INFRA-RED ABSORPTION SPECTRA OF PYRIMIDINE NUCLEOTIDES IN H_2O AND D_2O SOLUTION*, **

by

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Infra-red absorption spectra have been widely used as a means of compound identification and of structure analysis in many fields of organic and biochemistry[1–5]. Applications to pyrimidine and purine chemistry[6–12] and to the study of nucleic acid derivatives[13–17] have been limited by the complexity of the structures involved and the consequent difficulty of band assignment.

The demonstration by GORE *et al.*[18], of the utility of infra-red absorption spectra of thin films of carboxylic and amino acids in water and in D_2O solution led to investigation in our laboratory of the possibility that similar spectra could be obtained with solutions of the highly soluble nucleosides and nucleotides. By variation of the pH (or pD) of such solutions, the associated variations in infra-red spectra could be correlated with structural changes in groups of known dissociation constant (amino, phosphate, and enolic groups). This correlation greatly facilitates the assignment of the various absorption bands.

A similar approach to the assignment of nucleotide infra-red absorption bands has been developed independently by BLOUT AND LENORMANT[19,20].

MATERIALS AND METHODS

Material. Thymidylic and deoxycytidylic acids were prepared by enzymic degradation of 1.6 g of thymus deoxyribonucleic acid and ion exchange fractionation[21,22]. Each of the fractionated nucleotides was readsorbed onto a short ion exchange column (Dowex-1-8x, 1 cm by 6 cm diameter) and eluted in small volume (approximately 100 ml) of 2 *M* ammonium acetate buffer, pH 4.7. These eluates were frozen and lyophilized to remove water and ammonium acetate. The residual ammonium nucleotide was taken up in water solution and converted to the sodium salt by passage through a column of Dowex-50 (2 cm × 2 cm diameter) in sodium form. Nessler tests were performed on the nucleotide-containing effluent to demonstrate the absence of ammonium ion. These solutions were again lyophilized to obtain the dry sodium nucleotides, which were stored in a dessicator. Both nucleotides are believed to be 5′ phosphates[16,23] as they are dephosphorylated by the enzyme 5′-nucleotidase.

Thymidine and deoxycytidine were purchased from California Biochemical Foundation.

Cytidylic acid was purchased from Nutritional Biochemical Corporation. This material was demonstrated by chromatographic analysis[24] to consist of 36% cytidine-2′-phosphate (cytidylic acid a[16]) and 58% cytidine-3′-phosphate (cytidylic acid b). One gram of the commercial cytidylic

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acid was fractionated on a column of Dowex-1-8x resin, 10 cm × 6 cm diameter. The resultant nucleotide fractions were concentrated by readsorption onto shorter columns (1 cm × 10 cm diameter) of the same resin, and eluted with 2 *M* ammonium acetate buffer, pH 4.7. The ammonium acetate was removed by lyophilization and the nucleotides converted to sodium salts as described above.

Uridylic acid was purchased from Nutritional Biochemicals Corporation. This material was demonstrated by chromatographic analysis[24] to consist of at least 98% uridine-3′-phosphate (uridylic acid b) and was used directly for these spectra.

To obtain uridine-2′-phosphate (uridylic acid a), 750 mg of commercial uridylic acid were dissolved in 100 ml of 0.1 *N* HCl and boiled for one hour. The resultant mixture of uridine-2′- and uridine-3′-phosphates was neutralized with NaOH and fractionated on a column of Dowex-1-8x (11 cm × 9 cm diameter), using 0.08 *M* sodium acetate buffer, pH 5.2 as the eluent[24].

33% was recovered as uridine-2′-phosphate. This material was concentrated, lyophilized, and converted to the dry sodium salt as described for the cytidylic nucleotides. Two such preparations were made.

Uridine-5′-phosphate and uridine were purchased from Nutritional Biochemicals Corporation.

D_2O was obtained from the Stuart Oxygen Company, San Francisco, on allocation from the United States Atomic Energy Commission.

Infra-red spectra. All infra-red spectra were obtained on double-beam, recording instruments, with a water, or D_2O, film in the balancing beam. The great majority of spectra were taken on the Perkin-Elmer Model 13 instrument, while a few spectra were obtained on the Baird Model B. NaCl prisms were used for all spectra.

The absorption cells consisted of two plates of AgCl separated by a 12.5 μ silver foil spacer*. The plates were held between silver plated brass clamps.

For spectra in H_2O solutions, 10 mg of the sample were dissolved in 25 λ of H_2O. This solution was then placed in the well of a one-drop glass electrode (Beckmann No. 290–82) and the pH adjusted to the desired value with 6 *N* HCl or 6 *N* NaOH (added in 2 or 3 λ aliquots).

For spectra in D_2O solution, the sample was first deuterated by repeated solution in D_2O, followed by evaporation to dryness. Ten mg of sample would be dissolved in about 100 λ of D_2O. This was then taken to dryness with a jet of heated nitrogen. This process was repeated three or four times. In this way it is probable that all labile H atoms (in OH or NH bonds) were replaced by D atoms.

The deuterated sample was then taken up in 25 λ D_2O. This volume was placed in the well of the one-drop electrode and the pD adjusted with DCl (1.7 *N*) or NaOD (6.7 *N*)**. DCl was prepared from PCl_3 and D_2O, followed by distillation in a N_2 stream, through a dry ice trap, into D_2O. NaOD was prepared by addition of metallic sodium to D_2O. Both reagents were standardized by titration, DCl against standard NaOH, and NaOD against potassium acid phthalate.

The following spectra were taken:

Deoxycytidine	in H_2O at pH 3.5, and 7.0; in D_2O at pD 3.0.
Deoxycytidine-5′-phosphate	in H_2O at pH 2.5, 5.0, 7.0, and 11.0; in D_2O at pD 2.5, 5.1, 7.0, and 11.5.
Cytidine-2′-phosphate	in H_2O at pH 5.6, 8.6, and 11.0; in D_2O at pD 5.5, 8.8, and 10.8.
Cytidine-3′-phosphate	in H_2O at pH 5.5, 8.6, and 11.0; in D_2O at pD 5.5, 8.1, and 11.0.
Thymidine	in H_2O at pH 6.7.
Thymidine-5′-phosphate	in H_2O at pH 3.5, 6.9, 9.0 and 10.8; in D_2O at pD 3.3, 7.0, 9.0, and 10.6.
Uridine	in H_2O at pH 4.0; in D_2O at pD 3.7.
Uridine-2′-phosphate	in H_2O at pH 3.6, 5.0, 7.0, 9.0, and 10.7; in D_2O at pD 3.6, 4.7, 7.2, 9.0, and 10.6.
Uridine-3′-phosphate	in H_2O at pH 3.5, 5.1, 6.7, and 10.7; in D_2O at pD 2.4, 7.4, and 10.8.
Uridine-5′-phosphate	in H_2O at pH 3.5, 7.0, 9.1, and 10.7; in D_2O at pD 3.5, 6.9, 9.1, and 10.7.

