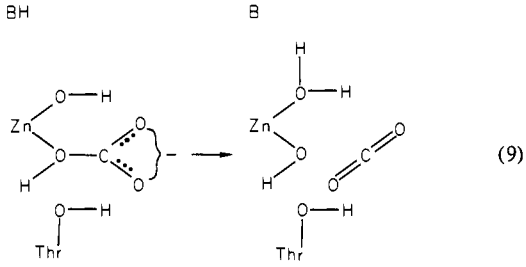
C–O bond breaking at this stage would convert HCO_3^- to CO_2 but would leave an unstable $Zn^{II}(OH)_2$ complex in the active site. Reprotonation of the Zn^{II}–OH ligand by BH, coincident with C–O bond breaking, avoids the formation of such highly basic transition state structures.



(9)

The enzyme is now in its basic form, as required by the reaction stoichiometry. A proton-transfer step, to reprotonate the enzyme from external medium, and loss of H_2O from Zn(II) complete the HCO_3^- catalytic cycle. The importance of proton-transfer steps in our suggested mechanism is clear. The active site of CA is structurally rather open; the alkyl carbonates, alkyl carboxylates, and HCO_3^- bind to and inhibit CA with internally consistent, comparable K_i values (see above). Yet the alkyl carbonates, despite a degree of interaction in the active site comparable to that of HCO_3^-, exhibit no detectable substrate activity. The possibility that strict steric restraints on the positioning of HCO_3^- vs. the alkyl carbonates in the active site might determine substrate activity has no support in the literature of CA; alternate substrates of great variation in structure are accommodated in catalysis,[1] and anions of diverse structure inhibit CA with K_i values generally uncorrelated with anion size.[1,5] These strongly suggest an essential role for the HCO_3^- proton in CA catalysis.

Conclusion

We have shown in our previous study that a simple rapid equilibrium analysis accounts quantitatively for the anionic inhibition of BCA-catalyzed CO_2 hydration and HCO_3^- dehydration over the full range of enzyme catalytic activity. In the present study we further demonstrate that the inhibition of BCA-catalyzed CO_2 hydration by HCO_3^- can also be quantitatively analyzed by the same rapid equilibrium scheme. The complete absence of catalysis of alkyl carbonate decomposition by BCA and the demonstration of a distinct inhibitory pattern for HCO_3^- strengthen the assertion that the proton of HCO_3^- performs a critical function in substrate binding and turnover.

This simplified analysis leads us to suggest that kinetic K_m values for CO_2 and HCO_3^- might in fact represent substrate dissociation constants. While a pH-independent substrate dissociation constant for CO_2, an uncharged species, is not unreasonable, a pH-independent substrate dissociation constant for HCO_3^- seemed heretofore to fly in the face of a substantial literature documenting the pH dependence of anion binding determined by both physical and kinetic techniques. Given, however, our recent observation[5] that the binding affinity of anions to the BCA–CO_2 complex changes only by a factor of 2 between pH 6 and 9, a pH-independent binding affinity for HCO_3^- became a considerably more reasonable possibility. We feel that the identification of pH-independent K_m values for CO_2 and HCO_3^- determined in kinetic experiments with substrate dissociation constants as demanded by a simple analysis of CA catalytic parameters should be entertained as an important conceptual simplification.

The results we have presented above and the mechanism we propose present an appealing structural model for the mechanism of CA catalysis. The implications of these structures lead to fruitful mechanistic insights into a number of diverse observations on this fascinating catalyst. We believe that our suggestions may provide some guidance in the search for a molecular mechanism for carbonic anhydrase catalysis.

Acknowledgment. We are grateful to the National Science Foundation and the National Institutes of Health for partial support of this research. T. L. Deits acknowledges the support of the Chevron Corporation for a predoctoral fellowship during the course of this research and the American Cancer Society for a postdoctoral fellowship during the preparation of this manuscript. We thank Drs. Conrad T. O. Fong, Robert R. Miksch, Kenneth W. Raymond, and Simo Sarkanen for valuable discussions and Donald B. Moore for his diligent assistance with computer interfacing.

Registry No. CA, 9001-03-0; HCO_3^-, 71-52-3; CO_2, 124-38-9; $HOCO_2^-$, 71-52-3; $CH_3OCO_2^-$, 49745-25-7; $CH_3CH_2OCO_2^-$, 49745-26-8; $CH_3(CH_2)_2OCO_2^-$, 84073-84-7; $CH_3(CH_2)_3OCO_2^-$, 44746-83-0; $CH_3(CH_2)_4OCO_2^-$, 84073-85-8.

Flexibility of Nucleic Acid Conformations. 1. Comparison of the Intensities of the Raman-Active Backbone Vibrations in Double-Helical Nucleic Acids and Model Double-Helical Dinucleotides Crystals

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Abstract: Raman spectroscopic measurements have been made on crystals of three dinucleotides whose sugar–phosphate conformation is precisely known from X-ray diffraction measurements. These are uridylyl(3′–5′)adenosine monophosphate (UpA), guanylyl(3′–5′)cytidine nonahydrate (GpC), and sodium thymidylyl(5′–3′)thymidylate 5′-hydrate (pTpT). The first two dinucleotides belong to the A-genus conformation with a C3′-endo ribose ring pucker and exhibit the typical frequency and intensity of the A-genus Raman marker band. On the other hand pTpT belongs to the B genus (C2′-endo furanose ring conformation). For this latter crystal, the conformationally dependent B-genus Raman marker band at 833 cm^{-1} is much more intense than that found in ordinary B-DNA in fibers or in solutions. These results are discussed with references to recent potential energy calculations. It is suggested that the deoxyribose rings in B-DNA are less rigid than in either A-DNA or ordered RNA. Some flexibility of the furanose rings is suggested to be responsible for the complete absence of either C2′- or C3′-endo marker bands for the dinucleotides in solution at room temperature.

