Conformation of Pseudouridine and Pseudouridine 5'-Phosphate. Ultraviolet and Nuclear Magnetic Resonance Study*

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ABSTRACT: 2,'3'-*O*-Isopropylidenepseudouridine 5'-acetate has been synthesized and its ultraviolet spectrum shown to be the same as that of a 5'-unsubstituted pseudouridine. Contrary to earlier proposals it is concluded, that the presence of a 5'-hydroxyl is without influence on the ultraviolet spectrum and distribution of tautomeric monoanions in pseudouridine.

Pseudouridine 5'-phosphate is shown to exist in aqueous solution in the *anti* conformation. This is concluded from nuclear magnetic resonance spectroscopy of pseudouridine and pseudouridine monophosphate, showing a specific interaction of the 5'-phosphate with the C_6-H uracil proton, which is shifted downfield in pseudouridine monophosphate as compared with the parent pseudouridine. On the basis of the *anti* conformation of pseudouridine monophosphate, the large difference in ultraviolet absorption spectra between the nucleoside and its 5'-phosphate, which is encountered only in the pseudouridine–pseudouridine monophosphate pair, is interpreted by an increased electron density at C_6 in pseudouridine monophosphate, as compared with pseudouridine. This increased electron density is in turn responsible for the tautomeric shift in the dissociation equilibrium between the two uracil monoanions of pseudouridine monophosphate, corresponding to loss of the N_1- or the N_3-protons.

Among the nucleic acid components, pseudouridine (5-β-D-ribofuranosyluracil), ψ,[1] proved to be very attractive for speculations as to its role in RNA. It is present in the loops of the cloverleaf model of transfer RNA (for references, see Dudock *et al.*, 1969), and in the case of tyrosine transfer RNA it is also part of the anticodon (Madison *et al.*, 1966). Very often it is present as a terminal ψ–A pair in double-stranded regions at the beginning of a loop. Although the most tempting structure of 1,5-diribosyluracil as a component of RNA (Lis and Lis, 1963, 1964) or as an intermediate in enzymatic transformation of uridine to pseudouridine (Pollak and Arnstein, 1962; Lis and Lis, 1962) has been disproved by the synthesis of this diriboside (Dugaiczyk, 1965; Brown *et al.*, 1965; Dugaiczyk and Eiler, 1966),[2] the unique structure of pseudouridine has retained its potential for an active role in transfer RNA in the mechanism of protein biosynthesis.

The riboside is found not only in ribonucleic acids. An interesting amino acid derivative (3-alanyl-5-ribosyl-6-aminouracil) was isolated from germinating pea seedlings (Lambein and Van Parijs, 1968), and in *Arthrobacter polychromogens*, pseudouridine was identified as part of a water-soluble blue pigment (Knackmuss *et al.*, 1969).

The nucleoside is very prone to conversions into other nonnatural isomers (Cohn, 1960; Chambers *et al.*, 1963), which probably accounts for the difficulties in obtaining pure samples. Although the ultraviolet spectra of pseudouridine (Cohn, 1960; Michelson and Cohn, 1962; Ofengand and Schaefer, 1965; Venkstern and Baev, 1965) and of its 5'-phosphate (Goldberg and Rabinowitz, 1961) have been published by several investigators, a close examination of the data convinced us that most of them are unsatisfactory for spectroscopic interpretation. This can be seen from Figure 2 of Ofengand and Schaefer (1965), where two distinct isosbestic points are recorded around 240 mμ, and only one is expected, or from Figure 5 of Michelson and Cohn (1962), where no isosbestic points are obtained around 215 mμ, when one is expected. This can also be inferred from the discrepancies in spectroscopic pK_a determinations of the nucleoside, reported as far apart as 8.97 (Ofengand and Schaefer, 1965) and 9.6 (Cohn, 1960).

From the difference in ultraviolet spectra of the ring monoanions (pH 12) of pseudouridine and its 5'-phosphate, Chambers *et al.* (1963) and Chambers (1966) interpreted the structure in terms of an intramolecular hydrogen bond in the nucleoside between the 5'-hydroxyl and the 4-carbonyl oxygen (I, same as XLI in Chambers, 1966). This hydrogen bond was made responsible for stabilizing the 1-anion (I) and not the 3-anion (II), and its necessary absence from pseudouridine 5'-phosphate was thought to account for the different spectrum. The interpretation implicitly postulates the *syn* conformation of this RNA component. We decided to obtain precise spectroscopic data on carefully purified and crystallized pseudouridine and its relevant derivatives, and to provide more direct evidence with regard to the conformation of the nucleotide by extending the studies to aqueous dioxane solutions of lower dielectric constant and to nuclear magnetic resonance spectroscopy. It will be seen, from what follows, that our results do not support the structure (I) proposed by Chambers (1966), and that in aqueous solutions pseudo-

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[1] The following abbreviations are used: ψ, pseudouridine; ψMP, pseudouridine monophosphate; UMP, uridine monophosphate; A, adenosine.

[2] In a private communication Dr. M. G. Burdon had informed us that the λ_{max} reported by their group (Brown *et al.*, 1965) as 256 mμ is a printing error, and the value obtained is 265 mμ. On a sample provided by Dr. Burdon we have confirmed the spectral and chromatographic identity with our (Dugaiczyk and Eiler, 1966) 1,5-diribosyluracil (λ_{max} 266 mμ), which had been independently synthesized in a different route.