* 12.5 μ silver foil was obtained from Handy and Harman Company, 82 Fulton Street, New York, New York.

** The Beckman one-drop electrode was calibrated for measurement of pD with buffers of computed pD made with deutero-phosphate, deutero-acetate, deutero-formate, and deuterated ammonium salts. The dissociation constants measured by SCHWARZENBACH *et al.*[25], were used for this computation. The results were on good agreement with those reported later by FISHER AND POTTER (AEC No. MDDC 715), and, to sufficient accuracy, the pD was equal to the pH meter reading plus 0.15 unit.

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RESULTS

The infra-red absorption bands are tabulated in Tables I, II and III, which refer to the spectra of the uracil, thymine and cytosine nucleotides respectively*. A typical series of spectra (of deoxycytidine-5'-phosphate) in H_2O and in D_2O at various values of pH and pD is shown in Figs. 1 and 2.

TABLE I

INFRA-RED SPECTRA OF URIDYLIC ACIDS

$\bar{\nu}$	*Absorption band*	*Remarks*
765 cm^{-1}	S in Ur at pD 4	Un-ionized ring band
	M in Ur2p, Ur3p, Ur5p at pD 4, 7	
780	VW in Ur2p, Ur3p, Ur5p at pD 7	Ionized ring band
	M in Ur2p, Ur3p, Ur5p at pD 11	
795	M in Ur5p at pD 4, 7, 11	Ur5p band
810	S in Ur at pD 4	Alkaline displacement
	M in Ur2p, Ur3p, Ur5p at pD 4, 7	
	M in Ur2p, Ur3p, Ur5p at pD 11, but displaced to 820 cm^{-1}	
900	VW in Ur at pD 4	
	W in Ur2p, Ur3p, Ur5p at pD 7, 11—may be present at pD 4, but hidden by 915 cm^{-1} band	
907	M in Ur2p at pH 11	Ur2p in alkali
915	Absent in Ur at pH 4	$ROPO_2H^-$ band
	M in Ur2p, Ur3p, Ur5p at pH 4, only; broad band	
920	W in Ur2p at pH 7, 11; may be present at pH 4, but hidden by 915 cm^{-1} band	Ur2p band
940	M in Ur3p, Ur5p at pH 7, 11; may be present at pH 4, but hidden by 915 cm^{-1} band	Ur3p, Ur5p band; analogous to 920 cm^{-1} band of Ur2p
950	M in Ur at pH 4	Analogous to 920, 940 cm^{-1} bands
975	W in Ur2p, Ur3p, Ur5p at pH 4	$ROPO_2^=$ band
	S in Ur2p, Ur3p, Ur5p at pH 7, 11	
990	W in Ur at pH 4	
1000	W–M in Ur5p at pH 4	
1010	W in Ur2p at pH 4, 7, 11; shifts to 1025 cm^{-1} at pH 7, 11	
	W in Ur3p at pH 4, 7, 11	
	Absent in Ur, Ur5p	
1045 (D_2O)	M–S in Ur, Ur2p, Ur3p, Ur5p at pD 4, 7, 11—shifts to 1060 in H_2O at pH 4, 7 but not 11	1045 (D_2O) ⟷ 1060 (H_2O) or alkali
1085	M in Ur at pH 4	Phosphate band plus $ROPO_2^=$ in 5'-phosphate plus common uridine band
	S in Ur5p, at pH 4; VS at pH 7, 11	
	S in Ur2p, Ur3p, at pH 4, 7, 11	
1110	S in Ur at pH 5	$ROPO_2^-$ band plus common uridine band
	M in Ur2p, Ur3p, Ur5p at pH 4	
	VS in Ur2p, Ur3p, Ur5p at pH 7, 11	
1135 cm^{-1}	M in Ur, Ur2p, Ur3p, Ur5p at all pH	
1190	M in Ur5p at pH 4, only	$ROPO_2H^-$ in 5'-phosphate

* The following abbreviations are used in the tables: Ur = Uridine, Ur2p = Uridine-2'-phosphate, Ur3p = Uridine-3'-phosphate, Ur5p = Uridine-5'-phosphate; Td = Thymidine, Td5p = Thymidine-5'-phosphate; Cd = Deoxycytidine, Cd5p = Deoxycytidine-5'-phosphate, Cr2p = Cytidine-2'-phosphate, Cr3p = Cytidine-3'-phosphate. The strengths of absorption bands are indicated as VS (very strong), S (strong), M (moderate), W (weak), and VW (very weak). In those spectroscopic regions in which both H_2O and D_2O spectra can be observed, a band listed as present at a particular pH is also present at the equivalent pD, unless otherwise indicated.