Earlier work in this laboratory on the correlations that exist between the X-ray determined conformation and the Raman spectra of fibers of deoxyribonucleic acid clearly show the existence of certain Raman bands that are indicative of the A, B, and C

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forms of DNA.[1,2] These bands were quantitatively assigned to conformationally dependent vibrations of the sugar–phosphate backbone chain by using a Wilson-GF calculation.[3] A simplified force field was developed for the polymer backbone by using force constants obtained from earlier work on simple phosphate esters.[4] More recent comparisons of the Raman spectra and the X-ray structures of DNA fibers and film have confirmed the original Raman observations.[5] A much more refined and complete normal coordinate calculation has essentially confirmed the earlier quantitative assignments with minor modifications.[6] These Raman bands, which we will call marker bands for the A-, B-, and C-type conformations, occur at characteristic frequencies and possess characteristic intensities. For the A-genus polynucleotide structure,[1,2,5] a strong sharp band in the region 807–815 cm^{-1} is found that is indicative of the C3′-endo conformation of the ribose rings. For the B and C forms, weaker, broader bands are found at 835 and 875 cm^{-1}, respectively. The 835-cm^{-1} band has been assigned to the asymmetric –O–P–O– stretching vibration of the sugar–phosphate chain when the deoxyribose rings are in the C3′-exo or C2′-endo conformation.[3,6]

The fact that DNA in solution shows essentially the same Raman spectrum as DNA in the fiber at 95% relative humidity is probably the most rigorous experimental proof that ordered DNA in solution is of the same B-genus conformation as that in the fiber.[1,2] Similarly the existence of the A-genus marker band in the Raman spectra of all ordered ribonucleic acid structures clearly shows that ordered ribonucleic acids belong to the A genus. For ribonucleic acids there is a slight shift in frequency of the marker band from 807 cm^{-1} in A-DNA to 814 cm^{-1} in RNA; however the intensity characteristics are identical.[1,2,5,7,8] (For recent reviews, see references 7 and 8.)

It should be noted that the labels A and B strictly refer to the conformational characteristics of DNA fibers at 75% and 95% relative humidity, respectively,[1,2,5,9] Thus the degree of this conformation in any nucleic acid in solution can only be determined by a quantitative comparison of the intensity of the marker bands relative to the corresponding intensities in fibers of A DNA and B DNA. In order to compare intensities between DNA and RNA most investigators have chosen the PO_2^- symmetric stretching as the internal reference band.[8,10,11] On the basis of quantitative measurements in a number of laboratories it is now well established that double-helical DNA in solution is identical with DNA fibers at 95% relative humidity, while ordered RNA in solution is essentially identical with DNA fibers at 75% relative humidity.

Recently, calculations based on semiempirical potential energy functions have indicated that the flexibility of the furanose ring in nucleic acids may be much greater than heretofore expected and that B DNA as it exists in fiber and solution may not be as rigidly in the C3′-exo or C2′-endo conformation as the rigid models built on X-ray fiber evidence would seem to indicate.[12] However, other calculations using different potential energy functions predict a less flexible furanose ring.[13a,b] Unfortunately, none of the published Raman spectroscopic work on nuclei acids[7,8] appears to be able to help in the testing of these calculations of ribose or chain flexibility. All previous correlations relating secondary DNA structure with the frequencies and intensities of Raman marker bands have been made from measurements on DNA fibers or films.[1,2,5] As a consequence, the extent to which the Raman marker bands accurately represent the actual conformations of nucleic acid polymers is directly dependent upon the correctness of the conformational models derived from an analysis of the fiber X-ray diffraction data.[9]

However, these considerations of flexibility that result in an alteration in the conceptual basis of the model for double-helical DNA also significantly influence the assignments and interpetations of the Raman marker bands. Thus it appears worthwhile to place the conformational assignments of the Raman marker bands upon a firmer foundation by obtaining Raman spectra from dinucleotide crystals in which the dinucleotide conformation is rigidly fixed due to crystal packing forces and is precisely known by X-ray crystallography. A comparison of the crystal Raman spectra with those obtained from nucleic acid fibers allows for both an assessment of the correctness of the Raman assignments and an assessment of the degree of conformational flexibility in nucleic acid polymers. To accomplish this task, we have prepared crystals and obtained Raman spectra of three dinucleotides whose structure, conformation, and sugar pucker have been determined by X-ray diffraction.

The three dinucleotides we have chosen for crystal Raman measurements are uridylyl(3′–5′)adenosine monophosphate (UpA), whose crystal structure was determined by Sussman et al.,[14] guanylyl(3′–5′)cytidine nonahydrate (GpC), whose crystal structure was determined by Rosenberg et al.,[15] and sodium thymidylyl(5′–3′)thymidylate 5′-hydrate (pTpT), whose crystal structure was determined by Camerman et al.[16]

Materials and Methods

UpA crystals were prepared by the method of Sussman et al.[14] UpA was dissolved in a 10^{-3} M HCl solution. Crystallization occurred by allowing the solution to undergo slow evaporation.

GpC crystals were prepared in accordance with the procedures of Rosenberg et al.[15] A 5 mM solution containing 20 mM sodium cacodylate and 16% 2-methyl-2,4-pentanediol was allowed to undergo equilibration with a 75% 2-methyl-2,4-pentanediol solution. Crystals appeared in a few weeks. Sodium thymidylyl(5′-3′)thymidylate 5′-hdyrate, pTpT, was crystallized from a 50% ethanol water solution as described by Camerman and co-workers.[16] All crystals were grown in Kimax melting point capillaries that allowed for direct data acquisition, eliminating the need of transferring crystals from their mother liquor.

