Solvent Effects on Nucleotide Conformations. I. A Proton Magnetic Resonance Study of the Effect of Electrolytes on Uracil Nucleotides and Nucleosides in Aqueous Solution[1]

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Abstract: The pmr spectra of aqueous solutions of uracil, uridine, deoxyuridine, uridine 3′-monophosphate (3′-UMP), and uridine 5′-monophosphate (5′-UMP) have been studied as a function of electrolyte concentration. The addition of a "solvent-structure breaking" salt such as $Mg(ClO_4)_2$ or $NaClO_4$ to solutions of the nucleosides and nucleotides was found to result in significant upfield shifts of the uracil H_6 resonance. In the cases of uridine and 5′-UMP, these salt-induced shifts are accompanied by a decrease in the ribose H_1'–H_2' coupling constant. These observations are interpreted in terms of a salt-induced conformation change, involving the orientation of the uracil base about the glycosidic bond and the puckering of the furanose ring. In view of an apparent correlation between the salt-induced shifts of the uracil H_6 resonance and the "solvent-structure breaking" properties of the salt, it is proposed that the addition of salt modifies the solvent structure of the solution, which in turn can affect the average orientation of the base about the glycosidic bond. Due to nonbonded interactions between the base and the furanose ring, alteration of the base conformation can induce a conformational change in the furanose ring to an extent commensurate with its rigidity. In uridine and 5′-UMP, it was shown that the observed changes in the H_1'–H_2', coupling constant are correlated with the salt-induced H_6 shifts. The absence of a detectable conformation change in the furanose rings of deoxyuridine and 3′-UMP was attributed to the greater rigidity of the furanose moiety in these cases.

During the past few years, a great deal of research has been devoted to the investigation of the forces which determine the preferred conformation of dinucleotides and higher oligonucleotides. Base-stacking interactions have been foremost among the forces investigated, and they appear to be influential in maintaining parallel and stacked configurations of the planar purine or pyrimidine bases in these molecules.[3-9] These interactions alone, however, cannot account for the rather specific conformations assumed by the bases relative to the ribose moieties to which they are attached. In the ApA molecule, for example, the *anti–anti* stacked conformation was shown to be preferred over the other possible stacked conformations, and it has been suggested that this conformational preference is due to the rotational barriers between the adenine base and ribose ring about the glycosidic bond.[8] That nonbonded interactions between these moieties are indeed important in determining the base conformation is evidenced by the fact that even in many mononucleotides the *anti* conformation is preferred.[10-12]

More recently we have begun to investigate the effect of electrolytes on the conformation of dinucleotides in aqueous solution. Preliminary measurements indicate that the addition of electrolytes does affect the conformation of these molecules. While it is reasonable to attribute these conformational changes to changes in the hydrophobic base-stacking interactions, it is possible that the allowed conformations of the base about the glycosidic bond are also affected by the solvent system. In view of this complication, it is clear that the nonbonded forces which determine the conformation of a nucleotide about its glycosidic bond, particularly its dependence on the solvent system, must be better understood.

With this objective in mind, we have studied the aqueous solution conformation of single nucleoside and nucleotide units under various perturbations to the solvent system. Since the solvent system may also affect any *intermolecular* base-stacking interaction between nucleosides and nucleotides, it is clear that it would be desirable to examine a simple case in which there is little base stacking or where base stacking does not alter the conformation of the nucleotide or nucleoside. Of course, in the latter case, the property monitoring the conformation must also be insensitive to what base stacking may exist. Uridine, deoxyuridine, uridine 5′-monophosphate (5′-UMP), and uridine 3′-monophosphate (3′-UMP) satisfy these requirements. These molecules self-associate only slightly at low concentrations (less than 0.02 *M*) and, as far as we can tell, any self-association which does exist does not alter the molecular conformation or affect any of the properties which we shall use in this work to monitor the conformation of the molecule.

In the past, salts such as $Mg(ClO_4)_2$, $NaClO_4$, NaOAc, and $[CH_3(CH_2)_3]_4NBr$ have been used to perturb the solvent system in investigations of conformational stability of macromolecules such as ribonuclease.[13,14] Their influence on the stability of

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Table I. Summary of the Chemical Shifts and Coupling Constants Observed for Uracil and Related Nucleosides and Nucleotides[a]

	H_6, ppm	H_5, ppm	$\lvert J_{H_6-H_5}\rvert$, cps	$H_{1'}$, ppm	$\lvert J_{H_{1'}-H_{2'}}\rvert$, cps
0.02 *m* uracil, pD 7.2	−7.955	−6.227	7.7		
0.01 *m* uridine, pD 8.3	−8.294	−6.338	8.1	−6.353	4.5
0.01 *m* deoxyuridine, pD 7.5	−8.279	−6.337	8.2	−6.730	6.7[b]
0.01 *m* 5′-UMP, pD 8.4	−8.554	−6.442	8.1	−6.437	4.8
0.01 *m* 3′-UMP, pD 8.1	−8.332	−6.351	8.1	−6.383	4.3

[a] Shifts are measured relative to a TMS capillary. [b] $0.5\lvert J_{H_{1'}-H_{2'}} + J_{H_{1'}-H_{2''}}\rvert$.

these macromolecules has been attributed to their "structure-breaking" and "structure-making" effect on the solvent.[14] Solvent structure contributes to the stability of macromolecules through its contribution to hydrophobic bonding.[15] These salts, therefore, should provide a convenient way of perturbing the aqueous solvent system.

Any conformation change in uridine, deoxyuridine, 5′-UMP, or 3′-UMP resulting from perturbations to the solvent system can easily be monitored by proton magnetic resonance spectroscopy (pmr). The chemical shifts for the various protons of uridine, deoxyuridine, 5′-UMP, and 3′-UMP are sensitive to molecular conformation. The chemical shift is a function of the local magnetic environment of the proton, and this magnetic environment is sensitive to the local electric and magnetic fields produced by other groups of the molecule. Likewise, spin–spin coupling constants are strongly conformation dependent and, in this work, the coupling constants between the various ribose protons are particularly suitable for monitoring the conformation of the ribose ring.[16] A study of the chemical shifts and coupling constants as a function of salt concentration should, therefore, yield information about the effect of solvent on the conformation of these nucleosides and nucleotides, and may lead to the elucidation of the nature of the forces which determine their conformation.

Experimental Section

Uridine and uridine 5′-monophosphate (disodium salt) were obtained from Calbiochem, Los Angeles, as the A grade materials. Uracil and uridine 3′-monophosphate (dilithium salt) were purchased from P. L. Biochemicals, Inc., Milwaukee, Wis. Deoxyuridine was obtained from Sigma Chemical Co., St. Louis, Mo. These compounds were dried over P_2O_5 under vacuum at room temperature and were used without further purification. Solutions were prepared in 99.7% D_2O supplied by Columbia Organic Chemicals, Columbia, S. C.

The salts $NaClO_4$, $Mg(ClO_4)_2$, and NaOAc were of reagent grade. Before use, they were dried under high vacuum at temperatures in excess of 100°. Tetramethylammonium chloride (TMACl) was obtained from Matheson Coleman and Bell. It was dried under high vacuum at 35°. Tetrabutylammonium chloride (TBACl) was prepared from the iodide salt (Eastman Organic Chemicals) by ion exchange. The salt was recrystallized from a CCl_4–hexane mixture and dried under high vacuum at 55°. Reagent grade NaCl was used without further treatment.