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TABLE I (continued)

$\bar{\nu}$	*Absorption band*	*Remarks*
1220	M in Ur at pH 4	
	M in Ur2p, Ur3p, Ur5p at pH 4, 7; appears to split into two bands, 1210 cm^{-1} (W) and 1230 cm^{-1} (M) at pH 11	
1280(H_2O)	S in Ur at pH 4	1280(H_2O) ← →1300(D_2O) or alkali
	M in Ur2p, Ur3p, Ur5p at pH 4, 7, 11 but displaced to 1290 cm^{-1} at pH 11. In D_2O, this band appears at 1300 cm^{-1} in all compounds at all pD.	
1320	M in Ur5p at pH 4, 7, 11	
	Absent in Ur2p, Ur3p	
1330	M in Ur at pH 4, Ur3p at 4, 7, 11	
1345	M in Ur2p in H_2O at all pH, in D_2O at pD 7, 11; not detected in D_2O at pD 4	Analogous to 1320 cm^{-1} and 1330 cm^{-1} band
1365	W in Ur3p, Ur5p at pH 4, 7	Ionized ring band
	S in Ur2p, Ur3p, Ur5p at pH 11	
1390	S in Ur2p at pH 3; can't read at higher pH	Ur2p band
1400	M–S in Ur, Ur3p, Ur5p at pH 4	
	M–S in Ur3p, Ur5p at pH 7; can't read at pH 11	
1415	S in Ur2p at pH 5, 7, 11	Ur2p band with $ROPO_2^-$
1420	S in Ur, Ur3p, Ur5p at pH 4	Paired with 1400 cm^{-1} band
	S in Ur3p, Ur5p at pH 7; can't read at pH 11	
1415–1500	S in Ur2p at pD 4, 5 only, in D_2O only	Appears in absence of hydrogen-bonding
	S in Ur3p, Ur5p at pD 4, 7, 11 in D_2O	
	S in Ur3p, Ur5p at pH 11 only, in H_2O	
	Absent in Ur at pD 4	
1460	VS in Ur, Ur2p, Ur3p, Ur5p at all pH	
1500	VS in Ur2p, Ur3p, Ur5p at pD 11	Ionized ring band.
1525	S in Ur3p only at pD 11	Ur3p in alkali
1560	M in Ur2p at pD 3.6; S at pD 4.7; VS at pD 7, 11	Ur2p band
1575 cm^{-1}	W in Ur2p, Ur3p, Ur5p at pD 11	Ionized ring band
1615	S in Ur at pD 4	
	S in Ur5p at pD 4, 7; shifts to 1605 cm^{-1} at pD 11 and is weaker	
	M at 1610 cm^{-1} in Ur3p at pD 4, 7, 11	
	M at 1610 cm^{-1} in Ur2p at pD 4, 7; shifts to 1600 cm^{-1} at pD 11	
1645	VS in all compounds at all pD; shifts to 1630 cm^{-1} in Ur3p, Ur5p at pD 11; shifts to 1640 cm^{-1} in Ur2p at pD 11	
1680	S in Ur3p at pD 4	Un-ionized ring band
	VS in Ur, Ur2p, Ur5p at pD 4; shifts to 1690 cm^{-1} in Ur2p	
	S in Ur2p, Ur3p at pD 7; VS in Ur5p	
	W in Ur2p, Ur5p at pD 11; absent in Ur3p at pD 11	

TABLE II

INFRA-RED SPECTRA OF THYMIDYLIC ACID

$\bar{\nu}$	*Absorption band*	*Remarks*
770 cm^{-1}	M in Td5p at pD 3, 7; W at pD 11	Un-ionized ring band
785	M in Td5p at pD 11, only	Ionized ring band
800	W in Td5p at pD 3, 7, 11	
870	W in Td5p at pD 3, 7, 11	
895	W in Td5p at pD 3, 7, 11	

TABLE II (continued)

$\bar{\nu}$	*Absorption band*	*Remarks*
920	S in Td5p at pH 3, only	$ROPO_2H^-$ band
932	M in Td5p at pH 7, 9; VW at pH 10.7; probably present at pH 3 but fused into 920 cm^{-1} band	Un-ionized ring band
975	Absent in Td at pH 7; absent in Td5p at pH 3	$ROPO_2^=$ band
	S in Td5p at pH 7, 11	
990	M in Td at pH 7; M in Td5p at pH 3, 7, 11	
1040 (D_2O)	M in Td, Td5p at all pH; shifts to 1060 cm^{-1} in H_2O	1040 (D_2O) $\longleftrightarrow$ 1060 (H_2O)
1085	Absent in Td	Phosphate band plus $ROPO_2^=$ in 5′-phosphate
	S in Td5p at pH 4, VS at pH 7, 11 (increase in strength at pH 7, 11 more easily seen in H_2O)	
1105	M in Td at pH 7	$ROPO_2^=$ band plus common thymidine band
	M in Td5p at pH 4; VS at pH 7, 11	
1140	W in Td at pH 7; W in Td5p in H_2O only at pH 3, only	
1190	W in Td at pH 7	$ROPO_2H^-$ band in 5′-phosphate plus common thymidine band (W) plus ionized ring band
	S in Td5p at pH 3; VW in Td5p at pH 7;	
	M in Td5p at pH 11	
1200	W in Td at pH 7	
	W in Td5p at pH 7, 11; may be hidden by 1190 cm^{-1} band at pH 3	
1230	M in Td at pH 7	
	M in Td5p at pH 3, 7, 11	
1280 (H_2O)	S in Td at pH 7	1280 (H_2O) $\longleftrightarrow$ 1300 (D_2O) or alkali
	S in Td5p at pH 3, 7, 11, but displaced to 1300 cm^{-1} at pH 11. In D_2O this band appears at 1300 cm^{-1} at all pD.	
1320	M in Td, Td5p at pH 3, 7, 11	
1360	S in Td5p at pH 11	Ionized ring band
1380	S in Td, Td5p at pH 3, 7, 11	Methyl deformation band
1418	M in Td, Td5p at pH 3, 7; can't read at pH 11	
1415–1510 cm^{-1}	VS in Td5p in D_2O at pD 3, 7, 11	Appears in absence of hydrogen-bonding
	VS in Td5p in H_2O at pH 11, only	
1475	S in Td at pH 7	
	VS in Td5p at pH 4, 7, 11	
1485	S in Td5p at pH 11, only	Ionized ring band
1580	VS in Td5p at pD 11, only	Ionized ring band
1600	W–M in Td5p at pD 11, only	Ionized ring band
1620	S in Td5p at pD 3, 7; absent at pD 11	Un ionized ring band
1650	VS in Td5p at all pD	
1670	S in Td5p at pD 3, 7; absent at pD 11	Un-ionized ring band