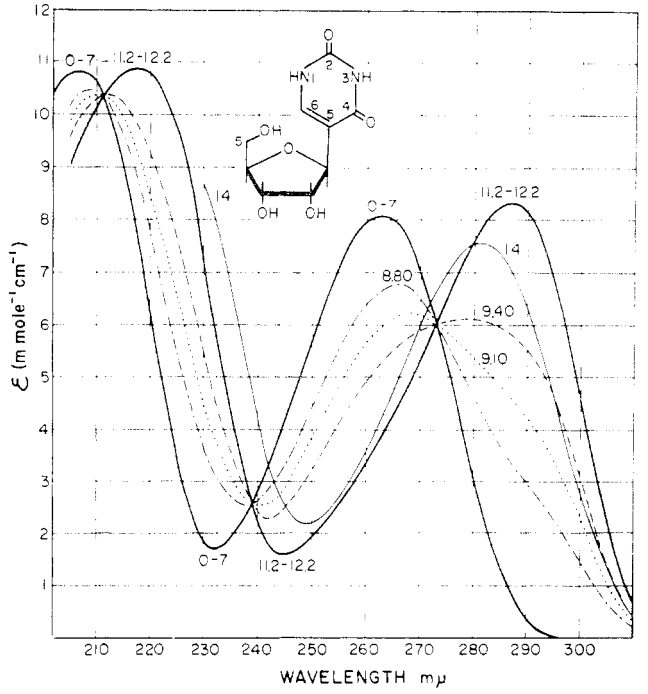


FIGURE 1: Absorption spectrum in aqueous solution of pseudouridine at indicated pH values.

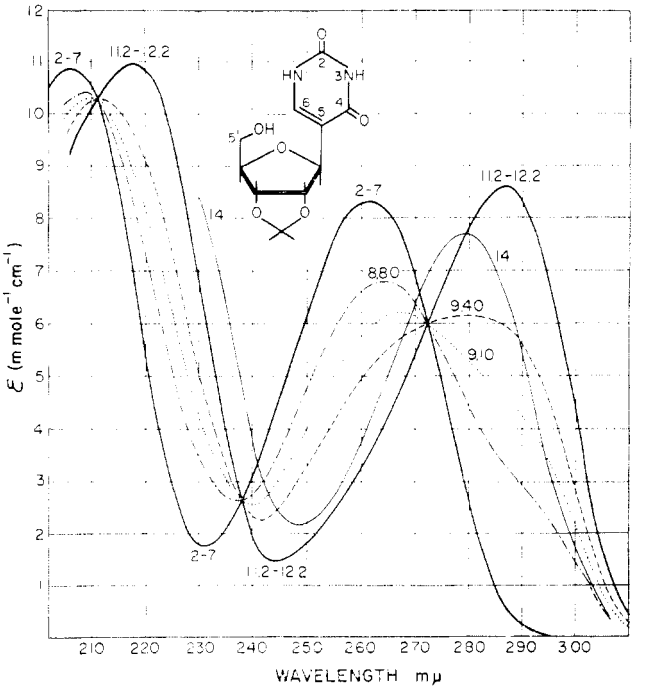
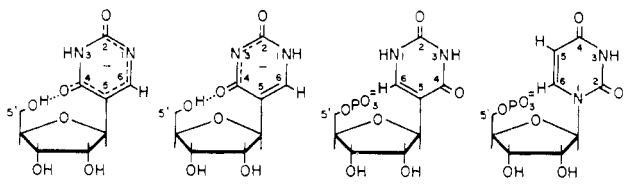


FIGURE 2: Absorption spectrum in aqueous solution of isopropylidene pseudouridine at indicated pH values.

uridine 5′-phosphate is correctly represented by the *anti* conformation (III) of this nucleotide.



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Experimental Procedures

Materials. The dioxane used was Merck Spectral Grade. D_2O (99.76% pure) was from Nuclear Research, Inc. Pseudouridine, its isopropylidene derivative, and pseudouridine 5′-phosphate were obtained as described earlier (Dugaiczyk and Eiler, 1969a). It was found that only after repeated crystallization (H_2O) could satisfactory spectra of pseudouridine be obtained. The melting point of the nucleoside was 230–231°, lit. (Shapiro and Chambers, 1961) mp 223–224°. Pseudouridine 3′-phosphate was obtained from Sigma. It contains several waters of crystallization and the anhydrous nucleotide is very hygroscopic, but it gives satisfactory spectra.

2′,3′-Isopropylidenepseudouridine 5′-Acetate. To 171 mg of isopropylidenepseudouridine (0.6 mmole) in 5 ml of anhydrous pyridine was added 0.47 ml of acetic anhydride (5 mmoles) and the mixture was set aside for 4 hr at 20°. Chromatographic controls (Whatman No. 1 paper) in water-saturated butanol showed that the reaction was essentially completed in 3 hr. Isopropylidenepseudouridine has an R_F of 0.65 in this solvent system, whereas its 5′-acetate has an R_F of 0.77. Anhydrous ethanol (5 ml) was added to the reaction mixture and, after 2 hr, the products were concentrated under reduced pressure. The residue was taken up in ethanol, reevaporated two more times, then applied as narrow bands on Whatman No. 1 paper, and submitted to the butanol–water solvent system. Two minor products were seen, both having R_F values higher than the desired acetyl derivative. Ultraviolet evidence suggested that the major of these by-products was an N_3-substituted derivative. This indicates that the reaction must be controlled in time in order not to obtain more ring substitution. After elution with methanol, 178 mg (91% yield) of chromatographically pure isopropylidene-pseudouridine 5′-acetate was obtained, which had mp 165–167°. Recrystallization from methanol gave 150 mg of the desired product, mp 170.5–171° (Kofler). *Anal.* Calcd for $C_{13}H_{16}N_2O_7$: C, 51.53; H, 5.56; N, 8.58. Found: C, 51.61; H, 5.73; N, 8.47.