The nucleotide or nucleoside concentration was 0.01 *M* in D_2O. The pD of each solution was measured with a Leeds and Northrup 7401 pH meter, equipped with miniature electrodes, and was taken to be the observed pH meter reading plus 0.4 (the standard correction).[17] The pD of all the nucleotide solutions was maintained at a constant value of 8.3 ± 0.3. Where adjustments were necessary, small amounts of 1 *M* DCl or 1 *M* NaOD were added. The salt concentrations quoted in this paper do not include the small amounts of sodium ion or lithium ion existing as the counterion to the uridylic acids, and the minute quantities of sodium or chloride ion introduced in the pD adjustments.

All spectra were run on a Varian HA-100 high-resolution nmr spectrometer, operated in the frequency sweep mode. A C-1024 time-average computer was used to enhance the signal-to-noise ratio. A TMS capillary provided the field/frequency lock signal and also served as an external standard. Chemical shifts were measured relative to the lock signal to ±0.001 ppm. However, in order to correct for changes in the bulk susceptibility of the solutions, all the electrolyte shifts reported are referred to an internal standard of either tetramethylammonium chloride or neopentyl alcohol.

Results

The uracil H_6, H_5, and ribose $H_{1'}$ protons of the uridine nucleoside and nucleotides give rise to distinct resonances in the pmr spectra, which are easily monitored. The assignment of these resonances is based upon the work of Jardetzky, *et al.*[18] The resonances of the other ribose protons appear near the water peak. This spectral region is quite complex, and we have made no attempt to monitor these signals. Hydroxyl- and NH-proton resonances were not observed because of exchange with the solvent.

The H_6 and H_5 resonances of the uracil base appear as doublets due to spin–spin coupling between these protons. In the ribose nucleoside and nuceotides, the $H_{1'}$ resonance often overlaps the H_5 resonance and is a doublet due to spin–spin coupling with the $H_{2'}$ proton of the ribose ring. In the case of deoxyuridine, however, the $H_{1'}$ resonance is a triplet due to coupling to both the ribose $H_{2'}$ and $H_{2''}$ protons. Since the chemical shift difference between $H_{2'}$ and $H_{2''}$ protons is small compared to the $H_{2'}$–$H_{2''}$ geminal coupling constant, only the average $H_{1'}$–$H_{2'}$ ($H_{2''}$) coupling constant, $0.5\lvert J_{H_{1'}-H_{2'}} + J_{H_{1'}-H_{2''}}\rvert$, can be measured.[19] The chemical shifts of these resonances and the various coupling constants in the molecules investigated are summarized in Table I.

Although data have been collected for the H_6, H_5, and $H_{1'}$ resonances, the principal spectral changes on the addition of electrolytes are observed in the position of the H_6 resonance and in the magnitude of the $H_{1'}$–$H_{2'}$ coupling constant. The results obtained for the various nucleosides and nucleotides are summarized as follows.

Uridine. The chemical shifts observed for the uracil H_6 resonance upon the addition of various salts to a 0.01 *m* uridine solution in D_2O at pD 8.3 are depicted in Figure 1. $Mg(ClO_4)_2$ produces the greatest effect, shifting the H_6 resonance 0.07 ppm upfield over a salt concentration range of 1 *m*. Similarly, 2 *m* $NaClO_4$ and 2 *m* NaCl shift the H_6 resonance upfield by 0.07

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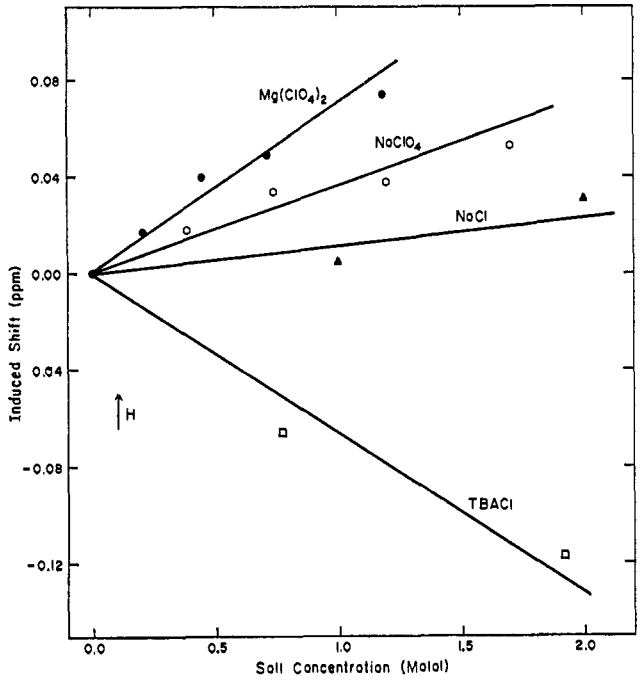

Figure 1. Salt-induced shifts of the H_6 resonance for a 0.01 *m* solution of uridine in D_2O at pD 8.3, 30°.

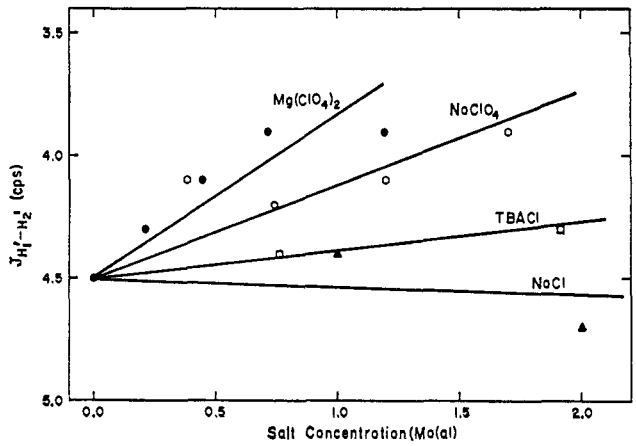

Figure 2. Salt-induced change of $|J_{H_1'-H_2'}|$ for a 0.01 *m* solution of uridine in D_2O at pD 8.3, 30°.

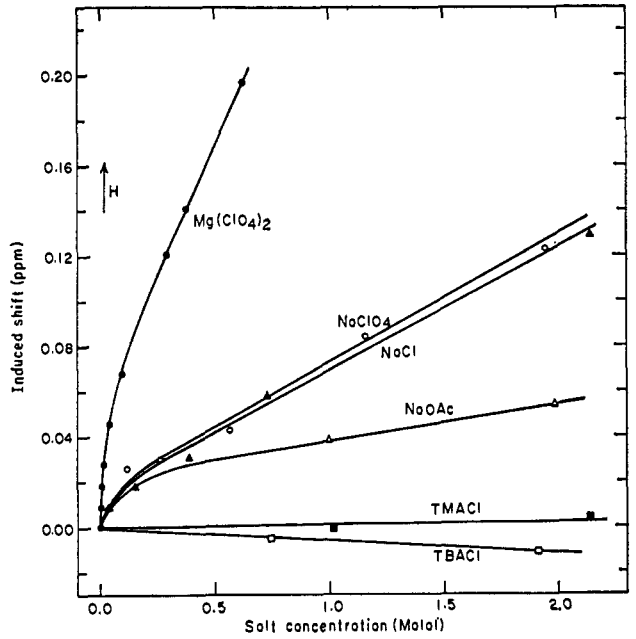

Figure 3. Salt-induced shifts of the H_6 resonance for a 0.01 *m* solution of 5′-UMP in D_2O at pD 8.4, 30°.

