OPTICAL ROTATORY DISPERSION, CIRCULAR DICHROISM, AND ABSORPTION STUDIES ON SOME NATURALLY OCCURRING RIBONUCLEOSIDES AND RELATED DERIVATIVES*

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In the past few years the investigation of the properties and function of nucleic acids has become a central problem in molecular biology. The double-helix configuration of the crystalline salts of deoxyribonucleic acid (DNA) has now been well established. However, the secondary structure of DNA and RNA in aqueous solutions remains obscure. Structural investigations on the nucleic acids and polynucleotides based on the interpretation of optical rotatory dispersion and circular dichroism measurements by means of excitation theory requires quite complete information on the ultraviolet optical properties of the isolated bases. We have studied the optical rotatory dispersion (ORD), circular dichroism (CD), and absorption properties of nucleosides that are related to the constituents of the nucleic acids and polynucleotides. Close examination of these measurements reveals considerably more detail than was evident in preliminary absorption studies on similar systems.[1-3] Our investigation hopefully will provide useful reference material for future work on the conformation and structure of the nucleic acids and related polynucleotides.

The recent paper of Clark and Tinoco[3] should be consulted for convincing evidence that the electronic states of all the bases are simply derived from those of benzene. Following Tinoco,[3] we shall label bands which appear to be derived from the benzene transition $A_{1g} \rightarrow B_{2u}$ and $A_{1g} \rightarrow B_{1u}$ as B_{2u} and B_{1u}, respectively. The more intense bands in the 180- to 220-mμ region will be related to the E_{1u} band in benzene. (Alternatively we could use the more descriptive Platt symbols.[4]) These bands are, of course, $\pi \rightarrow \pi^*$ transitions which are polarized in the plane of the base. The purine and pyrimidine chromophores, due to the nonbonding electron pairs of the N and O atoms, also exhibit $n \rightarrow \pi^*$ transitions which are polarized perpendicular to the base plane.

Ultraviolet absorption studies[1, 3, 5] of various substituted purines and pyrimidines indicate that the strong absorption band in the 250- to 280-mμ region is complex containing the B_{2u} and one or more $n \rightarrow \pi^*$ transitions. Furthermore, the studies reveal that a second in-plane transition (B_{1u}) is also located in the same region in some purines such as adenine. The two bands derived from the doubly degenerate $A_{1g} \rightarrow E_{1u}$ benzene transition are usually found in the 180- to 220-mμ region. The fact that $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ bands usually show opposite substituent, pH, and solvent effects helps to determine which type of transition is responsible for a particular Cotton effect or absorption band.[1, 6-8] For example, the $\pi \rightarrow \pi^*$ bands are usually not greatly affected by protonation, whereas the $n \rightarrow \pi^*$ bands undergo significant blue shifts. The absence of a significant pH effect and the close correlation between the observed Cotton effects and the intense absorption bands may be taken as evidence that a particular Cotton effect arises from one of the $\pi \rightarrow \pi^*$ transitions.

Experimental Procedures.—The optical rotatory dispersion measurements were performed on the Cary model 60 recording spectropolarimeter, using 10-second and 30-second response periods exclusively, and scan speeds of 1 to 5 mμ/min with the higher pen response period and the slower scanning speed for regions of high absorption. The circular dichroism measurements were performed with the Cary model 6001 circular dichroism accessory to the Cary 60 recording spectropolarimeter.[9] The absorption curves were measured with the Cary 14 spectrophotometer. The cell compartment in each instrument was flushed continually with dry prepurified nitrogen, thus eliminating the oxygen absorption bands in the far-ultraviolet region.

To ensure against artifacts in the optical rotation data, concentrations were chosen to be well under the instrument's peak toleration absorbance level of 3.0, and further dilution of each sample did not alter the shape of the dispersion curve. Similar precautions were taken for the circular dichroism measurements.