Sodium UpA and sodium GpC were purchased from Sigma Chemical Co. Sodium pTpT was obtained from P.L. Biochemicals, and 2-methyl2,4-pentanediol was a product of Mallinckrodt Chemical Co. All compounds were used without further purification.

Raman spectra were obtained by using conventional 90° scattering geometry. Details of the apparatus have been described previously.[2] The collection equipment is interfaced to a Varian 620-I computer, allowing for computer-controlled multiple scans and computer data acquisition and refinement. Raman excitation was accomplished with the 151.4-nm line of an argon ion 165 Spectra Physics laser. Crystal samples were illuminated with approximately 100 mW of laser power measured at the laser. Solution spectra were obtained by using 200 mW. The number of scans varied between 10 and 20 depending upon whether crystal or solution samples were used.

Results and Discussion

Raman spectra of a number of dinucleotides have previously been reported in aqueous solution at pH 7 by several workers.[17,18]

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Table I. Crystal and Solution Raman Data of A-Form Model Dinucleotides

UpA							5′UMP[c] pH 1.6	5′AMP[c] pH 0.5	GpC		
crystals		solution pH 3		solution pH 7[b]					crystal		
peak, cm^{-1}	I	peak, cm^{-1}	I	peak, cm^{-1}	I	assignment[a]	peak, cm^{-1}	peak, cm^{-1}	peak, cm^{-1}	I	assignment[a]
									603	27	
632	23	636	10	640	16	R	630	625			
									664	34	G
712	54	728	43	731	45	A		725	716	30	R
789	62	785	54	783	64	U	786	790	786	100	C
802	56	810	11	806	25	O–P–O	820	815	811	33	O–P–O
869	21	871	11	852	15	R	885		868	21	R
903	14			903	9	R		900	897	43	R, diol
						R		950			
999	20	1003	8	989	8	R	1000		1002	23	R
								1020			
1032	36			1038	14				1043	21	R
1078	38	1086	31	1082	14	PO_2^-	1090		1080	13	PO_2^-
1100									1112	11	
1158	10	1160	5	1134	5	R		1160	1181	23	G, C
				1177	7						
								1205			
1236	100	1233	100	1234	100	U, A	1234	1240	1244	65	C
				1307	43	A		1295	1294	48	C
1322	56	1329	55	1338	72	A		1330	1321	48	G
1373	58			1382	38	U	1400		1388	37	C, G
1404	64	1404	49	1400	28	A		1413			
1425	34			1421	16	A		1430			
1472	15	1475	5	1460	7	A, U	1475	1470			
				1482	20				1486	88	G
1513	47	1513	38	1508	18	A		1510	1530	12	C
1558	30	1504	37			A, U	1540	1563			
1599	7			1584	26	U	1620		1514	26	G
1620	10					A		1620			
1651	24			1078	32	U	1685				
1698	27					U					

[a] A = adenine, G = guanine, C = cytosine, T = thymine, R = ribose, diol = 2-methyl-2,4-pentanediol. [b] E. J. Kiser, Ph.D. Dissertation, University of Oregon, 1975, p 38. [c] R. C. Lord and G. J. Thomas, Jr., *Spectrachim. Acta, Part A*, **23A**, 2551 (1967).

In general the observed Raman bands may be divided into the ring vibrations of the purines or pyrimidines and the backbone vibrations of the sugar–phosphate linkage. The ring vibrations are easily identified by comparison of the frequencies with those reported from nucleotide monomers by Lord and Thomas, while the backbone vibrations are identified from comparison with the normal coordinate calculations[3,4,6] and measurements on simple phosphate diesters.[4]

Figure 1B shows the Raman spectrum of UpA crystals from a pH 3 aqueous solution. The corresponding acidic solution spectra is shown in Figure 1A. At this pH the N(1) position of adenine is protonated. As one can see by examining Table I, there exists substantial differences in the base vibrations of the dinucleotide compound compared to that which is normally encountered at pH 7. The UpA crystal and solution spectra correlate well to previous spectra of UMP and AMP under acidic conditions and reflect a superposition of the corresponding acidic mononucleotide spectra.[19] In the UpA crystal there are two UpA molecules per unit cell. One of the phosphate groups is hydrogen bonded strongly to the protonated adenine, while the other is not.[14] This appears to cause a splitting of the well-known PO_2^- symmetric stretching vibration at 1100 cm^{-1} into two crystal bands occurring at 1078 and 1100 cm^{-1}. We assign the former of these bands to the strongly hydrogen bonded PO_2^- and the latter band to the normal non hydrogen bonded PO_2^- group. As a consequence of the splitting, Raman band intensity measurements usually normalized to the 1100-cm^{-1} PO_2^- band intensity can be calculated by taking the sum of the intensities of the two parts of the split reference band.

According to the X-ray results of Sussman et al.,[14] the UpA crystal contains two UpA molecules per unit cell, which are in somewhat different overall conformations but which both possess

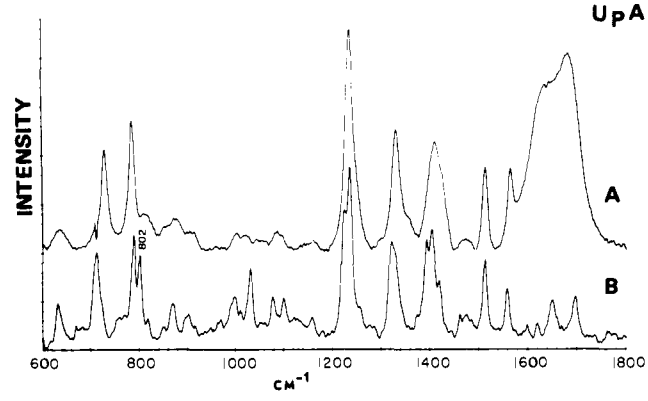

Figure 1. (A) Raman spectrum of aqueous solution of UpA at 20 °C, pH 3 (HCl acidified), 15 μg/μL. A 514.5-nm line (100 mW) of argon laser was used as excitation source. Spectrum is 20 scans at 1 cm^{-1}/s scan rate. (B) Raman spectrum of crystals of UpA grown from the above solution. Raman scattering conditions were identical, but only 10 scans were obtained.