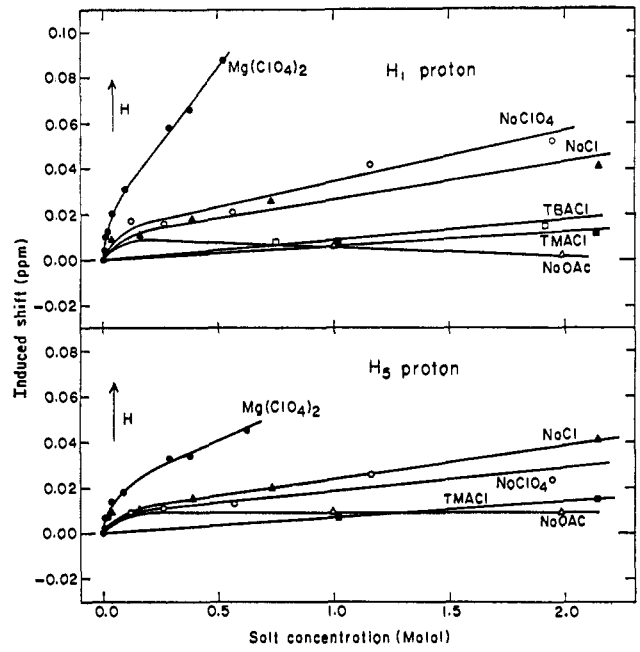

Figure 4. Salt-induced shifts of the $H_{1'}$ and H_5 resonances for a 0.01 *m* solution of 5′-UMP in D_2O at pD 8.4, 30°.

and 0.018 ppm, respectively. TBACl (2 *m*), however, causes a downfield shift of 0.13 ppm.

The H_5 and $H_{1'}$ resonances are also shifted by the addition of these electrolytes. However, the shifts are all less than 0.03 ppm over the entire range of salt concentrations investigated.

The $H_{1'}$–$H_{2'}$ coupling constant does, however, change significantly with the addition of some of these salts (Figure 2). $Mg(ClO_4)_2$, again, produces the greatest effect, with $|J_{H_1'-H_2'}|$ decreasing from 4.5 to 3.9 cps at a salt concentration of 1 *m*. A 0.7-cps decrease in the coupling constant was noted for 2 *m* $NaClO_4$, while NaCl and TBACl were found to have only a small effect.

5′-UMP. The chemical shifts of the H_6, H_5, and $H_{1'}$ resonances of a 0.01 *m* solution of 5′-UMP at pD 8.4 as a function of the concentration of the various salts under study are summarized in Figures 3 and 4. These salts can be seen to produce a pronounced shift of the resonance position of the H_6 proton. $Mg(ClO_4)_2$ has the greatest effect, inducing an upfield shift of 0.19 ppm over a salt concentration range of 0.6 *m*. At salt concentrations of 2 *m*, the observed electrolyte shifts for NaCl, $NaClO_4$, and NaOAc are 0.124, 0.125, and 0.054 ppm upfield, respectively. Tetramethylammonium chloride has little effect on the H_6 chemical shift over the entire concentration range studied, while the shifts observed in tetrabutylammonium chloride are slightly downfield (−0.012 ppm at 2 *m*).

In contrast to the nearly linear induced shifts observed with increasing salt concentration for the H_6 resonance of uridine, the corresponding shifts for 5′-UMP at low

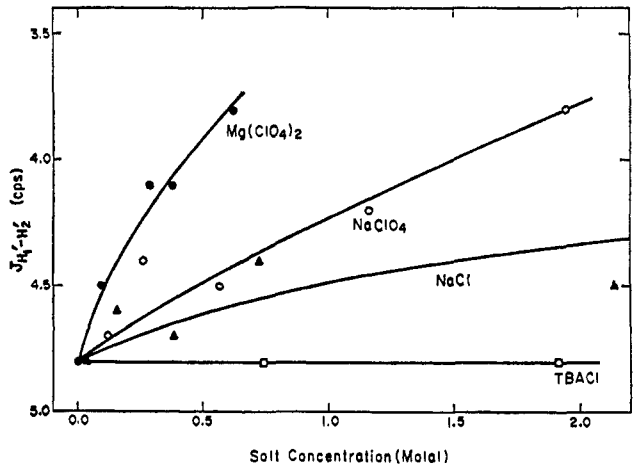

Figure 5. Salt-induced change of $J_{|H_1'-H_2'|}$ for a 0.01 *m* solution of 5′-UMP in D_2O at pD 8.4, 30°.

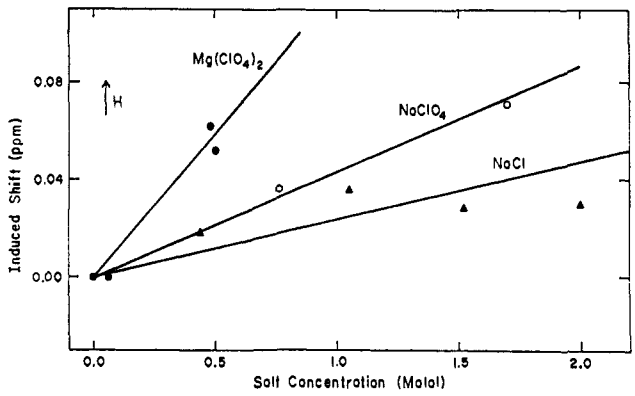

Figure 6. Salt-induced shifts of the H_6 resonance for a 0.01 *m* solution of 3′-UMP in D_2O at pD 8.1, 30°.

salt concentrations can be seen to be abrupt. This abrupt change is most pronounced in the case of magnesium perchlorate and is also evident for the sodium salts, although to a lesser extent. At high salt concentrations, however, the H_6 resonance appears to vary linearly with salt concentration. As we shall show, the abrupt shifts arise from binding of the cation to the negatively charged phosphate group.

The effects of these salts on the H_5 and H_1' resonances of 5′-UMP are again small compared to effects noted for the H_6 resonance. However these salt-induced H_5 and H_1' shifts are large compared to those observed for uridine.

In addition to shifting the H_6, H_5, and H_1' resonances, the same salts were again found to have an effect on the coupling constant between the H_1' and H_2' protons of the ribose ring. These results are summarized in Figure 5. Again, $|J_{H_1'-H_2'}|$ is generally reduced upon the addition of salt. $|J_{H_1'-H_3'}|$, for example, is lowered from 4.8 to 3.8 cps when the $Mg(ClO_4)_2$ concentration is increased from 0.0 to 0.6 *m*; 2 *m* $NaClO_4$ causes a 1.0-cps decrease. Tetrabutylammonium chloride, however, has no effect on $J_{H_1'-H_3'}$.

3′-UMP. In the presence of salts, similar electrolyte shifts are observed for the H_6 (Figure 6), H_5, and H_1' resonances of 3′-UMP as in uridine. The H_6 resonance, however, does not exhibit the abrupt shifts depicted by 5′-UMP at low salt concentration. The

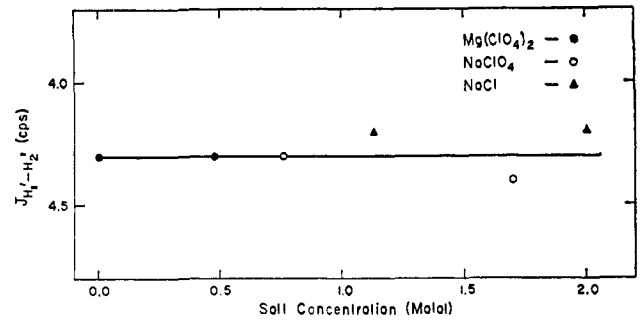

Figure 7. Salt-induced change of $|J_{H_1'-H_2'}|$ for a 0.01 *m* solution of 3′-UMP in D_2O at pD 8.1, 30°.

magnitudes of the salt-induced shifts observed for 3′-UMP are also smaller. At 0.6 *m* $Mg(ClO_4)_2$, the H_6 resonance is shifted 0.065 ppm upfield from its value in the absence of salt, and the corresponding shifts induced by 2 *m* $NaClO_4$ and NaCl are 0.09 and 0.05 ppm. Again these salts do not affect H_1' and H_5 chemical shifts significantly, the induced shifts being less than 0.03 ppm.