Instrumentation. Ultraviolet absorption spectra were obtained on Unicam single-beam SP-500 and double-beam SP-800 instruments, performing necessary controls at the short wavelengths (validity of Beer's law, stray light, and no or minimal absorption of the buffers used). Values of pH below 3 were obtained with HCl, 1 N HCl was taken as pH 0. The buffers used were phosphate and ammonia–ammonium chloride at final ionic strength 0.05. Finally, pH values above 11 were obtained with NaOH; 1 N NaOH was taken as pH 14.

Nuclear magnetic resonance spectra were recorded on A-60 Varian Associates spectrometer using a sweep width of 500 Hz and a sweep time of 250 sec. Chemical shifts were measured in D_2O solutions from an external Me_4Si capillary, with probe temperature of 31–32°. The spectra were recorded on 0.2 M solutions in D_2O after a threefold exchange with D_2O by

TABLE I: Spectrophotometric Data and Apparent pK_a Values of Pseudouridine and Derivatives.

Property	Pseudouridine at pH		Isopropylidenepseudouridine at pH		Isopropylidenepseudouridine 5′-Acetate at pH	
	0–7	11.2–12.2	2–7	11.2–12.2	2–7	11.2–12.2
λ_{max} (mμ)	207; 263	218; 287	206; 261.5	217.5; 286.5	205.5; 261	217; 286
ϵ_{max}	10,800; 8,100	10,900; 8,350	10,900; 8,350	10,950; 8,650	10,800; 8,350	10,900; 8,800
λ_{min} (mμ)	232	245	231	244.5	230	244
$\epsilon_{240}/\epsilon_{260}$	0.36	0.68	0.38	0.59	0.38	0.57
$\epsilon_{280}/\epsilon_{260}$	0.40	2.22	0.32	2.40	0.27	2.58
$\epsilon_{290}/\epsilon_{260}$	0.05	2.42	0.03	2.53	0.02	2.67
pK_a	9.10	>12.5	9.10	>12.5	9.10	>12.5
Isosbestic points	221.5; 239; 273	280	221; 238.5; 272	279.5	210; 238; 271.5	278

	Pseudouridine 3′-Phosphate at pH		Pseudouridine 5′-Phosphate at pH		
	7.0	11.5–12.5	0	7.0	12
λ_{max} (mμ)	206.5; 262.5	217.5; 286.5	263	207.5; 264	285
λ_{min} (mμ)	232	245	232	233	244
$\epsilon_{240}/\epsilon_{260}$	0.35	0.70	0.36	0.34	0.63
$\epsilon_{280}/\epsilon_{260}$	0.37	2.39	0.40	0.47	1.53
$\epsilon_{290}/\epsilon_{260}$	0.04	2.51	0.06	0.07	1.50
pK_a	9.40	>12.5		9.60	>13
Isosbestic points	211.5; 239; 273	280	232; 268	239.5; 275.5	247; 265; 290

evaporation under reduced pressure and after adjustment with NaOD to pD 8.0. The equation (Glasoe and Long, 1960) pD = meter reading + 0.4 was used.

Results

Ultraviolet Studies. The spectra of pseudouridine and isopropylidenepseudouridine in aqueous solutions are shown in Figures 1 and 2, respectively, and the spectral data are summarized in Table I. The main difference from earlier data can be seen in the short region of the spectrum, where a distinct isosbestic point is obtained, which in effect gives pK_a values in the short region of the spectrum, the same as obtained at longer wavelengths. Our noncrystallized samples, even chromatographically pure, showed the same continuous increase of absorption (pH 12) with decreasing wavelength as reported by Michelson and Cohn (1962). In addition, the maximum at 287 mμ (pH 12) for pseudouridine is too low, and the minimum at 245 mμ (pH 12) too high in most previously reported spectra. Michelson and Cohn (1962), in fact, pointed out the increased ϵ_{max} at 287 mμ (pH 12) of isopropylidenepseudouridine in contrast with that of pseudouridine. However, when pseudouridine is sufficiently purified by crystallization, then the spectral difference between pseudouridine and its isopropylidene derivative becomes practically nonexistent, except for the slight blue shift of the whole spectrum of isopropylidenepseudouridine by about 1 mμ. This can be seen by comparing Figures 1 and 2, and by the high 290:260 ratio at pH 12 (Table I). Exactly the same minimal shift of 1 mμ can be observed in the spectrum of uridine as a result of isopropylidene substitution.

The spectrum of isopropylidenepseudouridine 5′-acetate is represented by Figure 3 and in Table I. The only observable difference is the further minimal hypsochromic shift (about 0.5 mμ) of the whole spectrum as a result of substitution of the last free hydroxyl in the ribose moiety. Since the spectrum of the monoanion of this 5′-substituted derivative was most important to the present investigation, and because of a potential danger of hydrolysis of the 5′-acetate by alkali, the following control was performed. The substituted derivative was incubated at 20°, pH 12.5, and at time intervals aliquots were spotted for immediate paper chromatography. After 1 hr, no evidence of hydrolysis could be obtained. After 24-hr incubation, about 20% of the incubated material was recovered as isopropylidenepseudouridine, the rest being the starting 5′-acetate of isopropylidenepseudouridine. Since it takes only 2–3 min to record the spectrum, and the spectrum of the monoanion can already be obtained at pH 11.5, it can be safely concluded, that the spectrum represents the 5′-substituted derivative, and that substitution of the 5′-hydroxyl with acetate has no effect on the ultraviolet spectrum, in contrast to a similar substitution with phosphate.