two C3′-endo ribose rings. We see in the Raman spectra of the crystal a strong band characteristic of A form. This band appears at 802 cm^{-1} in UpA and is slightly shifted to lower frequency if compared to the canonical frequencies of A-type RNA and DNA that occur at 814 and 807 cm^{-1}, respectively.[1,2] The intensity ratio of the 802-cm^{-1} band relative to the sum of the two split PO_2^- bands is 1.5 ± 0.1, which is in almost perfect agreement with that published earlier in this laboratory for both A-type DNA and RNA as well as by Thomas and Hartman for a large variety of ordered RNA-type polymers.[10,11] It should be pointed out that though the two molecules per unit cell contain A-type ribose rings, neither molecule forms a helical segment. One can see in an examination of Table II, which contains a listing of the dihedral

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Table II. Values of the Conformational Angles for the Examined Dinucleotides and Models of A and B Nucleic Acid Structures

molecule	χ[O1-C1-N-C8(Pu) or C6(Py)]	δ[O3-C3-C4-C5]	ψ[C3-C4-C5-O5]	φ[C4-C5-O5-P]	ω[C5-O5-P-O3']	ω'[O5-P-O3'-C3']	φ'[P-O3'-C3'-C4']	ρ'[O3'-C3'-C4'-C5']	χ'[O1'-C1'-N'-C8(Pu) or C6(Py)]	ref
CpC	32	77	50	184	285	292	211	89	13	*a*
UpA1	37	85	55	203	82	81	206	86	12	*b*
UpA2	44	93	54	192	271	164	224	77	19	*b*
pdTpdT	34	151	43	179	288	163	255	157	27	*c*
A-RNA	14	95	49	186	294	294	202	95	14	*d*
A-DNA	26	83	45	208	285	313	178	83	26	*e*
B-DNA	82	156	36	214	314	264	155	156	82	*f*

[a] Rosenberg, J. M.; Seeman, N. C.; Day, R. O.; Rich, A., *J. Mol. Biol.* **1976**, *104*, 145. [b] Sussman, J. L.; Seeman, N. C.; Kim, S.-H.; Berman, H. *Ibid.* **1972**, *66*, 403. [c] Camerman, N., Fawcet, J. K.; Camerman, A. *Ibid.* **1976**, *107*, 601. [d] Arnott, S.; Hulkins, D. W. L.; Davies, S. D.; Fuller, W.; Hodgson, A. R. *Ibid.* **1973**, *81*, 107. [e] Arnott, S.; Chandrasekarnn, R.; Hulkins, D. W. L.; Smith, P. J. L.; Watts, L. *Ibid.* **1974**, *88*, 523. [f] Arnott, S.; Hulkins, D. W. L. *Ibid.* **1973**, *81*, 93.

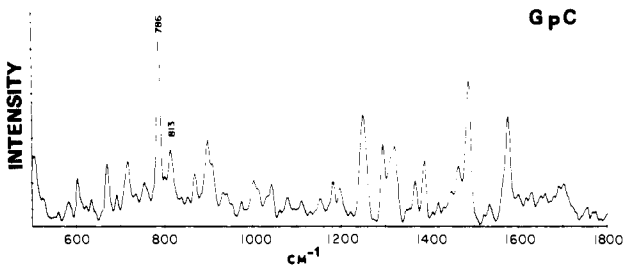

Figure 2. Raman spectrum of crystals of GpC prepared as described in text. Raman scattering conditions are identical with those in Figure 1. The crystallization solvent contains 2-methyl-2,4-pentanediol. The bands due to this compound have been removed by computer subtraction.

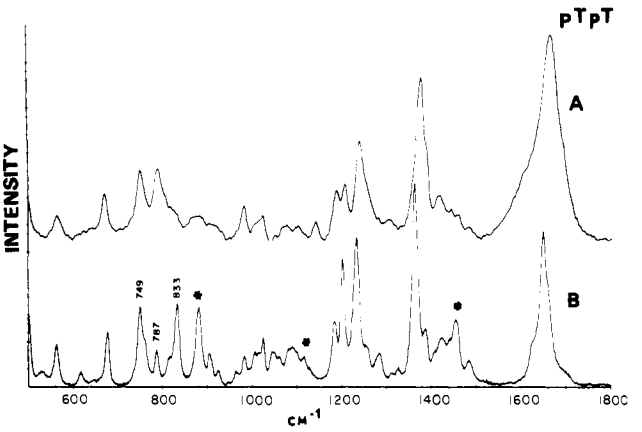

Figure 3. Raman spectra of pTpT. (A) Aqueous solution, pH 7. (B) Crystals grown from 50% ethanol solutions. The asterisks show the presence of ethanol bands.

angles along the phosphate backbone for the various structures discussed in this paper, that the phosphorus–oxygen–ribose bonds are twisted away from those of the idealized A form. This results in placing the molecule designated UpA[2] in an extended conformation and other molecule UpA[1] in a kinked or bent conformation. This conformational difference from canonical A-type DNA possibly accounts for the appearance of the band at 802 cm^{-1} at a frequency shifted lower than one might have anticipated from the sole knowledge that both sugars are in the C3′-endo conformation.