In contrast to the coupling constant changes observed for 5′-UMP and uridine, the H_1'–H_2' coupling constant in 3′-UMP appears to be insensitive to the added electrolytes, $Mg(ClO_4)_2$, $NaClO_4$, and NaCl. This behavior is depicted in Figure 7.

Deoxyuridine. Deoxyuridine was investigated in order to ascertain the possible influence of the 2′-hydroxyl group on the electrolyte shifts observed for uridine, 3′-UMP, and 5′-UMP. It was found that the addition of salts again shifts the H_6 resonance upfield from the aqueous solution value. The shifts, however, are somewhat smaller than those observed for the ribose nucleoside and nucleotides. For 2 *m* $NaClO_4$ the upfield shift is 0.042 ppm, and for 1 *m* $Mg(ClO_4)_2$ the observed shift is 0.030 ppm. The salt-induced shifts of the H_1' and H_5 resonances are again of smaller magnitude, being 0.02 ppm or less over the salt concentration range investigated.

The average H_1'–H_2' coupling constant ($0.5|J_{H_1'-H_3'} + J_{H_1'-H_3''}|$) appears to be unaffected by the addition of salt to the deoxyuridine solution.

Uracil. A similar study was undertaken for uracil to ascertain any direct effects which the electrolytes might have on the chemical shifts of the ring protons. Both the H_5 and H_6 resonances were found to be shifted upon the addition of salt. In salt solutions of $NaClO_4$, NaCl, and $Mg(ClO_4)_2$, these resonances are shifted downfield. At 1 *m* salt, the electrolyte-induced shifts are 0.008, 0.028, and 0.019 ppm, respectively, for the H_6 resonance, and the corresponding electrolyte shifts for the H_5 resonance are 0.012, 0.017, and 0.028 ppm, respectively. TBACl (1 *m*) produces a downfield shift of 0.047 ppm for the H_6 resonance and an upfield shift of 0.023 ppm for the H_5 resonance.

Discussion

The addition of salt to aqueous solutions of uracil nucleosides and nucleotides can affect the chemical shifts of the base protons either through direct interactions between the ions and the nucleoside or nucleotide molecule, or through indirect effects of the salt on the solvent structure. In addition to possible specific ion binding both to the uracil ring and to the phosphate

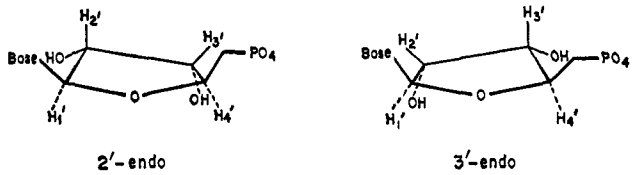

Figure 8. 2'-*endo* and 3'-*endo* ribose conformations.

group of the ribose moiety, the added electrolyte most certainly alters the structure of the solvent, which in turn can influence the solvation of the uracil base as well as the conformation of the nucleoside or nucleotide molecule.

Interpretation of the conformational changes induced by the addition of salts to aqueous solutions of the uracil nucleoside and nucleotides necessitates the assignment of some initial aqueous solution conformation to these molecules. The uracil base can assume several rotational conformations relative to the ribose moiety about the glycosidic bond, and there are also several possible ribose conformations. That the rotational conformation of the base and the conformation of the ribose ring can influence one another might be expected, since nonbonded interactions presumably play an important role in deciding the average conformation of each moiety.[10]

The $H_{1'}$–$H_{2'}$, coupling constant provides a means of monitoring the average conformation of the ribose ring. The ribose rings of pyrimidine nucleosides and nucleotides, in crystal structures at least, are most commonly found in either the 3'-*endo* or 2'-*endo* conformations where either the 3'-carbon atom or the 2'-carbon atom is out of the plane formed by the other four atoms of the ring and is on the same side of the plane as the base and the 5' linkage (Figure 8).[20] Theoretical values for the $H_{1'}$–$H_{2'}$ coupling constant when the ribose ring is in the 2'-*endo* and 3'-*endo* conformations are 6.9 and 1.7 cps, respectively.[16] On the basis of these limiting values, it appears that uridine, 5'-UMP, and 3'-UMP, which exhibit $H_{1'}$–$H_{2'}$ coupling constants of 4.5, 4.8, and 4.3 cps, respectively, all have average ribose conformations intermediate between 2'-*endo* and 3'-*endo*, with the conformations in uridine and 3'-UMP pushed more toward the 3'-*endo* side. In the case of deoxyuridine, the theoretical values for the average coupling constant, $0.5|J_{H_{1'}-H_{2'}} + J_{H_{1'}-H_{2''}}|$, in the 2'-*endo* and 3'-*endo* limits are 6.4 and 4.8 cps, respectively. The observed coupling constant is 6.7 Hz, it thus appears that the conformation of the deoxyribose ring in deoxyuridine is 2'-*endo*.

Considerable evidence has been accumulated in recent years, both from crystallographic studies[10] and from solution nmr studies,[11,12] to indicate that the rotational conformation of the pyrimidine base in the pyrimidine nucleosides and nucleotides is preferentially *anti*. In the case of 5'-UMP, the conformation of the uracil base in solution can be inferred from the chemical shifts of the H_5 and H_6 base protons relative to the corresponding shifts in uridine. The H_5 and H_6 resonances in 5'-UMP appear appreciably downfield (see Table I), and it can be concluded from this observation that the uracil base assumes a rotational conformation about the glycosidic bond such that the part of the uracil ring bearing these protons is in close proximity to the doubly charged phosphate group (Figure 9). This deduction is substantiated both by protonation studies and by the diamagnetic ion binding studies presented in this work. Protonation of the phosphate group over the pD range between 8 and 5 has been shown to result in an upfield shift of 0.15 ppm for the H_6 resonance. Qualitatively, this upfield shift can be understood in terms of the decreased electric field effect accompanying the reduction in the effective charge of the phosphate moiety upon phosphate protonation. Similar upfield shifts are observed on the binding of magnesium ions to 5'-UMP in aqueous solution. In the case of uridine, deoxyuridine, and 3'-UMP, there is less direct pmr evidence to indicate the preferred solution conformation of the uracil base about the glycosidic bond. In these molecules, the phosphate moiety is either absent or, in the case of 3'-UMP, constrained to a position far removed from the base. Perturbations of the phosphate therefore have little effect on the resonance positions of the uracil base protons. There is, however, no *a priori* reason not to expect a similar *anti* conformation for the uracil base in these molecules, since the rotational conformation of the base about the glycosidic bond is largely determined by nonbonded interactions between the base and the ribose ring. Esterification of the nucleoside to the phosphate group at either the 3' or 5' position of the ribose ring is not expected to have a pronounced effect on the rotational conformation of the base, even though some perturbation undoubtedly occurs indirectly through the effect of the phosphate substitution on the conformation of the ribose ring.