The spectrum of 5′-ψMP is shown in Figure 4; some of the data is recorded in Table I. The following points of interest may be noted: (a) No isosbestic point around 212 mμ; (b) change of spectrum in the pH region 0 to 7 in contrast to pseudouridine, which has identical spectra at these two pH values; (c) curves representing first ring dissociation form isosbestic points with curve "7," not "0;" (d) distinctly different picture at pH 12, known from earlier studies by Goldberg and Rabinowitz (1961); (e) acid-weakening effect on the uracil moiety by 0.5 pH unit.

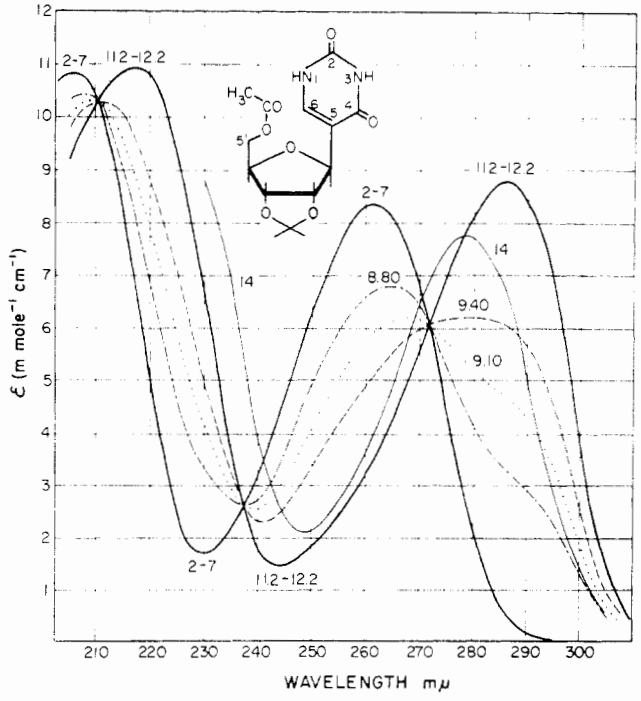

FIGURE 3: Absorption spectrum in aqueous solution of isopropylidene pseudouridine 5′-acetate at indicated pH values.

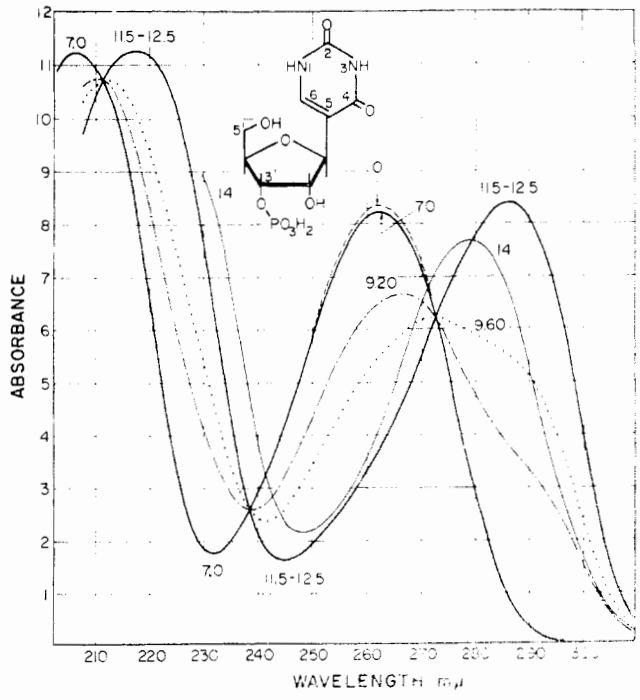

FIGURE 5: Absorption spectrum in aqueous solution of pseudouridine 3′-phosphate at indicated pH values.

The spectrum of 3′-ψMP is shown in Figure 5; relevant data are summarized in Table I. The spectrum is again analogous to that of pseudouridine, the effect of the 3′-phosphate being both quantitatively smaller, and qualitatively distinct from that of the isomeric 5′-phosphate. (a) The isosbestic point at 212 mμ appears again. (b) Change of pH from 0 to 7 is accompanied not as much by a shift of the spectrum as more by a slight decrease of ϵ_{max} at 263 mμ. The tautomeric distribution of the uracil monoanions is the same as in the parent nucleoside, despite the change of pK_a. (d) The acid-weakening effect on the ring is smaller, only 0.3 pH unit. The spectra at pH 14 of both 5′- and 3′-ψMP are again similar, because they represent the second dissociation from the ring, which abolishes differences in tautomeric equilibrium present in the ring monoanions.