Perhaps a better choice as a model for double-helical RNA are crystals of GpC, since this dinucleotide crystalizes as a short segment of an A-type double helix.[16] A solvent-subtracted Raman spectrum of this dimer is illustrated in Figure 2. The crystallization solvent system contains a strong Raman-scattering nonaqueous material, namely 2-methyl-2,4-pentanediol. Due to the intensity of the diol bands, solvent subtraction was required in order to obtain the GpC spectrum. This yields a spectrum with less than desirable signal to noise. However, a band at 813 cm^{-1} which lies between the A-DNA frequency at 807 cm^{-1} and A-RNA frequency at 814 cm^{-1}, is plainly visible. Assessment of an intensity ratio relative to the PO_2^- band is not permitted due to strong contributions of the diol in the 1100-cm^{-1} region of the Raman spectrum. When the diol bands are subtracted out the signal to noise deteriorates, as can be seen by the uneven base line in this spectrum. For this reason, we have not tried to reference the intensity of the 813-cm^{-1} band relative to the 1100-cm^{-1} band; however, it does appear that the C3′-endo ribose ring conformation is primarily responsible for this sharp intense marker band, which occurs in the 802–815-cm^{-1} region, but that the exact frequency within this range depends on other conformational factors.

Information concerning the C2′-endo idealized B-type sugar ring pucker can be obtained from an examination of the Raman spectra of pTpT. Figure 3 shows the crystal and solution Raman spectra of pTpT. The crystal spectrum shows the presence of a strong band at 833 cm^{-1} that is absent in solution. Both deoxyribose ring puckers are in the canonical C2′-endo form; this strong

band can be assigned to the sugar–phosphate in this conformation. It is interesting to compare the intensity of the band at 833 cm^{-1} of the pTpT dimer to that found in ordinary DNA fibers upon which X-ray diffraction identification of the B structure has been made.[2] The 833-cm^{-1} marker band in B-type calf thymus DNA is about one-third the height of the 1100-cm^{-1} PO_2^- stretching band; i.e., $I_{835}/I_{1100} \approx 0.33$.[1,2] The ratio cannot be measured exactly in the pTpT crystals because of the ethanol peak at about 1120 cm^{-1}. However, if we ignore this ethanol band, which only serves to increase the 1100-cm^{-1} band, we can calculate a lower limit for the intensity ratio I_{833}/I_{1100} for pTpT. Measurements using the data in Figure 3 of this intensity ratio give a value I_{833}/I_{1100} of about 2.3. This ratio is then increased from .33 to 2.3 in going from B-DNA to crystalline pTpT. This corresponds to an increase in the intensity of the 833-cm^{-1} C2′-endo marker band of a factor of about 7 for pTpT crystals over that found in B-DNA fibers. (See also Table III.)

Before discussing further the significance of these Raman intensities, it seems worthwhile to analyze these marker band frequencies in terms of specific torsional angles of the furanose rings. Previous work in several laboratories assign the 813-cm^{-1} band to the diester phosphate symmetric stretch for the A-genus backbone.[7,8] We may write this diester as R_1–O–P(O_2^-)–O–R_2 where R_1 is attached through its 3′-hydroxyl and R_2 is attached through its 5′-hydroxyl. Experiments with 3′-guanosine monophosphate and 5′-guanosine monophosphate in their ordered helical forms at pH 5 show that the 814-cm^{-1} C3′-endo marker band exists for the 5′-GMP but not for the 3′-GMP.[20] Thus in the diester formula written above R_1 may be replaced by a hydrogen atom; it is the ring pucker in the 5′-ester linkage that appears to determine the value of the 813-cm^{-1} marker band. This is reasonable since, as we have already discussed, this band is found in ordered, helical polyribonucleotides, and a dinucleotide is not, strictly, a unit cell of such a polymer. The polymer structure may be written as H(–O–P(O_2^-)–O–R_i)$_N$–H, where R_i is the ith ribonucleotide and N is the degree of polymerization. Since the Raman selection rules require that Raman-active vibrations involve vibrations of the unit cells vibrating in phase, it becomes apparent that only one furanose ring can be involved in this normal mode. In view of the experiments on 3′- and 5′-GMP[20] this must be the ribose attached by its 5′ position. This observation is important since, as we shall see for the diribonucleotides, both ribose rings do not have exactly the same geometry.

Table II gives the values of all of the conformational angles for the dinucleotides and the corresponding DNA structures. As may be seen, there is an excellent correlation between the observed δ values for the O3′–C3′–C4′–C5′ torsional angle of the furanose ring attached at the 5′ end and the Raman marker band frequencies. There are essentially two values for this torsional angle: the value of 151–156° for the C2′-endo ring pucker of both B-DNA and the pdTpdT dimer and the values of 77–95° for the collection of C3′-endo ring pucker conformations that include the two distinct UpA molecules, the GpC molecule, and both A RNA and A DNA. There is no other torsional angle that will give such a correlation. The average value of the two UpA's is 81.5°, which is even lower than the value of 83° for A-DNA. This is another possible reason why the UpA has the somewhat lower than usual frequency of 802 cm^{-1}. Since A-RNA has both the largest value of δ (95°) and the highest A-genus marker band frequency (814 cm^{-1}), while the average δ of UpA is only 81.5° and it has the lowest vibration frequency (802 cm^{-1}), there may be a correlation between δ and the A-genus marker band frequency. It is very likely that the 802-cm^{-1} band of UpA corresponds to a mode of the crystal unit cell involving the in-phase symmetric diester stretch of both UpA molecules in the unit cell.