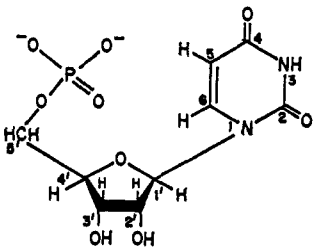

Figure 9. 5'-UMP

While it appears that the orientation of the pyrimidine ring with respect to the ribose moiety in the uracil nucleosides and nucleotides is most likely to be preferentially *anti*, this rotational conformation describes a range of torsion angles about the glycosidic bond. In this work, it is necessary to define the base conformation more precisely, and in order to do this, we have adopted the nomenclature of Donohue and Trueblood.[21] The angle of rotation about the glycosidic bond between $C_{1'}$ of the ribose and N_1 of the pyrimidine base is specified by the torsion angle ϕ_{CN}, which is defined as the dihedral angle between the plane of the base and the plane formed by the $C_{1'}$–$O_{1'}$ bond of the furanose ring and the $C_{1'}$–$N_{1'}$ glycosidic bond. The torsion angle, ϕ_{CN}, is 0° when C_6 of the pyrimidine base is eclipsed with the ether oxygen ($O_{1'}$) of the furanose ring, and

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(21) J. Donohue and K. N. Trueblood, *J. Mol. Biol.*, 2, 363 (1960).

positive angles are measured when the base is rotated in the clockwise direction when viewing from $N_{1'}$ to $C_{1'}$. The *anti* conformation expected for the pyrimidine nucleosides and nucleotides describes a range of torsion angles centered at $\phi_{CN} \sim -30°$, and X-ray crystallographic studies have shown that this range can extend from -5 to $-65°$.[10]

Because of the close proximity of the uracil H_6 proton to the ribose moiety, variations in the average base conformation within the above limits of torsion angles might be expected to result in small variations in the chemical shift of this proton. The small differences between the chemical shifts of this proton in uridine, deoxyuridine, and 3′-UMP may in part be due to small variations in the base orientation in these molecules. Experimentally, the effect of the ribose ring on the resonance position of the H_6 proton can be ascertained by comparing the H_6 chemical shifts in uracil and uridine. The H_6 resonance of uridine is 0.34 ppm downfield from the corresponding resonance in uracil. In other words, attachment of the ribose moiety to N_1 of the uracil base causes an appreciable downfield shift of the H_6 resonance. This does not appear to be caused by an inductive effect due to an alkyl-type substituent, since a nearly equal shift of 0.30 ppm[18] is observed when the methyl group in N_1-methylcytosine is replaced by the ribose moiety in cytidine. The 0.3-ppm downfield shift induced by the ribose ring most likely arises from an electric field effect and/or a magnetic anisotropy effect due to some group in the ribose ring. In view of the close proximity of the uracil H_6 proton to the ether oxygen linkage, this ether group appears to be the most likely candidate. Both the electric field effect and the magnetic anisotropy effect due to the ether linkage can be estimated on the basis of the standard formulas,[22] and both effects can be expected to be sensitive to the rotational conformation of the base about the glycosidic bond. For torsion angles ϕ_{CN} in the vicinity of $\sim -30°$, the base conformation which is generally observed for the pyrimidine nucleosides in the crystalline state, the calculations indicate that the magnetic anisotropy effect is small and that the electric field effect of the ether oxygen can account for the observed ribose shift of the H_6 resonance. When $\phi_{CN} \approx -60°$, both the electric field effect and the magnetic anisotropy effect due to the ether oxygen were found to be negligible. Although such calculations have often been found to be quantitatively unreliable, it is, nevertheless, clear from these considerations that the chemical shift of the uracil H_6 proton would be expected to be sensitive to the conformation of the base relative to the ribose moiety about the glycosidic bond. Specifically, the H_6 resonance is expected to move to higher fields when the uracil base assumes average rotational conformations corresponding to more negative torsion angles.

At first glance, one might also expect the chemical shift of the ribose or deoxyribose $H_{1'}$ proton to give a similar indication of the rotational conformation of the base due to the close proximity of this proton to the 2-keto group of the uracil ring when the base conformation is *anti*. Both the bond dipole and the magnetic anisotropy of the carbonyl group are expected to influence the chemical shift of this proton with the dominant electric field effect being of the order of several tenths of 1 ppm downfield. The dependence of the carbonyl effect on the base orientation is not difficult to ascertain. Since rotation of the base toward a torsion angle ϕ_{CN} of $\sim -90°$ brings the keto oxygen in closer proximity to the $H_{1'}$ proton, a downfield shift is to be expected when the average orientation of the base tends toward more negative torsional angles within the range of 0 and $-90°$. However, in the ribose nucleosides and nucleotides, the 2′-OH group of the ribose ring also contributes to the $H_{1'}$ shifts, and this contribution is most likely dependent upon the conformation of the ribose ring. The 2′-OH group results in increased shielding of the $H_{1'}$ proton, as can be ascertained by comparing the chemical shifts of the $H_{1'}$ protons in the ribose and deoxyribose nucleosides in Table I. Conformational changes within the ribose ring, which alter the dihedral angle between the 1′-CH and 2′-CO bonds and the distance between the anomeric hydrogen and the 2′-OH group should therefore be reflected in the chemical shift of the $H_{1'}$ resonance. A change in the ribose ring conformation from 2′-*endo* to 3′-*endo*, the two most commonly found ribose conformations, for example, would be expected to result in an upfield shift, since the 2′-hydroxyl group is closer to the $H_{1'}$ proton when the ribose conformation is 3′-*endo*. The factors which affect the $H_{1'}$ shifts are therefore quite complex, and the interpretation of these shifts is not necessarily straightforward since the two contributions which we have considered here can add or subtract, depending on the detailed nature of the conformational changes within the nucleoside or nucleotide molecule. Moreover, there are probably other factors influencing the $H_{1'}$ shifts, such as the local solvent structure, the solvation of 2-keto oxygen, the 2′-OH group, and the ether oxygen of the ribose ring, etc.

With the above background, we can now discuss the salt-induced effects observed in this work. In view of the low nucleoside or nucleotide concentrations, we do not believe that the observed effects arise from enhanced or reduced stacking of the uracil bases due to modification of the solvent system. Even though uracil–uracil interaction is not expected to be reflected in the uracil H_5 and H_6 resonances due to the small magnetic anisotropy of the uracil ring,[23] changes in the base-stacking interaction might be reflected in the $|J_{H_{1'}-H_{2'}}|$ coupling constants. We note, for example, that in 3′-AMP and 5′-AMP, where the base-staking interaction can be monitored by the chemical shifts of the base protons, the $|J_{H_{1'}-H_{2'}}|$'s are also concentration dependent, these coupling constants varying about 1–1.5 cps over the concentration range of ~0.01 to ~0.2 *m*.[24] However, in the present systems, we find that both the uracil H_5, H_6 shifts, and the ribose $H_{1'}$–$H_{2'}$ coupling constants are independent of concentration over the concentration range of 0.01–0.08 *m*.

Our results with uracil would seem to rule out the importance of any effects related to specific ion binding to the uracil ring and to changes in the solvation of the base as a consequence of modifications in the solvent structure. With the exception of TBACl, the salt-

(22) R. F. Zürcher in "Progress in Nuclear Magnetic Resonance Spectroscopy," Vol. 2, J. W. Emsley, J. Feeney, and L. H. Sutcliffe, Ed., Pergamon Press, Oxford, 1967, p 205.

(23) M. P. Schweizer, S. I. Chan, and P. O. P. Ts'o, *J. Am. Chem. Soc.*, 87, 5241 (1965).