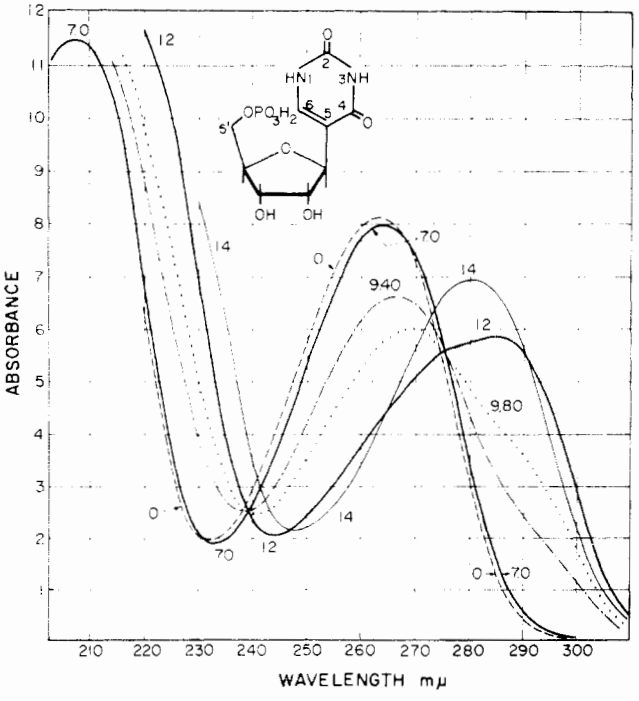

FIGURE 4: Absorption spectrum in aqueous solution of pseudouridine 5′-phosphate at indicated pH values.

From earlier studies by Shugar and Fox (1952), by Fox and Shugar (1952), and from our results, the following observation is proposed with respect to the maxima and isosbestic points in the short region of the spectrum of uracil derivatives.

The spectrum has an apparent harmonic character as a function of wavelength. This point is best observed on 5-formyluracil (Dugaiczyk and Eiler, 1969a) or 5-nitrouracil (Shugar and Fox, 1952), because the spectra of the latter two derivatives are shifted toward observable regions of present-day instruments. The harmonic character of the spectrum allows predictions somewhat beyond the observable region.

The change of the spectrum representing the monoanion with respect to that of the neutral form depends on the contribution of both tautomers and has the following characteristics. (a) The spectrum of a pure N_1-anion (N_3 substituted) is displaced in wavelength, giving repetitiously isosbestic points on both shoulders of the spectrum representing the neutral form. (b) The spectrum of a pure N_3-anion (N_1 substituted) shows mainly changes in ϵ_{max}. The maxima of the monoanion are alternately below and above those of the neutral form, and isosbestic points are formed only on one shoulder of the curves (the "blue" one). It is

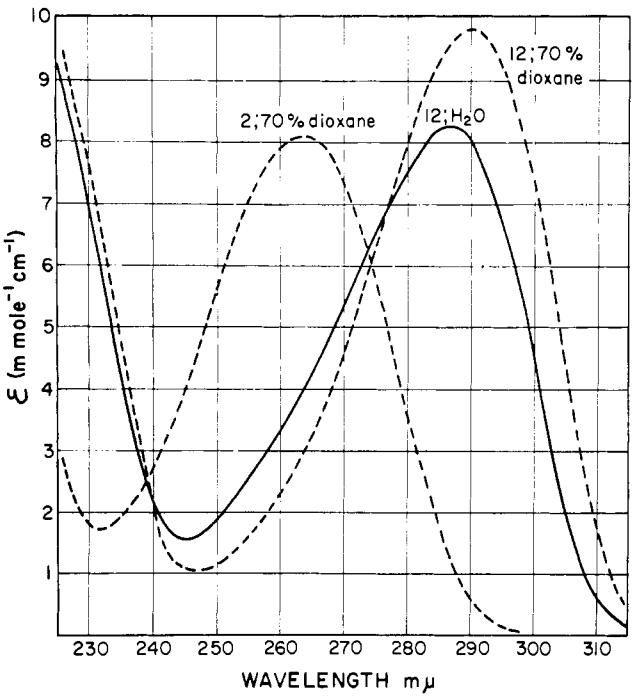

FIGURE 6: Absorption spectrum of pseudouridine in H_2O and in 70% aqueous dioxane at pH 2 (0.01 N HCl) and 12 (0.01 N NaOH).

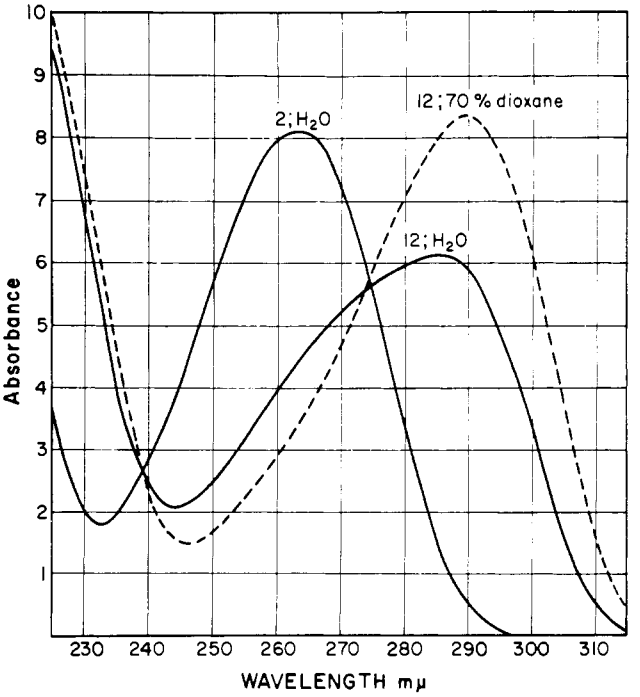

FIGURE 7: Absorption spectrum of pseudouridine 5′-phosphate in H_2O and in 70% aqueous dioxane at pH 2 (0.01 N HCl) and 12 (0.01 N NaOH).