All compounds were prepared and purified in the laboratory of Professor Roland K. Robins or were commercial samples of high purity. Details of the preparation and characterization of these nucleosides are given elsewhere.[10]

Results and Discussion.—*Pyrimidine nucleosides:* The band assignments in uridine (I) and thymidine (II) are readily correlated with the spectrum of benzene owing to the absence of the pyridine-type nitrogen capable of $n \rightarrow \pi^*$ excitation. Also the nonbinding electrons on the keto oxygens have been shown by polarized adsorption studies to contribute very little to the absorption spectra above 220 mμ.[5] Figures 1 and 2 present the ORD, CD, and absorption spectra of uridine and thymidine at several pH values. No pH effect is observed as expected over the 1 to 7 pH range. The CD curve, which gives a much better resolution of the observed Cotton effect than the ORD curve, shows four Cotton effects for both
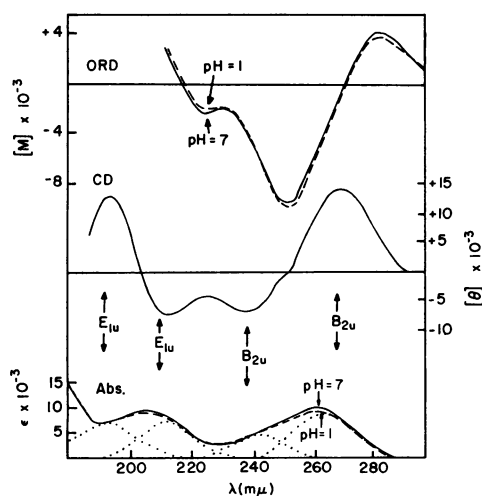

Fig. 1.—The ORD, CD, and absorption spectra of uridine at pH 7 and pH 1. Dotted line on absorption curve shows the resolved absorption bands.
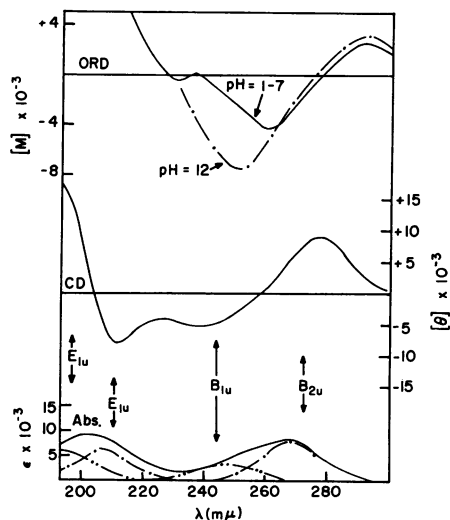
Fig. 2.—The ORD, CD, and absorption spectra of thymidine. The resolved absorption bands are also shown by the dash-dot lines.

uridine and thymidine over the range studied. For uridine the maxima are at 267, 240, 210, and 190 mμ. The absorption curves of uridine and thymidine in the past have been resolved into absorption bands at about 260 and 200 mμ.[11] However, with the aid of the CD spectra the absorption curve can be resolved into four absorption bands (see Figs. 1 and 2) that can be related to the B_{2u}, B_{1u}, and the E_{1u} bands of benzene. According to Caldwell and Eyring,[12] the two bands with E_{1u} parentage in benzene derivatives should have rotational strengths of opposite sign and nearly equal magnitude, as is observed in Figures 1 and 2.

Figure 3 displays the ORD, CD, and absorption spectra of cytidine. Levene and Simms[13] have reported a pK_a for cytidine of 4.2. The structure of the neutral form is III as determined by X-ray diffraction,[15] the ultraviolet absorption spec-

I   II   III   IV
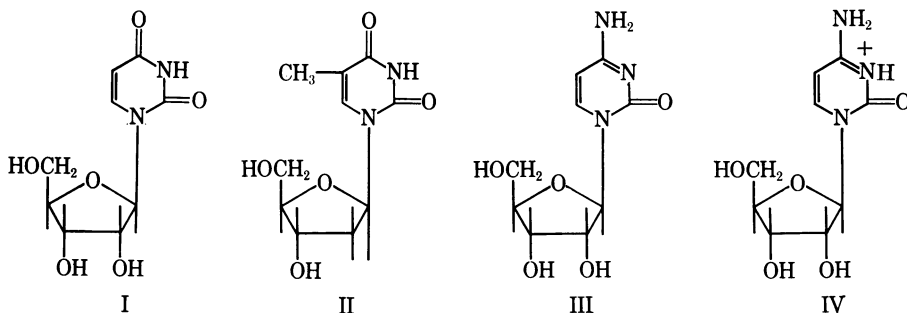
trum,[16] and the infrared spectrum,[17] and the structure of the protonated form is IV as determined by the recent infrared studies by Shimanouchi.[14] The protonated form is not capable of $N \rightarrow \pi^*$ excitation in the spectral region above 220 mμ. Careful examination of the CD curves of cytidine at pH 7 and pH 1 suggest that the large positive band at about 280 mμ is the results of superposition of two positive Cotton effects both of which are shifted to the red upon protonation. Conse-
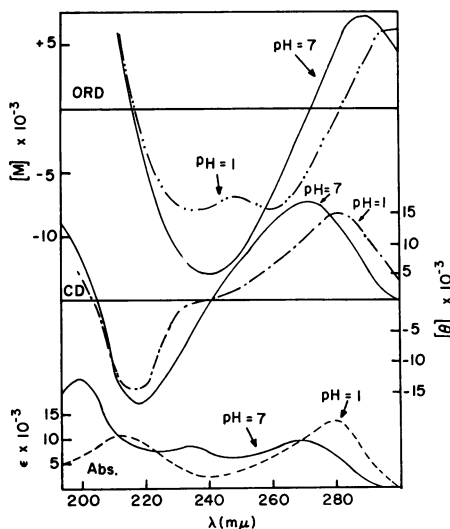