Although the frequencies correlate well with structure between various oligo- and polynucleotides, for pTpT and B-DNA the intensity is very surprising (see Table III). It may be checked by using the 750-cm^{-1} ring mode of thymine as an internal standard. Since pTpT and calf thymus contain differing amounts

(20) Small, E. W.; Peticolas, W. L. *Biopolymers* **1971**, *10*, 1377.

Table III. Crystal and Solution Raman Data of pdTpdT

crystals		solution		
peak, cm^{-1}	relative I	peak, cm^{-1}	relative I	assignment[a]
504	19	503	15	
619	7			
677	27	670	30	T
749	39	750	48	T
787	14	790	49	
833	41			O–P–O
881	39			EtOH
904	16			R
983	17	983	23	R
1025	24	1023	18	R
1047	4			EtOH
1091	18			PO_2^-, EtOH
1185	31	1188	32	
1203	61	1206	36	T
1234	73	1236	61	T
1286	17			EtOH
1363	100	1378	100	T
1455	32			EtOH
1649	77	1663	132	T

[a] T = thymine, R = ribose.

of thymine relative to the number of phosphate diester groups, it is necessary to calculate the relative intensity of the 833-cm^{-1} band compared to the 750-cm^{-1} band in a manner that compensates for this difference and results in a relative intensity of the backbone vibration that is independent of the thymine content. To do this, we first measure the ratio of the intensity of the 833-cm^{-1} to the 750-cm^{-1} band, I(833 cm^{-1})/I(750 cm^{-1}), in both calf thymus DNA and pTpT. These measured intensity ratios are then multiplied by the ratio of the number of thymines to the number of phosphate furanose groups in each sample, [T]/[O–P–O]. This gives us the normalized intensity ratio, I_R, as

$$I_R = \frac{(I_{833}/[\text{O–P–O}])}{(I_{750}/[\text{T}])} = \frac{I_{833}[\text{T}]}{I_{750}[\text{O–P–O}]} \quad (1)$$

In pTpT there are two thymines and two phosphates connected at the 5′ position. As Lu et al. have shown,[6] the 1100- and 833-cm^{-1} bands are only normal modes of the –O–PO_2^-–O–C5′ furanose chemical repeat group. The terminal furanose phosphate will contain this normal mode only if the terminal phosphate is protonated to give H–O–PO_2^-–O–C5′-furanose. Such an ionization state requires only two sodium ions per pTpT. If the terminal phosphate is not protonated, then the terminal phosphate will be of the form PO_3^{-2}–O–C5′-furanose. In this case, the molecule will possess the well-known Raman-active PO_3^{-2} mode at 980 cm^{-1}.[8] The actual ionization state of pTpT in the crystal is unclear. Camerman et al.[16] have suggested on the basis of their X-ray work that there are only two sodiums per pTpT molecule but they are unable to locate them both. The Raman spectrum of the crystals shows the 980-cm^{-1} band indicative of the PO_3^{-2} group that would require three sodium ions per pTpT. Thus on the basis of the Raman data there are two thymines for one Raman active [–O–P–O–5′–] group while if the X-ray suggestion is correct they are in the ratio 1:1. Thus we can set limits on I_R: $1 \leq I_R \leq 2$.

In calf thymus DNA the observed intensities of the 833-cm^{-1} and 750-cm^{-1} bands are also about equal, but in this case thymine constitutes only 28% of the total number of bases. Consequently, the normalized intensity ratio is about 0.3. This later figure is the predicted I(830)/I(750) ratio for a hypothetical B-form DNA molecule containing only thymine bases. We find that I_R(pTpT)/I_R(DNA) lies between 3.5 and 7. Thus there appears to be little doubt that in pTpT there is a remarkable 3.5- to 7-fold increase in the intensity of the B-genus DNA marker band at 833 cm^{-1} over that found in all previously studied B-type DNA fibers or solutions.

Having established a pronounced difference between the intensities of the B-genus marker band in crystals of pTpT and

B-DNA, we must inquire as to the reason for this difference. To do this properly, we must first review some observations on the behavior of both the A-genus and B-genus Raman bands upon disordering of the secondary structure of oligo- and polynucleotides. As we have seen in Figures 1 and 3, the solution spectra of dinucleotides at room temperature show no marker bands whatsoever. This is an interesting result because it might seem to indicate that there is no preference in solutions for either the C3′-endo or C2′-endo furanose ring conformation. If there were such a preference for these two conformations over intermediate conformations, we would expect that the solutions of single-stranded oligo- or polynucleotide should show both C3′-endo and C2′-endo marker bands. Furthermore, from the relative intensity of the two marker bands, one could in principle obtain the relative population of each of the two conformers. The total absence of any Raman marker bands in solutions of disordered polynucleotides or nucleic acids[7,8,20] could be interpreted as if there is no measurable preference for either the ribose or deoxyribose rings to form either C3′- or C2′-endo ring puckers. Such an interpretation would lead to the conclusion that the ordering of the furanose rings in A- and B-genus DNA and in nucleotide crystals is not due to a low potential energy of the ring for these pronounced ring puckers. The specific C3′- and C2′-endo ring conformations might be due to forces applied to the furanose rings from sources external to the rings themselves. Thus it might be crystal-packing forces or other steric forces that act on the furanose rings which gives rise to either the A- or B-genus (C3′- or C2′-endo) ring pucker and the consequent appearance of the Raman marker bands. This interpretation of the Raman results leads to essentially the same conclusions as those obtained earlier by Levitt and Warshel from their potential energy calculations.[12]

If we consider the intensities of the A- and B-genus marker bands to be indicative of the degree of rigidity of the furanose rings, then we can use these intensities to obtain a quantitative estimation of the rigidity (or flexibility) of ordered double-helical portions of RNA and DNA. Thus we will take the intensities of the A- and B-genus marker bands in these dinucleotide crystals to be the intensities for the completely rigid furanose rings in the C3′- and C2′-endo, respectively.