expected, that in the spectrum of uridine, for example, the second maximum of the monoanion should be formed above the one at 205 mμ of the neutral form, and a second isosbestic point should be found around 185 mμ, corresponding to the first one at 245 mμ. (c) In a tautomeric mixture, the more N_3-anions are present, the less displaced is the spectrum with respect to that of the neutral form, until their maxima are sufficiently close but alternately below and above each other, and consequently isosbestic points are found only on one shoulder. Spectra of compounds that have a fair amount of N_1-anions, like 5-formyluracil or 5-carboxyuracil (Dugaiczyk and Eiler, 1969a),[3] are still sufficiently displaced to form isosbestic points on both shoulders of their curves. On the other hand uracil, or 5′-ψMP have already sufficient N_3-anionic character, that their shortwave maxima are to be around 205 mμ, above the maxima of the neutral forms. Consequently, the shortwave isosbestic point is shifted onto the "blue" shoulder and should be found around 185 mμ.

The correctness of this speculation remains to be seen. Nevertheless the above reasoning, together with the fact that pseudouridine has distinctly more N_1-anionic character than uracil (the maximum at 287 mμ being high and displaced from that at 263 mμ) has led us to expect that the second isosbestic point should be still formed on the "red" shoulder of the maximum at 207 mμ. This is presently demonstrated to be the case for several pseudouridine derivatives, which have isosbestic points at 212 mμ.

Solvent-Induced Shift in Tautomeric Equilibrium. It was shown by Wierzchowski *et al.* (1965) that in the 1-anion of uracil the charge is distributed along the bond system $(O{=}{-}C_2{-}{=}N_1{-}{=}C_6{-}{=}C_5{-}{=}C_4{-}{=}O)^-$, (as in I), whereas the charge in the 3-anion is distributed along the bond system $(O{=}{-}C_2{-}{=}N_3{-}{=}C_4{-}{=}O)^-$ (as in II). In both of the tautomers the charge is distributed to the 4-carbonyl group. In view of these results, it is hard to see why a hydrogen bond between the 5′-hydroxyl and the 4-carbonyl oxygen should stabilize the 1-anion (I), as proposed by Chambers (1966), and not the 3-anion (II). It is further shown by the same investigators, that in water–dioxane mixtures the equilibrium between the two tautomeric forms of the uracil anion can be shifted in favor of the less polar 1-anion. We found that the same holds true for pseudouridine and its 5′-phosphate, which is shown by their spectra in 0.01 N NaOH in 70% aqueous dioxane (Figures 6 and 7). From the large increase of the maximum around 290 mμ (pH 12) of pseudouridine in dioxane solutions (Figure 6), it is concluded that the tautomeric equilibrium is shifted much further in favor of the 1-anion. The new spectrum in dioxane now resembles that of an N_3-substituted uracil, where anion formation must involve loss of a proton from N_1. It follows, that in aqueous solution the monoanion of pseudouridine does not correspond to a predominantly single tautomeric form, but represents a mixture of the 1-, and the 3-anions of uracil, with fair amounts of both forms. As can be seen from comparison of Figures 1 and 6, dioxane affects only slightly the spectrum of the neutral form of pseudouridine, amounting to a bathochromic shift of the whole spectrum by about 1.5 mμ, with no change in the molar extinction coefficient. This may be due to hydrogen bonding of dioxane to the ribose hydroxyls, because this shift is even smaller in the case of isopropylidenepseudouridine and 5′-ψMP, and is totally absent in the case of

[3] In the above work, the values of ϵ_{max} for 5-carboxyuracil in Table I represent a monohydrate, whereas Figure 3 represents the anhydrous compound. See Dugaiczyk and Eiler (1969b).

TABLE II: Influence of the Phosphate Group (pD 8.0) on the Chemical Shifts in Isomeric Uracil Nucleosides and Nucleotides in D_2O.[a]

Compound	δ H-6, ppm	J, Hz	δ H-5, ppm	J, Hz	δ H-1′, ppm	J, Hz	δ H-5′, H-5″, ppm
Uridine	8.32	d; 8.0	6.37	d; 8.0	6.38	d; 4.0	4.33 m
5′-UMP	8.57	d; 8.0	6.46	d; 8.0	6.47	d; 4.0	4.47 m
Δδ	−0.25		−0.09		−0.09		−0.14
Pseudouridine	8.13	s	Absent		*b*		4.25 m
5′-ψMP	8.37	s	Absent		*b*		4.47 m
Δδ	−0.24						−0.22
3′-ψMP	8.18	s	Absent		*b*		4.30 m
Δδ	−0.05						−0.05

[a] Chemical shifts were measured from external Me_4Si to better than ±0.01 ppm except for the H-5′, H-5″ resonances, which are difficult to discern and partially overlap with the unresolved multiplet of the 2′, 3′, 4′-protons. [b] Hidden in the HDO artifact (~5.2 ppm). s, singlet; d, doublet; m, multiplet.

uracil. The spectra in the two different solvent systems (Figures 6 and 7) do not show sharp isosbestic points in contrast to those in one solvent only (Figures 1 and 4), and despite the fact that they were obtained on the same samples of pseudouridine or 5′-ψMP. But since dioxane changes the ratio of the two tautomeric uracil anions, the absorption curves are not expected to give well-defined, single isosbestic points in the two solvents.