FIG. 3.—The ORD, CD, and absorption spectra of cytidine at pH 7 and pH 1.
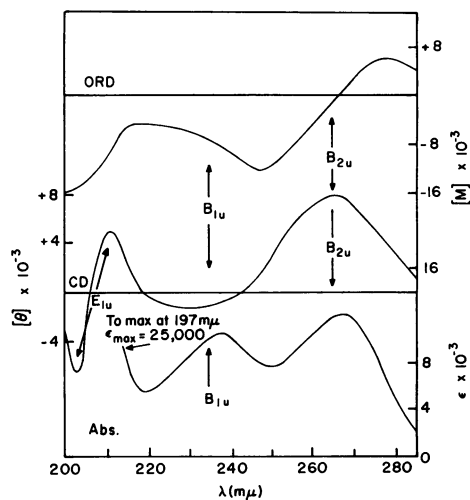
FIG. 4.—The ORD, CD, and absorption spectra of V at pH 7. Measurements at pH 1 gave identical results.

quently it appears reasonable to believe that the B_{2u} and B_{1u} transitions lie in the 240- to 280-mμ region at pH 7 and in the 250- to 286-mμ region at pH 1. The first E_{1u} band gives a negative Cotton effect at about 215 mμ. The changes in the CD spectrum in the 240- to 200-mμ region due to protonation suggest the presence of an $n \rightarrow \pi^*$ transition at about 230 mμ. The negative Cotton effect due to this transition is removed from the spectrum upon protonation at site N-3. Thus the explanation of the pH effect on the ORD and CD curves of cytidine is that the B_{2u} and B_{1u} Cotton effects are red-shifted upon protonation while the $N \rightarrow \pi^*$ Cotton effect is shifted to the far ultraviolet. The changes in the absorption curves can be attributed to the same effects. This requires that the 235-mμ maximum in the pH 7 absorption curve be assigned to an $n \rightarrow \pi^*$ transition.

Purine nucleosides: Optical rotatory dispersion and circular dichroism studies of purine nucleosides such as guanosine and adenosine are hampered by a much less favorable anisotropy factor than that exhibited by the pyrimidine nucleosides. For example, the signal-to-noise ratio at the peak and trough of the 250-mμ Cotton effect of guanosine is about 1 to 1. Fortunately a series of 9-β-D-ribofuranosyl-3,5'-purine cyclonucleosides possessing the guanine, adenine, and several other important purine chromophores has recently been prepared.[10] The signal-to-noise ratio exhibited by the purine cyclonucleosides is 5 to 1 or better.

The absorption, ORD, and CD curves of 2',3'-O-isopropylidine-3,5'-xanthosine cyclonucleoside (V), observed at pH 7 and pH 1, are given in Figure 4. The absorption curve of V at pH 7 and pH 1 indicates an absorption maximum at 265 mμ. The rotatory dispersion curve at both pH values shows a positive Cotton effect in this range with the midpoint located at about 265 mμ. Similarly, the circular dichroism curve reveals a positive Cotton effect with maximum at 265 mμ. Because of the correlation of the Cotton effects with the intense absorption band and because of the absence of pH effects (we have measured a pK_a of 3 for V for the protonation of N-7) on the Cotton effects it is probable that the 265-mμ Cotton effect arises from the B_{2u} transition.