The C3′-endo marker band (805–815 cm^{-1}) in the crystalline diribonucleotides, A-form DNA fibers, and ordered double-helical RNA structures all have approximately the same intensity when compared either to the PO_2^- symmetric stretch at 1100 cm^{-1} or to other common Raman-active ring modes. From this result, we conclude that A-form DNA fibers, double-helical sections of RNA, and the diribonucleotide crystals all possess highly rigid furanose rings in the C3′-endo conformation. On the other hand, the fact that wet fibers of DNA (humidity >90%) and aqueous solutions of DNA possess a marker band at 830 cm^{-1} that is only 15–30% as strong as that found in the rigid dinucleotide crystals indicates the presence of a great deal of flexibility in the ordinary DNA chain. Indeed, double-helical DNA in fibers and solutions possess deoxyribose rings that are almost completely without a specific C3′ or C2′ conformation. The relatively weak marker band at 835 cm^{-1} in ordinary DNA indicates a slight preference for the C2′-endo furanose ring conformation.

The above conclusions are based upon the assumption that the Raman marker bands possess the same frequency and intensity throughout the range of conformations described either as C2′-endo or C3′-endo and that the absence of a marker band is due to population of states that lie between the two minimum energy conformations on the pseudorotational pathway. Such a conclusion is consistent with the calculations of a 0.6-kcal/mol potential energy barrier between the two minima,[12] but is inconsistent with the calculations of either a 2- or 4-kcal potential energy barrier.[13] Another possibility is that the loss in intensity of the Raman marker bands for dinucleotides in solution is due to conformational variations in the furanose ring about the local minimum described by either the C2′-endo or C3′-endo conformations. Even when a 2- or 4-kcal potential barrier is used, the potential energy wells extend over a sizable range of the pseudorotational pathway. If we assume that observable Raman-active marker band modes exist only within a very narrow conformational range within the pseudorotational pathway such as are found in the dinucleotide crystals, then the reduction in intensity could be due to a low relative population of the furanose rings in solution in this narrow pseudorotational range. The magnitude of this reduction in intensity can be estimated by using Boltzmann statistics from an examination of the potential energy curves describing pseudorotation.

Altona and Sundaralingam[21] have used two parameters, the pseudorotational phase angle P and the maximum torsional angle τ_m, to describe the pseudorotational pathway that represents a continuous progression through all of the possible furanose puckered ring conformations. The pseudorotational angle and the maximum torsional angle are related to the torsional angles of the furanose ring, $\tau_i (i = 0,1,2,3,4)$, by following equation:

$$\tau_i = \tau_m \cos (P + (i - 2)144°) \tag{2}$$

Since the potential energy curves describing the conformational state of the furanose rings are in terms of the phase angle of the pseudorotation of the furanose ring, it is necessary to calculate the pseudorotation parameters for the dinucleotides examined in this work. From the X-ray-determined internal torsional angles of the furanose ring, one can calculate P by means of the following relation:

$$\tan P = \frac{(\tau_4 + \tau_1) - (\tau_0 + \tau_3)}{2\tau_2(\sin 36 + \sin 72)} \tag{3}$$

When the value of P calculated in eq 3 is used, the parameter τ_m may be calculated by using eq 2, where $i = 2$. The calculated pseudorotation parameters for the Raman-active ribose rings (those attached to the phosphate at the 5′ position) of UpA_1 and UpA_2 are $P = 16°$, $\tau_m = 35°$ and $P = 6°$, $\tau_m = 31°$, respectively. For GpC the pseudorotation parameters are $P = 17°$ and $\tau_m = 42°$. Thus the observed range in P represented by these dinucleotides in the C3′-endo conformation $P = 6$–$17°$ or about 0.03–0.10 in units of P (radians)/π. Similarly, the furanose conformation in pTpT is described by a value of 173° for P, or $P/\pi = 0.96$. A value of +29° is found for τ_m.

If we make the assumption that the Raman marker bands only exist for the values of $P/\pi = 0.03$–0.13 (the C3′-endo band at 813 cm^{-1}) and $P/\pi = 0.95$–1.05 (the C2′-endo band at 833 cm^{-1}), then we can calculate the intensity of these bands for both ribo- and deoxyribodinucleotides in solution by using the potential energy curves describing the furanose rings. The potential energy calculation of Levitt and Warshel[12] predicts that the barrier between the C2′- and C3′-endo conformations for both ribose and deoxyribose rings is 0.6 kcal. In this situation, the population of conformations will be spread out over the pseudorotational space at room temperature (kT = 0.6 kcal). The potential energy calculation of Olson[13] predicts a 4-kcal potential barrier for ribose rings and a 2-kcal barrier for deoxyribose rings. When the statistical weights in the paper of Olson are used, the relative population of conformers in the above P ranges can be extracted. (See Figures 3 and 4, ref 13). The expected solution intensity of the Raman marker bands relative to the PO_2^- internal standard at 1100 cm^{-1} can be calculated by

$$I_{max}^{\nu}[\sigma(P_2)/\sum\sigma(P_2)]$$

where $\sigma(P_i) = \exp[-E(P_i)/kT]$ and I_{max}^{ν} is the observed intensity relative to the band at 1100 cm^{-1} in the crystal that we will take as the largest possible marker band intensity. The calculated Raman marker band intensities for ribose dinucleotides in solution are 0.1 (I_{max}^{813}) for the C3′-endo band and 0.14 (I_{max}^{833}) for the C2′-endo band. For deoxyriboses the predicted bands intensities in solution are 0.04 (I_{max}^{814}) and 0.21 (I_{max}^{833}), respectively. These calculated intensities would fall close to the base-line noise of our Raman spectra and would be difficult to observe. Thus with the assumption that the C3′- and C2′-endo furanose rings only exhibit

(21) Altona, C.; Sundaralingam, M. *J. Am. Chem. Soc.* **1982**, *104*, 8205–8212.