(24) J. H. Nelson, Ph.D. Thesis, California Institute of Technology, Pasadena, Calif., 1969, p 79.

induced shifts of both the H_5 and H_6 resonances of uracil are quite small, but more significantly, these shifts are opposite in direction (downfield instead of upfield) to those noted for the corresponding nucleosides and nucleotides. Moreover, in the uracil nucleosides and nucleotides, only the H_6 resonance appears to be significantly shifted upon the addition of salt to the solutions, indicating that the perturbation in the magnetic environment of the H_6 proton must be inherent in the close proximity of this proton to the ribose or ribose phosphate moiety. In the case of 3′-UMP and 5′-UMP, specific cation binding to the negatively charged phosphate group is expected with $Mg(ClO_4)_2$, and to some extent with the sodium salts. However, only in the case of 5′-UMP is the phosphate close enough for specific ion binding to directly affect the chemical shifts of the uracil H_6 proton. It thus appears that the addition of salt to the nucleoside or nucleotide solutions has resulted in some perturbation of the ribose moiety and/or a change in the average conformation of the uracil base about the glycosidic bond. In the case of uridine and 5′-UMP, the observed changes in the $H_{1'}$–$H_{2'}$ coupling constant upon the addition of salt do indicate a change in the conformation of the ribose ring. However, even though a salt-induced upfield shift of the H_6 resonance is noted in 3′-UMP, a concomitant change in the $H_{1'}$–$H_{2'}$ coupling constant is not observed for this molecule. These observations would seem to suggest that the salt-induced H_6 shifts are not the direct result of conformational changes in the ribose moiety, and with the exception of 5′-UMP, we can also rule out contributions due to specific ion binding. With these possibilities eliminated in the case of uridine, deoxyuridine, and 3′-UMP, there remains only one other reasonable interpretation of the salt-induced upfield shifts of the uracil H_6 resonance in these molecules. That is, the addition of salt to these nucleoside and nucleotide solutions produces a change in the average rotational conformation of the uracil base about the glycosidic bond and the resultant variations in the position of the H_6 proton relative to the ribose ring oxygen give rise to the observed shifts.

The different variations in the salt-induced shifts with salt concentration among the various nucleosides and nucleotides are noteworthy and serve to distinguish 5′-UMP from uridine, deoxyuridine, and 3′-UMP. Whereas the induced shifts are within experimental error linear with respect to the salt concentration over the range of concentration investigated for uridine, deoxyuridine, and 3′-UMP, the salt-induced shifts in the case of 5′-UMP, by contrast, vary abruptly at low salt concentrations, even though the variation again becomes linear as the concentration is increased. In the case of 5′-UMP, specific cation binding to the phosphate group leads to upfield shifts of the uracil H_6 resonance, since the ion binding in effect decreases the effective charge of the phosphate and its deshielding effect on the H_6 proton. The effect of this complex formation on the chemical shift of the H_6 resonance can be estimated from its pH dependence. Since the first protonation of the doubly charged phosphate in 5′-UMP over the pD range 8–5 shifts the H_6 resonance upfield 0.15 ppm, and the second protonation of phosphate at a pD of 1.2 results in an additional 0.12 ppm, one might expect, upon the formation of a 1:1 ion–nucleotide complex, an upfield shift of the H_6 resonance by 0.27 ppm for a divalent ion, and by 0.15 ppm for a univalent ion. However, even for a divalent cation such as Mg^{2+} which is expected to bind quite strongly to the negatively charged phosphate group, the complex formation is not expected to be complete when the nucleotide concentration is of the order of 0.01 *m*, due to the increasing importance of activity coefficients for the ionic species at the salt concentrations necessary to drive the reaction toward completion. The observed chemical shift therefore represents a weighted average of the shifts for the complexed and uncomplexed molecules; that is

$$\delta_{obsd} = \delta_u f_u + \delta_c f_c \qquad (1)$$

where f_c is the fraction of nucleotide complexed, f_u is the fraction uncomplexed, and δ_u and δ_c denote the chemical shifts of the uncomplexed and complexed molecules, respectively. When the shifts are not complicated by salt-induced conformational changes, both δ_u and δ_c are independent of the salt concentration. The following expression for the salt-induced shift can then be obtained from mass-action considerations for the formation of 1:1 cation–nucleotide complexes.

$$\delta_{obsd} - \delta_u = \frac{1}{2}(\delta_c - \delta_u)\left\{\left(1 + \frac{M}{N} + \frac{\gamma_c}{\gamma_M\gamma_N KN}\right) - \left[\left(1 + \frac{M}{N} + \frac{\gamma_c}{\gamma_M\gamma_N KN}\right)^2 - \frac{4M}{N}\right]^{1/2}\right\} \qquad (2)$$

Here *M* and *N* denote the stoichiometric concentration of the cation and the nucleotide, respectively, *K* is the formation constant of the complex, and γ_M, γ_N, and γ_c are the activity coefficients of the cation, nucleotide, and the complex, respectively.

We have applied eq 2 to the H_6 shifts of 5′-UMP induced by the addition of $Mg(ClO_4)_2$, but without success. In this treatment, we have assumed that $\gamma_c \approx 1$, and have estimated $\gamma_M\gamma_N$ from the known mean activity coefficients ($\gamma_\pm$) of $ZnSO_4$ at the same ionic strength.[25] While eq 2 does predict the abrupt salt-induced shifts at low Mg^{2+} concentrations, it does not reproduce the linear dependence noted at salt concentrations above 0.1 *m*. Instead, calculations made using reasonable values of the parameters ($\delta_c - \delta_u \approx 0.27$ ppm, $K = 100\ m^{-1}$) indicate that if specific ion binding were the only contribution to the observed salt-induced shifts, the induced shifts would level off at ~0.06 ppm at a Mg^{2+} concentration of ~0.1 *m*. The results of this analysis suggest that, as in the case of uridine, deoxyuridine, and 3′-UMP, the addition of salt to the 5′-UMP solution induces a change in the nucleotide conformation which affects the chemical shift of the H_6 resonance. If this were the case, then δ_c and δ_u would both be dependent upon the salt concentration. Under the assumption that the effects due to specific ion binding and those related to nucleotide conformation change are independent of each other, we can write

$$\delta_{obsd} = (\delta_c^0 + \delta_c')f_c + (\delta_u^0 + \delta_u')f_u \qquad (3)$$

where δ_c^0 and δ_u^0 denote the chemical shifts of complexed and uncomplexed nucleotide and δ_c' and δ_u' rep-

(25) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed, Reinhold Publishing Corp., New York, N. Y., 1958, p 564.

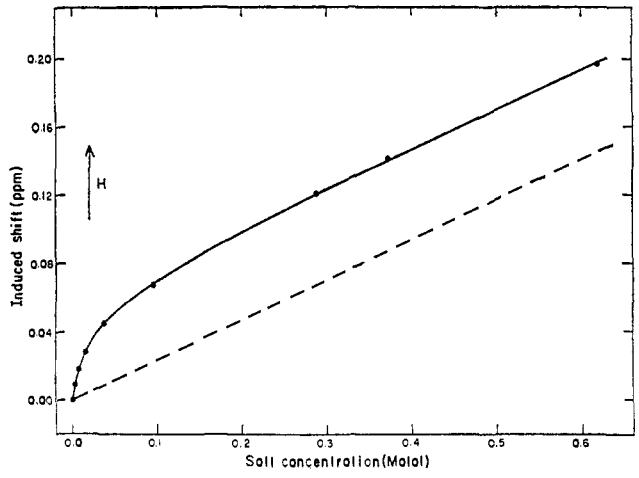

Figure 10. $Mg(ClO_4)_2$-induced shifts of the H_6 resonance for a 0.01 *m* solution of 5′-UMP in D_2O at pD 8.4, 30°.