From calculated spectra, Chambers (1966) had calculated the distribution of the monoanions of pseudouridine as 4:1 in favor of the 1-anion. From his Figure 4, used as a basis for these calculations, and which represents the absorbance ratio $A_{280}:A_{260}$ (pH 12) as a function of the mole fraction of 1-anion, the highest value for this 280:260 ratio obtained was 2.66. This figure should represent the pure 1-anion, but it is difficult to reconcile with the present data. From our Figure 6, the same 280:260 ratio can be obtained as high as 3.5, and the spectrum still does not represent the pure 1-anion.

The lack of the 4–5′ hydrogen-bond interaction in pseudouridine 5′-phosphate was proposed to account for the decreased ϵ_{max} around 290 mμ (pH 12) as compared with the same maximum of pseudouridine (Chambers *et al.*, 1963; Chambers, 1966). It can be seen from Figure 7 that this decreased maximum in the spectrum of pseudouridine 5′-phosphate (pH 12) can be restored by dioxane to the original ϵ_{max} of the pseudouridine anion. The high ϵ_{max} of the pseudouridine anion, therefore, cannot be taken as being specific for the presumed 4–5′ hydrogen bond.

Nuclear Magnetic Resonance Spectroscopy. With regard to the conformation of pseudouridine 5′-phosphate, direct proof was obtained from nuclear magnetic resonance spectroscopy, the pertinent data being summarized in Table II. From the chemical shifts it can be seen, that there is a specific interaction between the 5′-phosphate (pD 8.0) and the H-6 proton of the uracil ring, and this deshielding is of the same magnitude (0.24 to 0.25 ppm) for both 5′-UMP and 5′-ψMP, indicating the same intramolecular proximity in both of the isomeric 5′-phosphates. This result leads to the conclusion that there is the same conformation around the "glycosidic" C-C linkage of ψMP as around the usual N–C glycosidic bond of UMP, and that in aqueous solution both isomeric nucleotides must exist in the *anti* conformation (III), where the H-6 proton is in juxtaposition to the 5′-phosphate group. The *anti* conformation of most of the common nucleotides has been concluded from earlier nuclear magnetic resonance studies by Schweizer *et al.* (1968) and by Danyluk and Hruska (1968). A fairly large (~0.2 ppm) deshielding effect can also be observed on the H-5′, H-5″ protons owing to their neighboring location to the electron-withdrawing phosphate group in 5′-UMP and 5′-ψMP, but not in 3′-ψMP.

The phosphate group can exert its influence on the H-6 proton of the uracil ring only from the 5′position. As can be seen from Table II, in 3′-ψMP this shift of H-6 is five times smaller.

The introduction of a 5′-acetyl group to isopropylideneuridine and isopropylidene pseudouridine is accompanied by a small but specific shielding (+0.06 ppm; D_2O) of the H-6 protons in both of the isomeric nucleosides. The H-5 proton of the uridine derivative is unaffected within the measured accuracy of better than ±0.01 ppm. The result may be taken as an indication that also the acetyl group is close to C_6–H. This small, and opposite to the phosphate effect on H-6 may be correlated with the small increase of ϵ_{max}^{286} (pH 12) of the 5′-acetyl derivative of pseudouridine. Acetylation of isopropylidenepseudouridine is also accompanied by a 0.55-ppm downfield shift of the methylene resonance, a result which confirms acetylation of a primary alcohol.

Discussion

On the basis of the *anti* conformation of 5′-ψMP, shown by nuclear magnetic resonance spectroscopy, we must interpret its ultraviolet spectrum by direct interaction of the phosphate group with the uracil ring. It is clear from the results of Wierzchowski *et al.* (1965) on the tautomeric equilibria between the uracil monoanions and from our present studies, that the spectrum of the ring monoanion of ψMP (pH 12) represents a greater contribution of the 3-anion, as compared with pseudouridine, where the tautomeric equilibrium is shifted more in favor of the 1-anion. On this point of a shifted ionization pattern between pseudouridine and ψMP we are in agreement with Chambers (1966), al-

though his calculated 4:1 distribution of tautomers in pseudouridine is questioned. A disagreement arises in the explanation for the different distribution of ring monoanions between pseudouridine and 5′-ψMP.

We postulate that this shift of the tautomeric equilibrium is caused by an increased electron density at C_6 in 5′-ψMP as compared with pseudouridine, and not by a 4–5′ hydrogen-bond interaction. The increased electron density is the result of a polarization of the C_6–H bond, due to attraction of this proton by the 5′-phosphate anion. Consequently, a negative charge, now to be accommodated on the uracil ring due to dissociation of one of its N–H protons, will have the tendency to be distributed "away" from the electron-dense C_6, *i.e.*, along the bond system $(O{=}C_2{-}N_3{=}C_4{-}O)^-$, which corresponds to a favored dissociation of the N_3-proton. At pH 12, the *anti* conformation of the nucleotide leads to the largest separation of charges between the phosphate and the ring 3-anion.

Upon dissociation of the 5′-phosphate (pH 0 to 7), the ultraviolet spectrum of ψMP exhibits a bathochromic shift, which mimics the beginning of a dissociation of the N_1-proton. This 0 to 7 ultraviolet shift can be rationalized in the same way: both the dissociation of the phosphate and of the N_1-proton are accompanied by an increase in electron density in the same region of the uracil chromophore. 5′-UMP shows a similar, although smaller shift of its spectrum in the 0 to 7 pH region (to our knowledge, unpublished). Comparing the formulas of the two nucleotides (III and IV) will indicate the structural similarity: the proximity of the phosphate to C_6–H. On the other hand, removing the phosphate group from the C_6–H proximity, like in 3′-ψMP, abolishes completely its influence on the tautomeric distribution, although it still has an acid-weakening effect on the ring.