Raman marker bands over a very narrow range within each conformational minimum ($\Delta(P/\pi) = 0.1$), we can rationalize the nonexistence of these bands in the solution spectra of the dinucleotides UpA and pTpT. This assumption is required to accommodate the 2- or 4-kcal energy barriers. On the other hand, the 0.6-kcal barrier does not require this assumption.

In the above calculations, the assumption was made that the Raman bands are dependent upon the pseudorotational angle, P. While this is correct, the dependence must be an indirect one. In discussing the effect of furanose ring flexibility upon the observed Raman spectra one must keep in mind that both the normal coordinate calculations of the furanose ring frequencies assign the C2′-endo and the C3′-endo furanose conformational marker bands to displacements of atoms in the backbone chain.[3,6] The normal modes of vibration giving rise to the Raman marker bands are formally dependent upon the backbone torsional angle ρ about the C3′–C4′ bond. There is, however, a relationship between the backbone torsional angle, ρ, and the two pseudorotational parameters that is given as

$$\rho = (\tau_m - 3.8) \cos (P + 148°) + 119° \quad (4)$$

Because of the nature of the cosine function, a given variation in the angle P does not always give a similar variation in the angle ρ. Thus a region of the pseudorotational pathway that gives a small change in ρ for a large change in P would lead to little change in the conformational marker band for a large variation in P.

Sundaralingam and Westhoff[22] have pointed out that the vast majority of X-ray-determined ribose and deoxyribose ring conformations possess pseudorotational angles that fall within two regions. The observed C3′-endo conformations fall within the range $0° \leq P \leq 36°$ while the C2′-endo conformations fall within the range $144° \leq P \leq 180°$; in each case the range of P is 36°. It is of interest that the calculated potential energy minima also fall within these ranges.[12,13] Sundaralingam and Westhoff have used eq 4 to show that for the 36° interval in the C3′-endo region there is only a 6° variation in the backbone torsional angle, ρ, while for the same interval width in the C2′-endo region the value of ρ changes by 20°. Thus they conclude that this factor of three in the allowed angular variation of ρ in the two regions is responsible for the inherent greater flexibility of the C2′-endo furanose ring conformation over the C3′-endo ring conformation. This could also be the reason that DNA shows such a weak marker band relative to RNA. If the varition of ρ is only 6° in RNA and 20° in DNA, then it is reasonable to expect that ordered RNAs in general should resemble their crystal model dinucleotides more closely than does DNA.

Finally, it should be noted that we observe C3′-endo Raman marker bands for observed values of ρ that vary between 77°, 85°, and 93° (see Table II). However, these values of ρ all give about the same value of P because of observed changes in τ_m.[14-16] Unfortunately, no calculation of the potential energy as a function of ρ is given for the high potential energy calculation.[13] However, such a calculation might make it more difficult to rationalize the absence of the C3′-endo marker bands in solution if the calculated C3′-endo minimum did not greatly extend over this 77–93° range in ρ.

At present time, there are unfortunately no calculations of Raman normal-mode frequencies or intensities for the furanose–phosphate backbone as a function of the dihedral torsional angle ρ or the pseudorotation angle P. Our results point out the need for carefully constructed potential energy force fields as a function of P and ρ that can be used to calculate the observed Raman frequencies and intensities in solution and in the crystal.

This difference between the observed rigidity in A- and B-genus nucleic acids is reasonable if the following facts are considered. DNA only goes into the A-type conformation when it is subject to three-dimensional crystal forces. The 75% humidity fiber patterns show clearly that a three-dimensional laterally packed helical structure is present.[2] In 80% ethanol suspension, DNA is in A form but only as a three-dimensional colloidal aggregate.[23] Ordered RNA is only known to exist in the A form. This is presumably because of steric interactions involving the C2′-hydroxyl that force this conformation on the ribose ring. Thus it may be that the C3′-endo furanose conformation in oligo- and polynucleotides is affected by forces acting upon the furanose ring from sources external to the ring. In each case, these forces apparently hold the ring in a rigid conformation. On the other hand, in B-form DNA the external forces acting upon the deoxyribose ring appear to be weak and the ring appears to retain its inherent flexibility with a measurable amount (15–30%) of C2′-endo conformation. It seems likely that the difference in rigidity of RNA and DNA may play a role in their respective functions.

Since this work was completed, two papers have appeared that should be discussed in light of the above conclusions. The Raman spectrum of crystals of the hexamer dCGCGCG has been reported, as has its crystal structure.[24-26] The Raman spectrum shows bands at 810 and 865 cm^{-1} with a shoulder at 850 cm^{-1}. None of these bands is very strong. There are several possible reasons for this. It may be that the rings do not have rigidly held ρ values of 90 and 157° for the C3′-endo and C2′-endo ring pucker. The actual values of these dihedral angles according to Wang et al.[24,25] are 138 and 147° for the C2′-endo, which are well removed from the 157° of pTpT. For the C3′-endo the values of 99 and 94° are close to that of A RNA. On the other hand, Drew et al.[27] have found values of 141 and 122° for a similar Z-type structure formed from the tetramer dCGCG. To some extent these angle values depend upon the nature of the restrained refinement procedure so that there is some possible distortion from the actual values. Indeed the frequencies and intensities of the Raman marker bands may ultimately prove useful to X-ray crystallographers in the refining of their structures. It appears that relaxation of the C3′-endo ring pucker from the 157° torsional angle for ρ' can greatly diminish the intensity of the 835-cm^{-1} B-form marker band.

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