resent the modifications in the shifts of the complexed and uncomplexed molecules as a result of the salt-induced conformation changes. If we now assume that the salt-induced conformation changes are quite similar for both the complexed and uncomplexed nucleotide molecule, then

$$\delta_c' f_c + \delta_u' f_u = \delta_c' = \delta_u' = \delta' \quad (4)$$

and eq 3 simplifies to

$$\delta_{obsd} = \delta_c^0 f_c + \delta_u^0 f_u + \delta' \quad (5)$$

For small conformational changes, we expect δ' to be proportional to the salt concentration; hence

$$\delta' = kM \quad (6)$$

and we obtain the following expression for the salt-induced shifts.

$$\delta_{obsd} - \delta_u^0 = kM + \frac{1}{2}(\delta_c^0 - \delta_u^0)\left\{\left(1 + \frac{M}{N} + \frac{\gamma_c}{\gamma_M\gamma_N KN}\right) - \left[\left(1 + \frac{M}{N} + \frac{\gamma_c}{\gamma_M\gamma_N KN}\right)^2 - \frac{4M}{N}\right]^{1/2}\right\} \quad (7)$$

It is not difficult to see that eq 7 does predict the abrupt variations in the salt-induced shifts at low salt concentrations and the linear dependence observed at high salt concentrations. We have fitted the H_6 shifts of 5′-UMP induced by $Mg(ClO_4)_2$ to eq 7 without difficulty. k can be determined from the linear dependence at high salt concentrations, and the nonlinear part of the data, as given by the difference between the experimental points and the broken line in Figure 10, can be fitted by the second term in eq 7 using the following parameters: $K = 60\ m^{-1}$ and $\delta_c^0 - \delta_u^0 = 0.25$ ppm. Both of these values are reasonable. We have indicated earlier that the binding of a divalent ion to the phosphate in 5′-UMP would reduce the deshielding effect of the phosphate on the H_6 proton by 0.27 ppm. The formation constant of the Mg–5′-UMP complex can be compared with the value of 90 M^{-1} previously reported for the Mg–5′-AMP complex by other workers.[26] A similar analysis of the data for the sodium salts can also be made. However, since the binding constant for the Na^+ nucleotide complex is much smaller, the effects of specific ion binding are much less dramatic, and at high salt concentrations, the induced H_6 shifts are dominated by modification of the nucleotide conformation.

It is interesting to compare the shifts associated with the salt-induced conformation change for the various nucleosides and nucleotides. A comparison of the H_6 shifts for 3′-UMP, uridine, and deoxyuridine with the results obtained for 5′-UMP in Figure 3 indicates that the effects are much more pronounced for 5′-UMP than for the other molecules. At 0.6 *m* $Mg(ClO_4)_2$, *e.g.*, the shifts attributable to the salt-induced conformation change are 0.14, 0.07, 0.04, and 0.02 ppm for 5′-UMP, 3′-UMP, uridine, and deoxyuridine, respectively. This may indicate that the H_6 resonance in 5′-UMP is more sensitive to the orientation of the uracil base about the glycosidic bond because of the close proximity of the H_6 proton to the phosphate group. Or it may be that the 5′-UMP molecule is more susceptible to the salt-induced conformation change. In this connection, we note that the salt-induced shifts per unit salt concentration are in general larger for the nucleotides than for the nucleosides. If the H_6 shifts are indeed more sensitive to the base conformation because of the deshielding effect of the phosphate group, then we expect the shifts induced per unit conformational change to depend upon the effective charge of the phosphate group, in which case the assumption $\delta_u' = \delta_c'$ used in obtaining expression 7 may not be valid. However, since the fraction of nucleotide molecules complexed is not large even at the highest salt concentration studied (~25% in the case of $Mg(ClO_4)_2$), any departure from the assumption given by (4) is not likely to be discernible experimentally.

With the exception of TBACl, the salt-induced H_6 shifts, which we have attributed to modifications of the average rotational conformation of the uracil base about the glycosidic linkage, are all upfield. If our interpretation of the shifts is correct, then the direction of the shifts indicates that when the uracil base departs from its initial *anti* conformation upon the addition of salt to the nucleoside or nucleotide solution, it assumes average rotational conformations corresponding to more negative torsion angles. This conclusion, it turns out, provides a reasonable rationale for understanding the conformational changes induced in the ribose ring as indicated by the variations in the H_1'–H_2' coupling constant with salt concentration. Examination of CPK molecular models for the pyrimidine nucleosides and nucleotides indicates that while the base can assume a range of torsion angles near the *anti* conformation, the range of torsion angles allowed depends upon the ribose conformation due to nonbonded interactions between the H_6 hydrogen of the base and various atoms on the ribose ring. When the ribose ring is in its 2′-*endo* conformation, the steric interaction between the H_6 and H_2' hydrogens is particularly important, and the allowed torsion angles are limited to the range between ~−10 and ~−60°. This nonbonded interaction is partially relieved, however, when the ribose ring is in its 3′-*endo* conformation, thus permitting the allowed range of torsion angles to be

(26) M. M. Taqui Khan and A. E. Martell, *J. Am. Chem. Soc.*, **89**, 5585 (1967).

extended toward more negative values somewhat beyond −90°. These ideas regarding the interrelationship between the allowed base conformations and the conformation of the ribose ring are in general agreement with the conclusions obtained by Haschemeyer and Rich on the basis of their analysis of the crystal structure data for similar molecules.[10] Thus, the average rotational conformation of the base may be closely correlated with the type of ring puckering in the furanose residue, and, conversely, any perturbation which affects the average rotational conformation of the base may induce a conformational change in the ribose ring. The direction and the extent of the induced conformational changes will of course depend upon the nature of the nonbonded interactions and the rigidity of the furanose ring toward changes in the out-of-plane ring deformation. For example, in the case where two furanose ring conformations are in equilibrium, it is clear that changes in the nonbonded interactions between the base and the furanose residue can influence the relative stability of the two conformations, with the average ring conformation shifted toward the limit stabilized by the nonbonded interactions. In uridine and 5′-UMP, the observed ribose conformational changes toward the 3′-*endo* direction are therefore not contrary to expectation, since on the basis of the salt-induced shifts of the uracil H_6 resonance, we have surmised that the addition of salt induces a change in the rotational conformation of the base toward more negative ϕ_{CN}'s.

A plot of the changes in $|J_{H_1'-H_2'}|$ *vs.* the observed salt-induced H_6 shifts for 5′-UMP is depicted in Figure 11 and reveals a near-linear correlation with slope $\Delta J_{H_1'-H_2'}/(\delta_{obsd} - \delta_u^0)$ equal to 8 cps/ppm. The slope for a similar plot of the uridine data is somewhat larger, with $\Delta J_{H_1'-H_2'}/(\delta_{obsd} - \delta_u^0) \cong 10$ cps/ppm. Since the rigidity of the ribose rings in 5′-UMP and uridine is probably quite similar, the slightly smaller $\Delta J_{H_1'-H_2'}/(\delta_{obsd} - \delta_u^0)$ for 5′-UMP may merely reflect the somewhat greater sensitivity of the H_6 chemical shifts to the average torsion angle ϕ_{CN} of the base about the glycosidic bond. No salt-induced ribose conformation change was observed in 3′-UMP, even though a sizable salt-induced shift was observed for the uracil H_6 resonance of this molecule (0.065 ppm in the presence of 0.6 *m* $Mg(ClO_4)_2$). This result probably reflects the increased rigidity of the ribose ring when the 3′-OH group is substituted by the bulkier phosphate group. No noticeable change in the average $H_1'-H_2'(H_2'')$ coupling constant was detected in the case of deoxyuridine. However, the salt-induced shifts observed for the H_6 resonance are also quite small. The value of the average $H_1'-H_2'(H_2'')$ coupling constant indicates that the conformation of the deoxyribose ring is close to 2′-endo, and it may be that removal of the 2′-OH group significantly stabilizes the 2′-*endo* conformation in deoxyuridine.