TABLE III

INFRA-RED SPECTRA OF CYTIDYLIC ACIDS

$\bar{\nu}$	*Absorption band*	*Remarks*
760 cm^{-1}	S in Cd, Cr2p, Cr3p, Cd5p at pD 3	Ionized ring band plus a band which appears in the deoxy-nucleotide, but not in the ribose-nucleotides until the rupture of hydrogen bonds by alkali
	M in Cd5p at pD 5, 7, 11	
	VW in Cr2p, Cr3p at pD 5, 7	
	M in Cr2p, Cr3p at pD 11	
780	Absent in all compounds at pD 3	Un-ionized ring band
	W–M in Cr2p, Cr3p, Cd5p at pD 5, 7, 11	
790	W–M in Cd, Cr2p, Cr3p, Cd5p at all pD	Weaker than 780 cm^{-1} band in Cr2p; same strength as 780 cm^{-1} band in Cr3p, Cd5p

TABLE III (continued)

$\bar{\nu}$	*Absorption*	*Remarks*
870	W in Cd5p only at pD 3, 5, 7, 11; strongest at pD 11	Cd5p band
906	M in Cr2p only, at pH 5, 7, 11	Cr2p band
920	W in Cd at pH 3; M in Cd5p at pH 3, 5; VW at pH 7, 11 M in Cr2p at pH 5; absent at pH 7, 11 S in Cr3p at pH 5; absent at pH 7, 11	$ROPO_2H^-$ band
940	M in Cd at pH 3, 7; M in Cd5p at all pH Absent in Cr2p; W in Cr3p at all pH	W or absent in ribose-nucleotides
975	Absent in Cd at pH 3, 7 Absent in Cr2p, Cr3p, Cd5p at pH 3 M in Cr2p, Cr3p, Cd5p at pH 5 S in Cr2p, Cr3p, Cd5p at pH 7, 11	$ROPO_2^-$ band
1000	W–M in Cd, Cd5p at all pH Absent in Cr2p, Cr3p	Deoxyribose band
1040 (D_2O)	M in all compounds at all pH; shifts to 1060 cm^{-1} in H_2O except in Cr3p at pH 11	1040 (D_2O) ⟷ 1060 (H_2O)
1090	Absent in Cd S in Cr2p, Cr3p, Cd5p at all pH	Phosphate band
1105	S in Cd at pH 3; W at pH 7 M–S in Cd5p at pH 3; S at pH 5, 7, 11; stronger at pH 7, 11, than at pH 5 S in Cr2p, Cr3p at pH 5, 7, 11; stronger at pH 7, 11, than at pH 5	Ionized ring band plus $ROPO_2^-$ band
1190	M in Cd5p at pH 3, 5; absent at pH 7, 11; fused with 1205 cm^{-1} band Absent in other compounds	$ROPO_2H^-$ in 5′-phosphate
1205	M in Cd, Cr3p, Cd5p at all pH; M at 1210 cm^{-1} in Cr2p at all pH; weaker, in all compounds at pH 7, 11, than at pH 5	
1240 cm^{-1}	W–M in Cd at pH 3, 7 Absent in Cd5p at pH 3; W at pH 5, 7, 11 Absent in Cr2p, Cr3p at all pH	
1290	M in Cd, Cr2p, Cr3p, Cd5p at all pH; shifts to 1280 cm^{-1} at pH 3; shifts to 1300 cm^{-1} in Cr3p, Cd5p at pH 11; shifts to 1310 cm^{-1} in Cr2p at pH 11	
1320	M in Cd at pH 3, 7 M in Cd5p at pH 3, 5, 7, 11; at 1312 cm^{-1} at pH 11 M in Cr2p at pH 5, 7, 11; absent in D_2O at pD 11 M in Cr3p at pH 5, 7, 11; absent in D_2O at pD 11; shift to 1312 cm^{-1} in H_2O at pH 11	Sensitive to hydrogen-bonding
1340	W in Cd at pH 3, 7 in H_2O only W in Cd5p at pH 3, 5, 7, 11 in H_2O; W at pD 7, 11 only, in D_2O M in Cr2p at pH 5, 7, 11 in H_2O; M in D_2O at pD 7, W in D_2O at pD 5, 11 M in Cr3p at pH 5, 7 in H_2O; M in D_2O at pD 7; W in H_2O, D_2O at pH 11; W in D_2O at pD 5	Sensitive to hydrogen-bonding
1365	M in Cd in H_2O at pH 7 M in Cd5p in H_2O only, at pH 5, 7, 11 Absent in Cr2p, Cr3p	Un-ionized ring band in deoxyribonucleotides, H_2O only
1380	M in Cr2p, Cr3p in H_2O only at pH 5, 7, 11	Un-ionized ring band in ribonucleotides, H_2O only
1390–1480	S diffuse band in all compounds in D_2O	D_2O band
1412	M in Cd, Cd5p at all pH S in Cr2p, Cr3p at all pH	Stronger in ribose-nucleotides

TABLE III (continued)

$\bar{\nu}$	*Absorption band*	*Remarks*
1445	W in Cd, Cd5p at pH 3, 5, 7; absent at pH 11	In deoxy-nucleotides only
1458	W in all compounds at all pH	
1490 (H_2O)	Absent at pH 3	Un-ionized ring band
	VS in all compounds at pH 5, 7, 11; shifts to 1505 cm^{-1} in D_2O	1490 (H_2O) $\longleftrightarrow$ 1505 (D_2O)
1530	M in all compounds at all pH; fused to 1505 band in D_2O	
1545	S in Cd, Cd5p at pD 3; absent in Cd5p at pD 5, 7; M in Cd5p at pD 11	Ionized ring band in 5′-phosphate
1560 cm^{-1}	M in Cr2p at pD 5, 7; S at pD 11	In ribose-nucleotides only
	M in Cr3p at pD 5, 7, 11; shifts to 1545 cm^{-1} at pD 11 in Cr2p, Cr3p	
1590	M in Cd at pD 3	In deoxy-ribose nucleotides, only
	M in Cd5p at pD 3, 5, 7, 11; shifts to 1585 cm^{-1} at pD 11	
1610	S in Cd5p at pD 5, 7, 11	Un-ionized ring band
	S in Cr2p; at 1620 cm^{-1} at pD 5; at 1625 cm^{-1} at pD 7; at 1600 cm^{-1} at pD 11	
	S in Cr3p; at 1620 cm^{-1} at pD 5; at 1625 cm^{-1} at pD 7, 11	
1648	VS in all compounds at all pD	
1702	S in all compounds at pD 3	Ionized ring band
	M in all compounds at pD 5; shifts to 1690 cm^{-1}	
	Absent at pD 7, 11	

Because of the absorption of water, all bands in the regions 650–900 cm^{-1} and 1500–1700 cm^{-1} are observed in D_2O spectra only. Because of the absorption of D_2O all bands in the region 1150–1280 cm^{-1} are observed in H_2O only. With almost all nucleotides, a strong but diffuse band was observed over the entire region 1380–1500 cm^{-1} in D_2O; this band so distorted the spectra that only the H_2O spectra could be used in this region. Other regions than those mentioned were observed in both H_2O and D_2O spectra. Differences between the two sets of spectra in these regions are noted in the tables.