The absorption curve of V shows another intense absorption band at 237 mμ which is probably derived from the B_{1u} benzene transition. In the CD spectrum a small negative Cotton effect is clearly resolved at about 235 mμ. The ORD curve gives little evidence of a Cotton effect in this region. The Cotton effect is there, of course, but it is submerged by the background rotations of other transitions.

The data below 220 mμ for V are considerably more complex. The absorption curve in the region 220 to 185 mμ shows a maximum at 197 mμ whereas the CD curve reveals two closely spaced Cotton effects of opposite signs in this same region. As in the case of uridine, the absorption band in this region is probably the result of the superposition of two bands of E_{1u} parentage. Once again the two bands derived from the E_{1u} benzene transition should have rotational strengths of opposite sign and nearly equal magnitude[12] as is observed in Figure 4.

The absorption, ORD, and CD spectra of 2',3'-O-isopropylidine-3,5'-inosine cyclonucleoside p-tolylsulfonate (VI) at pH 7 and pH 1 are given in Figure 5. The absorption spectrum of VI exhibits an absorption maximum at 260 mμ and a shoulder at 220 mμ. The contribution of the tolylsulfonate anion to the absorption curve was eliminated by using an equal molar concentration of sodium tolylsulfonate in the reference cell. The ORD curve at pH 1 seems to indicate the presence

<div align="center">V   VI   VII   VIII</div>
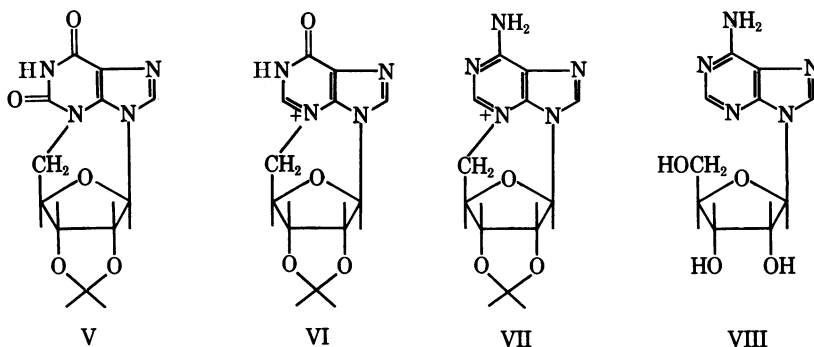
of a positive Cotton effect centered at about 260 mμ and a negative Cotton effect with midpoint at about 220 mμ. This interpretation ignores the presence of a trough at 280 mμ. The CD curve shows maxima at 276, 257, and 222 mμ and reveals quite clearly the true nature of both the ORD and absorption curves. The 260-mμ absorption band must be composite and the ORD curve should be resolved into a negative Cotton effect, a positive Cotton effect of somewhat greater magnitude, and a large negative Cotton effect at 276, 257, and 222 mμ, respectively.

The 276 and 257-mμ Cotton effects at pH 7 and pH 1 are virtually superimposable. This leads us to conclude that the bands are of B_{2u} and B_{1u} origin, respectively. Furthermore the CD data require that the 260-mμ absorption band be composite. A rough resolution has been attempted in Figure 5.

The CD and ORD curves of VI are sensitive to pH in the short-wavelength region. The long-wavelength portion of the 222-mμ Cotton effect in the CD spectra appears to decrease progressively as the pH is lowered; moreover, the effect is reversible, thereby ruling out arguments of acid decomposition. This effect is
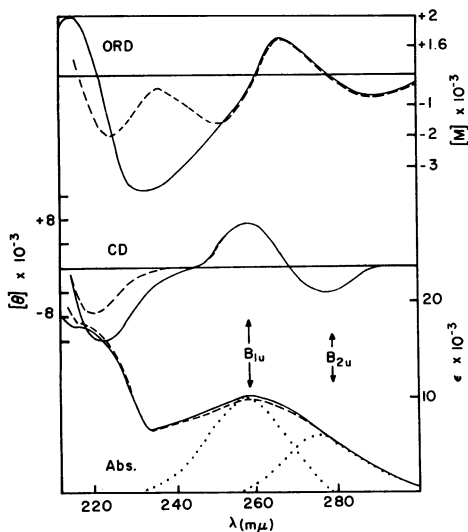