Except in the case of 5′-UMP, the salt-induced shifts observed for the H_5 and H_1' protons of the uracil nucleosides and nucleotides are quite small, and consequently do not deserve the same attention which we have devoted to the H_6 shifts. The ribose ring might be expected to exert some influence on the chemical shift of the H_5 proton; however, the H_5 proton being further removed, the effect of the ether oxygen is expected to be smaller, as can be seen by comparing the chemical shift difference between uridine and uracil for the H_5 and H_6 protons (0.11 ppm *vs.* 0.34 ppm). Small changes in the base conformation are then not readily monitored by the position of the H_5 resonance. Moreover, with the exception of 5′-UMP, the salt-induced shifts of the H_5 proton are all downfield, as in the case of uracil, illustrating the probable importance of changes in the local solvent structure and specific solvation of the uracil base in determining the small salt-induced H_5 shifts. In 5′-UMP, the negatively charged phosphate also deshields the H_5 proton somewhat (~0.11 ppm), and part of the salt-induced upfield shifts observed, particularly in the case of Mg^{2+}, is undoubtedly due to specific cation binding. As mentioned earlier, the factors which contribute to the salt-induced H_1' shifts are quite complex. On the basis of the salt-induced conformational changes conjectured for uridine and 5′-UMP, the contributions of the 2-keto effect and the 2′-OH effect would subtract and the salt-induced shifts would be expected to be small. The large salt-induced H_1' upfield shifts observed for the $Mg(ClO_4)_2$–5′-UMP system are somewhat surprising, but may reflect gross changes in the solvation of the ribose ether oxygen upon specific cation–phosphate binding in view of the close proximity of the negatively charged phosphate group to the ribose ether oxygen in this mononucleotide. In this connection, we note that the chemical shifts of the H_1' proton of 5′-UMP appears to be somewhat anomalous in that it is 0.08 ppm downfield from that of the H_1' proton of uridine (see Table I).

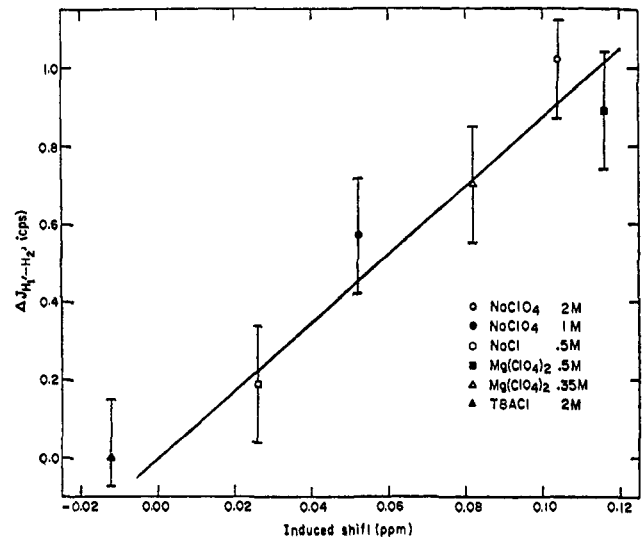

Figure 11. Correlation of salt-induced H_6 shifts with the salt-induced change of $|J_{H_1'-H_2'}|$ for various 0.01 *m* solutions of 5′-UMP in D_2O containing different electrolytes at pD 8.4, 30°.

At present, the manner by which the addition of salt to an aqueous solution of uridine, deoxyuridine, 3′-UMP, or 5′-UMP can affect the average orientation of the uracil base about the glycosidic bond is still open to conjecture. But one general observation about the data presented in this work does shed some light on this problem. That is, irrespective of the nucleoside or nucleotide molecule, the various salts investigated possess the same relative effectiveness in inducing the

H_6 shifts ($(MgClO_4)_2$ > $NaClO_4$ > NaCl > NaOAc, TMACl, TBACl). Variations in the magnitude of the effects observed for the four nucleoside and nucleotide molecules studied might be accounted for on the basis of minor differences in the local solvent structure about these molecules and/or small differences in the nonbonded interactions opposing the conformational change. The relative effectiveness of the various salts does not appear to correlate with the cation or the anion present in the solution. But, there appears to be a rough correlation with the solvent-structure "breaking" and "making" properties of the salts ($Mg(ClO_4)_2$ > $NaClO_4$ > NaOAc > NaCl > TMACl > TBACl).[13,14] These considerations suggest the possibility that the salts act indirectly through their effect on the water structure in influencing the rotational conformation of the uracil base about the glycosidic bond.

The results of this work indicate that electrolytes can have important effects on the conformation of nucleosides and nucleotides. The implications of these findings to the conformational properties of dinucleotides, oligonucleotides, and to polynucleotide structure, however, remain to be ascertained, since the vertical base-stacking interactions presumably also play an important role in stabilizing the base orientations and the conformation of each furanose residue in these molecules. Recently, there has been some controversy over the nature of the intramolecular base-stacking interaction in several pyrimidine–pyrimidine dinucleotides.[3,4] However, in these works, the molecules were examined under quite different experimental conditions of added electrolytes and ionic strengths. The observed discrepancies are therefore probably real rather than experimental artifacts, and may well reflect the effects of solvent structure on both the vertical stacking interaction and the conformational properties of the individual nucleotide units as demonstrated in this work. The intramolecular stacking interaction is generally considered to be weak in the pyrimidine–pyrimidine dinucleotides; hence subtle differences in the conformational properties of the nucleotide units might be expected to have a more profound influence on the overall conformation of the molecule.

Mechanisms of Photochemical Reactions in Solution. LX.[1] Photochemical Isomerization of 2,4-Hexadiene *Via* a Quantum-Chain Mechanism

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Abstract: An investigation of the benzophenone-sensitized photoisomerization of 2,4-hexadiene has been carried out in an attempt to infer if the preferred configuration of the lowest lying triplet state of the diene is in the form of a "1,4-biradical," which would permit isomerization about both double bonds, or an "allyl–methylene" system, which would permit isomerization about only one double bond per excitation. During the course of the investigation it was found that a quantum-chain mechanism, heretofore unreported for the type of system, is operative during the early stages of photoisomerization. The chain-carrying species is believed to be an electronically excited state of the diene. Although the chain process appears to occur with isomerization of only one end of the diene, under conditions designed to minimize the chain process, the data are consistent with a "1,4-biradical" or rapidly equilibrating "allyl–methylene" system.

For a fuller knowledge of the photochemistry of unsaturated hydrocarbons, it is necessary to have a better understanding of the properties of their electronically excited states.[4] Olson made a thorough theoretical study of the electronically excited states of *cis* and *trans* isomers of the substituted ethylenes, which is one of the first reported discussions in the nature of these species.[5] In 1939, Lewis and Calvin presented the "loose bolt" theory, which discusses the conversion of electronic oscillation to atomic vibrations and heat.[6] In another paper, Lewis, Magel, and Lipkin discussed further the conversion of electronic energy into rotational and vibrational energy.[7] They concluded that if ordinary *cis* and *trans* molecules are given increasing torsional vibration about the ethylenic double bond, the distinction between the two isomers will persist until the torsional energy approaches $\Delta H^{\ddagger}$ for thermal isomerization, above which true rotation will occur, and that it seems certain that *cis* isomers and some *trans* isomers excited to the lowest excited states will, before they decay to the ground state, probably acquire enough torsional energy to lose their identity

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