DISCUSSION

Vibrational assignments are suggested for many of the stronger bands in Tables I, II, and III. Because of the complexity of the compounds concerned these assignments must be regarded as tentative until complete spectra on more, and appropriately substituted, nucleotides are available.

Phosphate bands and phosphate position. A strong band at 915–920 cm^{-1} appears to be associated with the singly ionized phosphate radical ($ROPO_3H^-$). Upon secondary ionization ($ROPO_3^{=}$), this band disappears and new strong bands appear at 975 and 1105–1110 cm^{-1}. In addition all phosphorylated compounds have a strong band at 1085–1090 cm^{-1} at all pH values studied (pH 3–11).

Certain bands appear to be correlated with the phosphate position on the sugar ring. Such bands may be quite useful for purposes of identification. Thus both 2′-phosphates studied appear to have a band of moderate strength at 907 cm^{-1} at alkaline pH.

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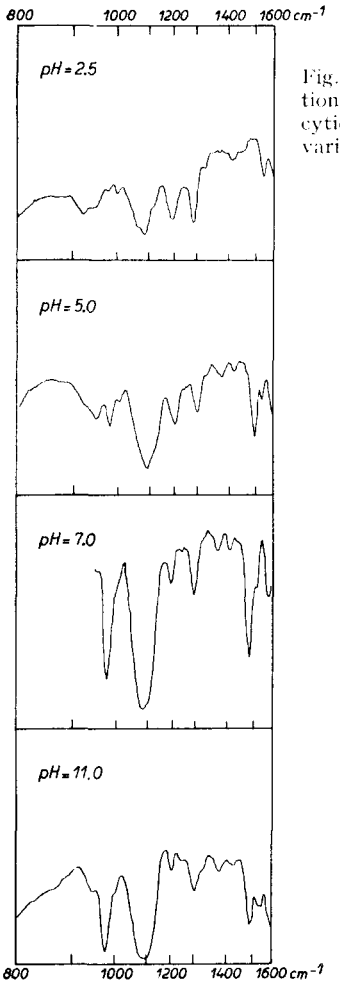

Fig. 1. Infra-red absorption spectra of deoxycytidylic acid in H_2O, at various pH.

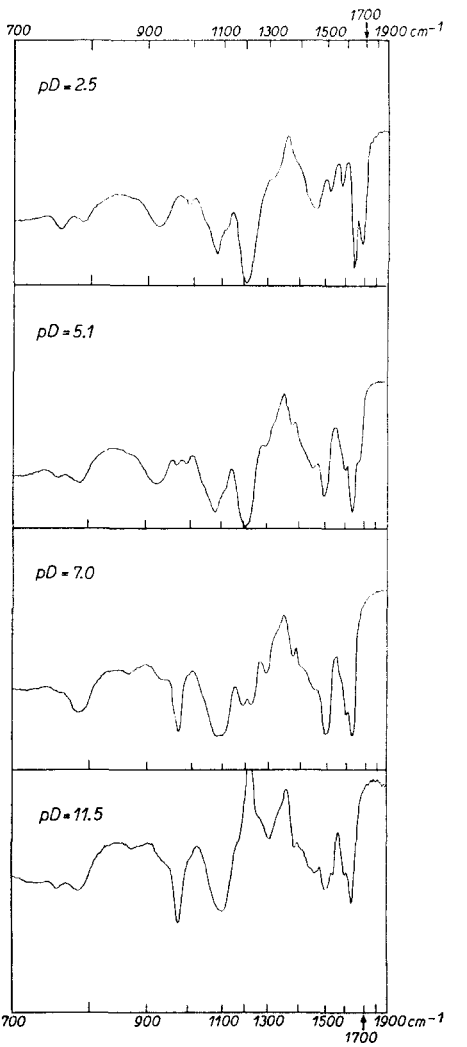

Fig. 2. Infra-red absorption spectra of deoxycytidylic acid in D_2O, at various pD.

All 5'-phosphates appear to have a band at 1190 cm^{-1} associated with singly ionized phosphate. Other bands, characteristic of specific phosphate positions in specific compounds are summarized in Table IV. These bands appear to indicate the presence of strong and structurally specific interactions between the phosphate groups and the pyrimidine rings.

In other instances, there appears to be a regular shift of the band position with removal of the position of phosphate attachment from the pyrimidine ring (*i.e.*, the 920, 940, 950 cm^{-1} band group and the 1320, 1330, 1345 group in the uridylic acids).

Previous studies by BELLAMY AND BEECHER[26,27] of the infra-red spectra of organic phosphates ascribed a band at 1030 cm^{-1} to the P→O→C linkage. In a later paper[28] it was suggested that this band arose from the C→O→ portion of the link (*vide infra*) and that a band at 980 cm^{-1} arose from the P→O→ portion. This latter band does not appear to be present in these ionized structures.

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TABLE IV

BANDS ASSOCIATED WITH SPECIFIC PHOSPHATE POSITIONS

2'-phosphate			*3'-phosphate*			*5'-phosphate*		
$\bar{\nu}$	$\bar{c}$	*pH*	$\bar{\nu}$	$\bar{c}$	*pH*	$\bar{\nu}$	$\bar{c}$	*pH*
906 cm^{-1}	Cr2p	5, 7, 11				795 cm^{-1}	Ur5p	4, 7, 11
907	Ur2p	11				870	Cd5p	3, 5, 7, 11
920	Ur2p	7, 11	940 cm^{-1}	Cr3p	3, 7, 11			
			940	Ur3p	7, 11	940	Ur5p	7, 11
						1085	Ur5p	7, 11
						1085	Td5p	7, 11
						1190	Ur5p	4
						1190	Td5p	3
1390	Ur2p	4				1190	Cd5p	3, 5
1415	Ur2p	7, 11						
1560	Ur2p	4, 7, 11	1525	Ur3p	11			

The strong band at 1250–1300 cm^{-1} ascribed to the P–O group does not appear in these ionic phosphate structures, as was observed by BELLAMY[27].