FIG. 5.—The ORD, CD and absorption spectra of VI measured at pH 7 (———) and pH 1 (— —). Dotted lines on absorption curve represent resolved absorption bands.
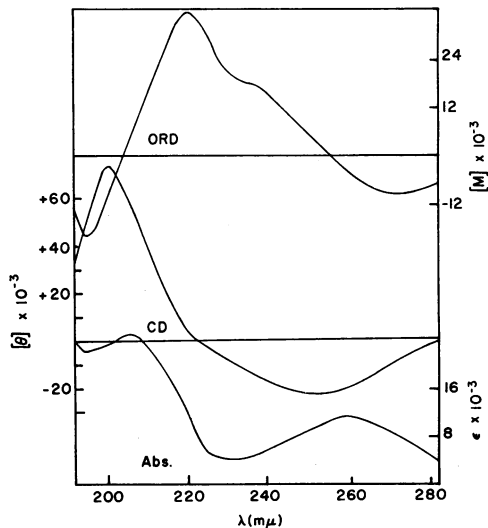
FIG. 6.—The ORD, CD, and absorption spectra of VII at pH 7. No significant change was observed at pH 1.

even more noticeable in the ORD measurement. A probable explanation is that an $n \rightarrow \pi^*$ Cotton effect is located in the 220–230-mμ region and is blue-shifted upon protonation of VI. The Cotton effect that remains is probably of E_{1u} origin.

The most interesting facet of the CD curve of VI is its comparison with the CD curve of V. The effect of removing the keto group from position-2 carbon of VII is to reverse the signs of the first three Cotton effects. This striking reversal of signs is predicted by our quantitative treatment.[18]

With the exception of a 3-mμ red shift and a 5 per cent hypochromism, the curves for 2′,3′-O-isopropylidene-3,5′-adenosine cyclonucleoside p-tolylsulfonate (VII) were identical at pH 7 and pH 1. In Figure 6 we note that only two Cotton effects are readily revealed although the possibility of some overlaid Cotton effects cannot be discounted. The absorption spectrum of VII is identical with that of adenosine (VIII) where the 260-mμ band is known to contain both the B_{2u}, B_{1u}, and an $n \rightarrow \pi^*$ transition according to the ultraviolet polarized absorption studies by Stewart and Davidson.[5] Their work shows that the two $\pi \rightarrow \pi^*$ transitions exhibited by the adenine chromophore in the 260-mμ region have an intensity ratio of approximately 10 to 1.

Furthermore, recent studies on the absorption[3] and ORD[18] of 6-substituted purine bases and purine nucleosides show that the B_{2u} Cotton effect and absorption band will dominate the ORD, CD, and absorption spectra of compounds such as VII and VIII which contain the adenine chromophore. This means that the Cotton effect measured at 260 mμ for structure VII is mainly of B_{2u} origin. The very strong CD band with maximum at 204 mμ is most likely of E_{1u} origin.

The absorption and CD spectra of 2′,3′-O-isopropylidine-3,5′-guanosine cyclonucleoside (IX) at pH 1 and pH 7 are given in Figure 7. The pH 7 form corresponds to the structural formula IX and the acid form probably exists protonated[10] at N-7 with its pK_a measured roughly in this laboratory to be about 3.0. The ef-

IX   X
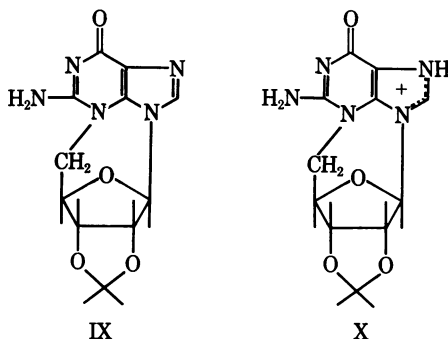
fect of changing from structure IX to structure X on the CD curve is quite remarkable. At pH 1 the CD curve shows a small positive Cotton effect at 263 mμ and negative Cotton effects at 242 and 210 mμ. These Cotton effects can quite reasonably be correlated with the B_{2u}, B_{1u}, and E_{1u} transitions of benzene. This assignment requires that the broad 250-mμ absorption band at pH 1 contain both the B_{2u} and B_{1u} transitions. The very intense band at 200 mμ is likely the result of superposition of the two E_{1u} absorption bands. At pH 7 the CD curve is negative throughout the 240- to 270-mμ region with a maximum at 248 mμ and a promi-
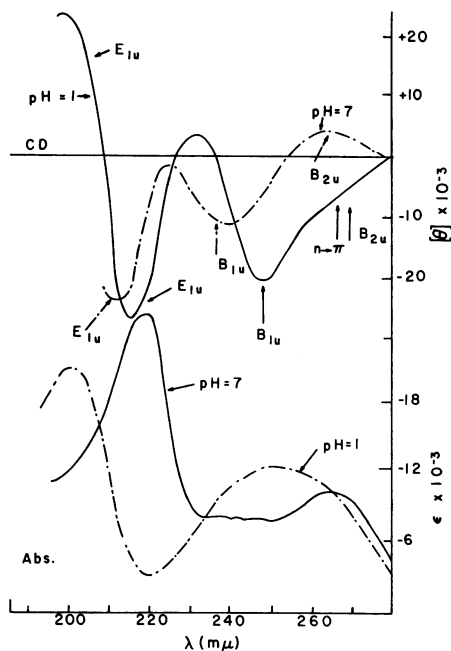
FIG. 7.—The CD and absorption spectra of IX at pH 7 and pH 1.