Our interpretation gains support from earlier data of Wempen and Fox (1964) on 6-halogeno-substituted uracil derivatives. They found that in 6-halogenouracils, anion formation involves loss of a proton almost exclusively from N_1, with subsequent electron distribution in the other "half" of the ring, *i.e.*, along the bond system $(O{=}C_2{-}N_1{=}C_6{-}C_5{=}C_4{-}O)^-$. According to our proposal, this is the result of the electronegative halogens, which polarize the C_6–X bond in the opposite way, thus diminishing the electron density at C_6, which in turn favors the accommodation of a negative charge along the bond system which embraces C_6. This effect of the 5′-phosphate on the ultraviolet spectrum (pH 12) of a nucleotide is specific for ψMP, even though the primary polarization of the C_6–H bond by the phosphate is the same also in UMP. In UMP however, as well as in all the other pyrimidine N_1-nucleotides, only the N_3-proton is available for subsequent dissociation, and consequently only one tautomeric species of the ring anion can be formed.

It should be pointed out that our nuclear magnetic resonance data do not prove whether the nucleotide is "locked" in the *anti* conformation or not. The observed 0.25-ppm shift of H-6 does not by itself indicate even a predominant *anti* conformation. The conclusions about *anti* conformation of UMP reached in previous nuclear magnetic resonance studies (Schweizer *et al.*, 1968; Danyluk and Hruska, 1968) involve the implicit assumption that there is a large rotational barrier present in these nucleosides. This assumption is amply supported by the available X-ray data (*e.g.*, Haschemeyer and Rich, 1967). There are no X-ray data on pseudouridine, but our nuclear magnetic resonance results can be taken as an indication that the rotational barrier between the *syn* and *anti* conformation of ψMP is practically the same as in UMP. The difference in length between the "glycosidic" C_5–C_1' bond in pseudouridine and the usual N_1–C_1' bond in other pyrimidines is apparently small to permit clearance of a rotational barrier. For uracil derivatives the N_1–C bond is 1.474 Å long, whereas the C_5–C bond is 1.510 Å long (Donohue, 1968). This makes a difference of less than 3%.

One may be tempted to further prove the specific effect of the 5′-phosphate by blocking its ionizable hydrogens, but this is a somewhat difficult task. The third pK_a of phosphoric acid is 12.4, but, as it is now generally recognized since the first study by Kumler and Eiler (1943), phosphoric esters are much stronger acids than phosphoric acid itself. A monomethyl ester of ψMP would have at pH 12 still a (full) negative charge on its phosphate, so its ultraviolet spectrum is expected to be still like that of 5′-ψMP and not like that of pseudouridine. On the other hand, exhaustive methylation to block both of the phosphoric hydroxyls, will lead to substitution of the ring NH group(s), giving undesired products. We feel that the 5′-acetyl derivative proved sufficiently the point of the negative charge as being responsible for the spectral changes.

In short, it is not the absence of the 5′-hydroxyl, but the presence of the 5′-phosphate which is responsible for the different spectra. The phosphate itself, due to its strong negative charge and the proximity to the ring, has an influence on the chromophore and cannot be taken as a model compound to demonstrate the absence of a presumed hydrogen bond. A 5′-acetyl derivative serves better for this purpose. As demonstrated in the present work by nuclear magnetic resonance and ultraviolet spectroscopy, the primary effect of the phosphate on the ring takes place in the acid-to-neutral region of pH, long before any dissociation of the ring protons starts. Pertinent to this problem of the influence of the 5′-phosphate on the pyrimidine ring is an observtion by Szer and Shugar (1966), that the reactivity of the NH groups is distinctly altered.

Having experimentally established the conformation of 5′-ψMP as *anti* at pD 8.0, and concluded the same conformation for pH 12 because of favorable separation of charges, let us return to the nucleoside. It would be very difficult to provide direct experimental evidence for or against the proposed internal hydrogen bond in aqueous solution. The infrared O–H stretching region can not be utilized, nor can nuclear magnetic resonance spectroscopy. Optical rotatory dispersion studies by Emerson *et al.* (1967) favored the *anti* conformation of natural pseudouridine, but the authors could not reach a conclusive answer. The only "evidence" put forward by Chambers (1966) for the proposed bond, the difference in uv spectra between 5′-ψMP and pseudouridine, is presently shown as not demonstrating this bond. The statement (Chambers, 1966), that only pseudouridine and derivatives that can form the 4–5′ hydrogen bond give the characteristic spectrum at pH 12, is not correct. A 5′-acetyl derivative gives the same type of spectrum at pH 12 as pseudouridine.

It may be pertinent to discuss in short the so-called α-pseudouridine and the A isomers, nonnatural products obtained from pseudouridine by strong acid or alkali treatment. Biologically they are not as much important, but their

structure is often taken as an argument for the conformation of pseudouridine. Much has been published about their chemistry (*e.g.*, Chambers and Kurkov, 1964), but the compounds have been obtained only as spots on chromatograms. To the best of our knowledge, none was crystallized, their melting points and elemental composition are unknown. "Structures" of such compounds cannot be taken as a proof of the structure of pseudouridine, or any other compound for that matter. As our present experience shows, a chromatographically pure compound may not be sufficiently pure to give a satisfactory spectrum.

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