MORALES AND CECCHINI[17], comparing the spectra of adenosine and adenosine-5'-phosphate dried from aqueous solution, observed the appearance of new bands at 870, 938, 987, 1082, and 1122 cm^{-1} in the phosphorylated compound. As the adenosine-5'-phosphate was neutralized before drying, the 1122 cm^{-1} band shifted toward 1100 cm^{-1}, the 987 cm^{-1} band became stronger and shifted toward 976 cm^{-1}, and the 938 cm^{-1} band became weaker. The 1082 cm^{-1} band may be correlated with our 1085–1090 cm^{-1} band, the 938 cm^{-1} band with our 920 cm^{-1} band for singly ionized phosphate, and the 1100 cm^{-1} and 976 cm^{-1} bands after neutralization, with our 1110 and 975 cm^{-1} bands for doubly ionized phosphate. The other bands of MORALES AND CECCHINI may be specific to the nucleotide concerned.

Infra-red spectra of various inorganic phosphates[3,27,28,29,30] provide many more bands than are observed with the nucleoside phosphates and no simple correlations can be made.

H_2O–D_2O *Shifts*. In those spectral regions that permit comparison of the spectra in water and in D_2O, three distinct effects are observed. The moderate 1060 cm^{-1} band in H_2O shifts to 1040 cm^{-1} in D_2O in all compounds. The strong 1280 cm^{-1} band in H_2O shifts to 1300 cm^{-1} in D_2O in the uracil- and thymine-containing compounds. The strong band at 1490 cm^{-1} in the cytosine containing compounds shifts to 1505 cm^{-1} in D_2O.

The slight shifts of the 1060 and 1280 cm^{-1} bands in D_2O are believed to be caused by the replacement of H atoms by D atoms, with the consequent weakening of hydrogen bonds to atoms involved in the vibrations which give rise to absorption bands. Weaker hydrogen bonds would raise the frequency of a stretching vibration and lower the frequency of a deformation vibration.

It is tentatively suggested that the 1040-1060 cm^{-1} band be assigned to a hydrogen bonded C-O stretching frequency[31–35] and the 1280–1300 cm^{-1} band to a hydrogen bonded C–O deformation frequency[3,18,32,34].

It is noteworthy that these two frequency shifts occur in H_2O at alkaline pH, as well as in D_2O (*vide infra*).

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Absorption in the 1490 cm^{-1} region appears to arise from a ring vibration in the un-ionized cytosine-containing compounds.

Deformation frequencies. BROWNLIE[8], and SHORT AND THOMPSON[9], have suggested that the 1220–1230 cm^{-1} band found in pyrimidine spectra may be an "in plane" C–H deformation. A band of moderate intensity is found in all compounds in this region (1205 cm^{-1} in cytosine-containing compounds, 1220 cm^{-1} in uracil-containing compounds, and 1230 cm^{-1} in thymine-containing compounds) and is tentatively assigned to this vibration.

The same authors found an absorption band at 810–825 cm^{-1} in all pyrimidines except tetra-substituted rings, which BROWNLIE[8] ascribes to a C–H out-of-plane vibration. A weak-to-moderate absorption band is found in all compounds studied in this general region (790 cm^{-1} in cytosine-containing compounds, 810 cm^{-1} in uracil-containing compounds, and 800 cm^{-1} in thymine-containing compounds), which is tentatively assigned to this vibration.

The moderate band at 1380 cm^{-1} in all thymine-containing compounds is almost certainly to be correlated with the symmetrical deformation frequency of the methyl group when attached to a C atom[3].

The deoxy-ribose-containing compounds examined have a moderate absorption band at 990–1000 cm^{-1} which is absent in the ribose-containing compounds.

1500–1700 cm^{-1} region. A summary of the absorption bands observed in this region (excluding those observed with only a particular position of phosphate attachment) is presented in Table V.

TABLE V

COMMON BANDS OF 1480–1700 cm^{-1} REGION

Uridylic		*Thymidylic*		*Cytidylic*	
*U**	*I*	*U*	*I*	*U*	*I*
	1500 cm^{-1}		1485 cm^{-1}	1505 cm^{-1}	
	1575 (W)		1580	1530	1530 cm^{-1}
1615	1615	1620	1600	1560	1560
1645	1645	1650	1650	1590	1590
1680		1670		1610	
				1648	1648
					1702

* U = un-ionized; I = ionized.

Any interpretation of these bands is contingent upon various assumptions concerning the existence of ketonic or enolic structures and of animo or imino structures. Evidence from both ultra-violet and infra-red spectroscopy[9,10,36,37,38,39] and from X-ray diffraction analysis[40,41] favors the assumption that ketonic structures are predominant, at least when the substances are in neutral solution or in crystalline form.

Evidence has been presented that singly-substituted amino pyrimidines are in the amine form[10], but no comparable evidence exists for cytosine and its nucleotides. The X-ray diffraction data of FURBERG indicate a very short C_4–N distance, suggesting at least an approximation to the imino form, when uncharged (in the crystal)*.

* Even though the shortened C–N distance (1.31 A) indicates considerable double band character, the N may still bond two hydrogen atoms, as is the case in adenine[42] (C–N distance = 1.30 A).

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Upon ionization (in acid) a shift to the ammonium form would be expected (Fig. 3).

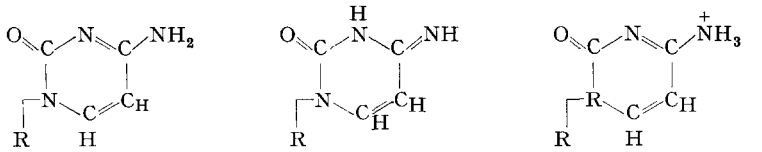

Fig. 3. Possible structures of the cytosine nucleotides.

In alkaline solution, both uridylic and thymidylic acids enolize and ionize. FOX AND SHUGAR[37] have presented considerable evidence that enolization takes place on the C_4 carbonyl of uridine (as contrasted to enolization of the C_2 carbonyl in uracil). By analogy, this result was presumed to be valid for thymidine, without, however, presentation of direct evidence.