nent shoulder at 258 mμ. The change in going from pH 1 to pH 7 (structure X to structure IX) in this region can be attributed to a 6-mμ red shift of the B_{1u} band and the return of an $n \rightarrow \pi^*$ transition to this region upon deprotonation of N-7. The position and rotational strength of the $n \rightarrow \pi^*$ transition is such as to completely hide the presence of the B_{2u} Cotton effect. The two very stout Cotton effects at 217 and 197 mμ of nearly equal amplitude but opposite signs are probably of E_{1u} origin.[12]

Conclusion.—The results of this paper should help clarify the true nature of the optical properties of the nucleic acids and polynucleotides and their oligomers and monomers. The results on the cyclonucleoside derivatives containing the guanine, xanthine, hypoxanthine, or adenine chromophore can be applied directly to the interpretation of the ORD, CD, and absorption measurements on polynucleotides containing these chromophores since the formation of the C-5′ to N-3 bond does not greatly alter the nature of these chromophores. Furthermore, the cyclonucleosides are convenient models for the study of substituent effects on the optical rotation and circular dichroism of nucleosides. A quantitative treatment of substituent effects on these systems will be forthcoming.

[1] Mason, S. F., *J. Chem. Soc.* (1954), p. 2071; (1959), p. 1240; (1959), p. 1247; (1959), p. 1253; (1960), p. 219.

[2] Voet, D., W. B. Gratzer, R. A. Cox, and P. Doty, *Biopolymers*, 1, 193 (1963).

[3] Clark, L. B., and I. Tinoco, Jr., *J. Am. Chem. Soc.*, 87, 11 (1965); L. B. Clark, G. G. Peschel, and I. Tinoco, Jr., *J. Phys. Chem.*, 69, 3615 (1965).

[4] Platt, J. R., *J. Chem. Phys.*, 17, 484 (1949).

[5] Stewart, R. F. and N. Davidson, *J. Chem. Phys.*, 39, 255 (1963); *Biopolymers Symp.*, 1, 465 (1964).

[6] Kasha, M., *Symposium on Light and Life*, (Baltimore, Md.: John Hopkins Press, 1961), p. 31.

[7] Krishna, V. G., and L. Goodman, *J. Chem. Phys.*, 33, 381 (1960).

[8] McConnell, H., *J. Chem. Phys.*, 20, 700 (1952).

[9] The circular dichroism measurements were performed on a prototype of this accessory that was made available to us through the courtesy of Dr. Dan Urry.

[10] Holmes, R. E., and R. K. Robins, *J. Org. Chem.*, 28, 3483 (1963); J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, 85, 193 (1963).

[11] DeVoe, H., and I. Tinoco, Jr., *J. Mol. Biol.*, 4, 518 (1962).

[12] Caldwell, D. J., and H. Eyring, *Ann. Rev. Phys. Chem.*, 15, 281 (1964).

[13] Levene, P. A., and H. S. Simms, *J. Biol. Chem.*, **65**, 519 (1925).

[14] Shimanouchi, T., *Adv. Chem. Phys.*, **7**, 435 (1964).

[15] Furbert, S., *Acta Cryst.*, **3**, 325 (1950).

[16] Marshall, J. R., and J. Walker, *J. Chem. Soc.* (1951), p. 1501.

[17] Tanner, E. M., *Spectrochim. Acta*, **8**, 9 (1956).

[18] Miles, D. W., Ph.D. thesis, University of Utah, Salt Lake City, Utah (1967).