K., Turner, D. H., Tinoco, Jr., I., von der Haar, R., & Cramer, F. (1976) *Nucleic Acids Res. 3*, 2233.