The marked similarity of the uridylic and thymidylic acids[1] infra-red spectra in the 1600–1700 cm^{-1} region at pH 7, and their obvious difference at pH 10.7 (Fig. 4) strongly suggest that the two enolize in different positions; if uridylic acid (by analogy to uridine) enolizes at C_4, it is suggested that thymidylic acid enolizes at C_2.

Assignments of individual bands in this spectral region can only be made by reference to comparable spectra of variously substituted pyrimidines. Unfortunately the possibilities of such comparison are limited by the paucity of spectra of N_1 substituted pyrimidines (which would be comparable to the nucleotides) and by the circumstance that most infra-red spectra of pyrimidines have been obtained with the material in the form of a crystalline powder (suspended in a Nujol mull). Such crystalline specimens often give rise to absorption bands caused by inter-molecular effects and hence not found in solution spectra[43].

The infra-red spectra of uridylic and thymidylic acids at pH 7 are generally similar to that of uracil[4,9] but displaced toward lower frequency. The group of three bands at 1700–1750 cm^{-1} in uracil may be compared to the 1690 cm^{-2} band of 1,3 dimethyl uracil and the 1680 cm^{-1} band or uridylic and thymidilic acids in this region and may be ascribed to the C_2 carbonyl (various degrees of hydrogen bonding in the crystals may give rise to the split band of uracil). The 1650 and 1670 cm^{-1} bands of uracil may be compared to the 1640 and 1660 cm^{-1} bands of 1,3 dimethyl uracil and to the 1620 and 1650 cm^{-1} bands of uridylic and thymidylic acids and may be ascribed to the $-C=C-C_4=O$ grouping*, **.

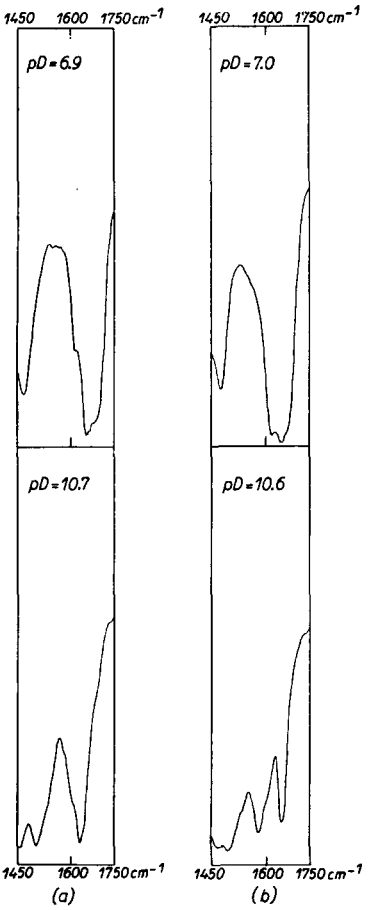

Fig. 4. Infra-red spectra in D_2O, at pD 7 and 11 of (a) Uridine-5'-phosphate; (b) Thymidine-5'-phosphate.

* That the C_5–C_6 link in uracil can be properly described as a double bond is questionable in view of the near equality of this bond length (1.41 A) with that of the C_4–C_5 link[40]. However, the

Upon enolization and ionization at the C_4 position (Fig. 5), the chain of conjugation $-C=C-\dot{C}=N-C_2-O$, become analogous to that in 2-keto pyrimidine[9]. The 1500, 1615, and 1645 cm^{-1} bands of ionized uridylic acid may be directly compared with the 1530, 1590, and 1630 cm^{-1} bands of the latter compound.

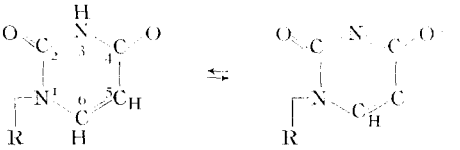

Fig. 5. Ionization of uracil nucleotides at C_4.

If thymidylic acid enolized (and ionized) at the C_2 position (Fig. 3) the conjugated structure would resemble that of 4-keto pyrimidine. This compound has infra-red absorption bands at 1520(S), 1590(S), 1605(W) and a pair of bands (S) at 1670 and 1710 cm^{-1}. It is suggested that these may be homologous with the 1485(S), 1580(S), 1600(W), and 1650(S) cm^{-1} bands of ionized thymidylic acid.

The lack of spectra of appropriately substituted cytosine derivatives, combined with uncertainty as to the amine or imine character of the C_4 substitution, makes interpretation of the cytidylic acid spectra in this region extremely uncertain. The disappearance, upon neutralization, of the 1700 cm^{-1} band which is probably produced by the $C_2=O$, may be correlated with the expected greater importance of imino type structures after neutralization, with corresponding changes in the electronic structure of the pyrimidine ring. The other bands in this region cannot be assigned at this time.

Keto-enol transitions. Although assignments of frequencies to specific groups within conjugated heterocyclic rings must, at present, involve a considerable degree of arbitrary judgment, frequencies can clearly be assigned to the un-ionized ketonic form or the ionized enolic form of the rings, on the basis of their variation upon shift of the pH from neutrality to alkaline (pH 11). In this way, both uridylic and thymidylic acids can be said to have a band at 765–770 cm^{-1} which is present only in the un-ionized, ketonic form. In addition, the 920–950 and 1680 cm^{-1} bands of uridylic acid and the 932, 1620, and 1670 cm^{-1} bands of thymidylic acid are present only with the unionized form.

Upon ionic enolization, these bands disappear to be replaced, in uridylic acid by bands at 780, 1365, 1500, and 1575 cm^{-1}, and in thymidylic acid by bands at 785, 1360, 1485, 1580, and 1600 cm^{-1}.

Amine (imine) -ammonium transitions. Similarly, bands which disappear, or appear, in the spectra of the cytidylic acids as the pH is raised from 3 to 5.5 can be assigned to the ammonium or amine (imine) form of the ring structure respectively. In this way, the bands at 760, 1105, 1545, and 1702 cm^{-1} can be assigned to the ammonium form, and bands at 780, 1365 or 1380, 1505 and 1610 cm^{-1} are associated with the amine (or imine) form.

Effects of intra-molecular hydrogen bonding. Numerous effects, of alkali, of D_2O solution as compared to H_2O solution, of the 2′-hydroxyl of the ribose-nucleotides, and

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ring structure may be changed upon substitution of the N_1 position. In cytidine[11], the C_5–C_6 link is reduced to 1.32 A.

** Infra-red spectra recently obtained by SCOTT[12], of 1-, 3-, and 1,3-dimethyl uracil and the corresponding 5,6 dihydro-compounds, in bromoform solution, support these assignments.

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of phosphate position appear to be more easily explained as consequences of intra-molecular hydrogen bonds. These bonds appear to be somewhat stronger in the cytosine-containing nucleotides than in the uracil- and thymine-containing nucleotides. Evidence of strong intra-molecular hydrogen bonding in phosphorylated compounds has been previously presented by BELLAMY[27].

In the spectra of the cytidylic acids three pairs of bands appear in which the position of the band in the desoxyribose-containing compounds is shifted from the position of the corresponding band of the ribose-containing compounds. The 1365 cm^{-1} bands of the former is considered to be equivalent to the 1380 cm^{-1} band of the latter (both are associated with the amine form of the ring) and similarly for the 1445 and 1412 cm^{-1} bands and the 1590 and 1560 cm^{-1} pairs of bands. These slight displacements of frequencies which probably represent ring vibrations are apparently caused by interactions between the ring and the 2'-OH or 2'-phosphate.

A variety of anomalous bands shifts in the spectra of the nucleotides are observed in alkaline solution; these may appear in D_2O and not in H_2O or vice versa. In the spectra of the cytidylic acids which do not dissociate in this pH region, it seems reasonable to ascribe these shifts in frequency to the weakening or rupture of intra-molecular hydrogen bonds by the presence of OH^- (or OD^-).

Similar effects may appear in the spectra of the uracil and thymine nucleotides but their detection is made difficult by the concomitant enolization and ionization in alkaline pH. By comparison of the spectra at pH 7 (ketonic), pH 9 (partially enolic) and pH 11 (enolic) it is possible to determine whether a particular band shift such as the 765–780 cm^{-1} shift in the uridylic acids is caused by the enolization and ionization (the 765 cm^{-1} band is weaker at pH 9 and absent at pH 11; the 780 cm^{-1} band is weak at pH 9 and strong at pH 11) or, as is the 810–820 cm^{-1} band shift, is caused by rupture of hydrogen bonds (the band is still at 810 cm^{-1} at pH 9 and shifts abruptly to 820 cm^{-1} at pH 11).

The strong, diffuse band which appears in the 1390–1500 cm^{-1} region of all these nucleotides in D_2O (and at alkaline pH in H_2O in the uracil- and thymine-containing nucleotides) is apparently a band which is easily shifted or destroyed by hydrogen-bonding. It is therefore absent (or much weaker) in H_2O than in D_2O, except at alkaline pH, and is absent in Ur2p even in D_2O when the phosphate acquires a double negative charge.

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The cooperation of the Iowa State College Institute for Atomic Research, which made available to us the recording infra-red spectrophotometers, is gratefully acknowledged. We are indebted to Mr. RICHARD HEDGES for assistance in the operation of the spectrophotometers.

It is a pleasure to note the valuable discussions concerning the interpretations of these infra-red spectra which were held with Dr. JESSE F. SCOTT and Dr. ROBERT RUNDLE.

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SUMMARY

Infra-red absorption spectra of several pyrimidine nucleosides and nucleotides have been obtained in H_2O and in D_2O solution. By correlation of the appearance and disappearance of absorption bands upon variation of the pH of these solutions with the known dissociation constants of amine, enolic, and phosphate groups, the assignment of bands to specific atomic groups has been greatly facilitated. Assignments are presented for various ionic phosphate frequencies, bond stretching and deformation frequencies, frequencies associated with amine-ammonium and keto-enol transitions, and frequencies associated with certain conjugated ring structures. Evidence is presented indicating the existence of strong intra-molecular hydrogen bonds. These data are correlated with previous infra-red and ultra-violet absorption and X-ray diffraction data pertaining to these compounds.

RÉSUMÉ

Des spectres d'absorption des rayons infra-rouges de plusieurs nucléosides et nucléotides pyrimidiniques ont été obtenus dans H_2O et dans une solution de D_2O. En établissant une correlation entre, d'une part, l'apparition et la disparation de bandes d'absorption selon la variation du pH de ces solutions et, d'autre part, les constantes de dissociation connues du groupe amino, du groupe énolique et des groupes phosphates, on a beaucoup facilité l'assignation de bandes à des groupes atomiques spécifiques. On présente ici des assignations pour les fréquences de l'ion phosphate, pour les fréquences de l'allongement et de la déformation de liaisons, pour les fréquences associées avec les transitions amine-ammonium et kéto-énol et pour les fréquences associées avec certaines structures de noyaux conjugés. On présente aussi des faits qui indiquent l'existence de fortes liaisons d'hydrogène intra-moléculaires. La correspondance de ces données avec des données plus anciennes, relatives à l'absorption de rayons infra-rouges et ultraviolets et la diffraction des rayons X par ces composés, est aussi indiquée.

ZUSAMMENFASSUNG

Infrarot-Absorptionsspektren mehrerer Pyrimidinnukleoside und Nukleotide wurden in H_2O- und D_2O-Lösung dargestellt. Durch Korrelation des Erscheinens und Verschwindens der Absorptionsbänder während der Änderung des pH dieser Lösungen mit den bekannten Dissoziationskonstanten der Amino-, Enol- und Phosphatgruppen wurde die Zuordnung der Bänder zu bestimmten Atomgruppen bedeutend erleichtert. Angeführt werden verschiedene ionische Phosphatfrequenzen, Bindungsstreckungs- und Verformungsfrequenzen, Frequenzen im Zusammenhang mit Amin-Ammonium- und Keto-Enol-Übergängen, sowie auch Frequenzen im Zusammenhang mit gewissen konjugierten Ringstrukturen. Es werden Tatsachen angeführt welche auf das Vorhandensein starker intra-molekularer Wasserstoffbindungen schliessen lassen. Diese Angaben sind zur Übereinstimmung gebracht mit früheren Angaben über Infrarot- und Ultraviolet-Absorption und Röntgenstrahlendiffraktion im Zusammenhang mit diesen Verbindungen.

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