however, attempts to dissociate these two labeling processes have not yet been conclusive.

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# Cyclo-pseudouridine and the Configuration of Pseudouridine

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Pseudouridine (5-ribosyluracil) C, the naturally occurring isomer, isolated from human urine, has been converted to the O-4:C-5′ anhydro (cyclo) pseudouridine by treating 2′,3′-*O*-isopropylidene-5′-*O*-*p*-toluenesulfonylpseudouridine with sodium *tert*-butoxide. The ether linkage of the product, O-4:C-5′-cyclo-2′,3′-*O*-isopropylidene pseudouridine, is readily hydrolyzed by dilute acid and by alkali to pseudouridine C, which, by virtue of its ability to form the anhydro compound, must have a $\beta$ configuration and be 5-$\beta$-D-ribosyluracil. From this it follows that pseudouridine B is the $\alpha$ anomer.

Pseudouridine, a component of many ribonucleic acids from a variety of sources (Dunn, 1959; Dunn *et al.*, 1960), differs from the other known naturally occurring nucleosides in that the ribosyl linkage is a carbon-carbon rather than a carbon-nitrogen bond (Cohn, 1959). The structure has been established as 5-D-ribosyluracil (Cohn, 1960) and this nucleoside has been synthesized (Shapiro and Chambers, 1961), but the configuration of the glycosyl linkage has not hitherto been demonstrated. Cyclonucleoside formation has been used to establish the $\beta$ configuration of other natural nucleosides such as the cytidines (Clark *et al.*, 1951; Andersen *et al.*, 1954), thymidine (Michelson and Todd, 1955), and uridine (Brown *et al.*, 1957). The same general method is applicable to pseudouridine, and we report here the accomplishment of such a cyclization and the properties of the product.

Pseudouridine C, the naturally occurring isomer (I in Fig. 1), isolated from human urine (Adler and Gutman, 1959; Adams *et al.*, 1960) by an improved method, was converted into the 2′,3′-*O*-isopropylidene derivative (II) by the method of Yung and Fox (1961). Treatment with *p*-toluenesulfonyl (tosyl) chloride gave the covalent 5′-tosyl derivative (III) which, upon treatment with sodium *tert*-butoxide (Letters and Michelson, 1961), yielded 2′,3′-*O*-isopropylidene O-4:C-5′-cyclopseudouridine (IV). This derivative possesses some of the properties (*e.g.*, acid lability) expected of such a compound (chromatographic and spectral data are given in Tables I and II). The O-4:C-5′ bond is readily hydrolyzed by mild alkali to give 2′,3′-*O*-isopropylidene pseudouridine and by dilute acid to give pseudouridine C.

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## Experimental and Results

*2',3'-O-Isopropylidene Pseudouridine* (Levene and Tipson, 1934).—To a solution of pseudouridine (I in Fig. 1), containing 0.5 g of approximately 75% pure material, the impurities being of a salt nature, in 2 cc of water and 4 cc of methanol was added 0.05 cc of concentrated hydrochloric acid followed by 20 cc of acetone. The solution was left at room temperature for several hours. (Additional methanol and water were added if the material precipitated from solution.) Another 30 cc of acetone was then added and the solution was again left at room temperature for 2 days. Anhydrous sodium bicarbonate was added and the mixture was shaken vigorously until neutral, then filtered, and the filtrate evaporated to dryness to give 464 mg of crude isopropylidene pseudouridine (II in Fig. 1). The material was recrystallized from methanol–acetone–petroleum ether to give needles (240 mg), m.p. 233–234° (uncorr.). A further 190 mg was recovered from the mother liquors. (Found, in material dried at 120° at $10^{-3}$ mm for 24 hours, N, 10.2; $C_{12}H_{16}N_2O_6$ requires N, 9.9%.)

The isopropylidene derivative has spectral forms (Fig. 2, *left*) resembling closely those of pseudouridine C (Cohn, 1960) but differing in two respects, *viz.*, a slight enhancement in the $\epsilon_{max}$ of both the neutral and singly ionized forms, and a higher $\epsilon_{max}$ in the alkaline form than in the neutral form. This is shown most clearly by

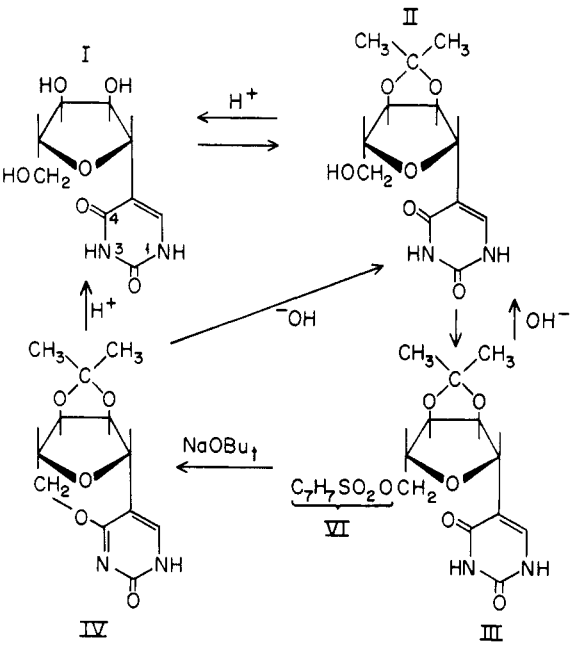


FIG. 1.—Route of synthesis and degradation of 2',3'-*O*-isopropylidene pseudouridine, its 5'-toluenesulfonyl derivative, and its O-4:C-5' cyclo derivative from pseudouridine (5-ribosyluracil). (The numbering system in the pyrimidine ring is that of The Ring Index, Second Edition, 1957, and the I.U.P.A.C. Commission on Nomenclature.) NaOBu$_t$ = sodium *tert*-butoxide.

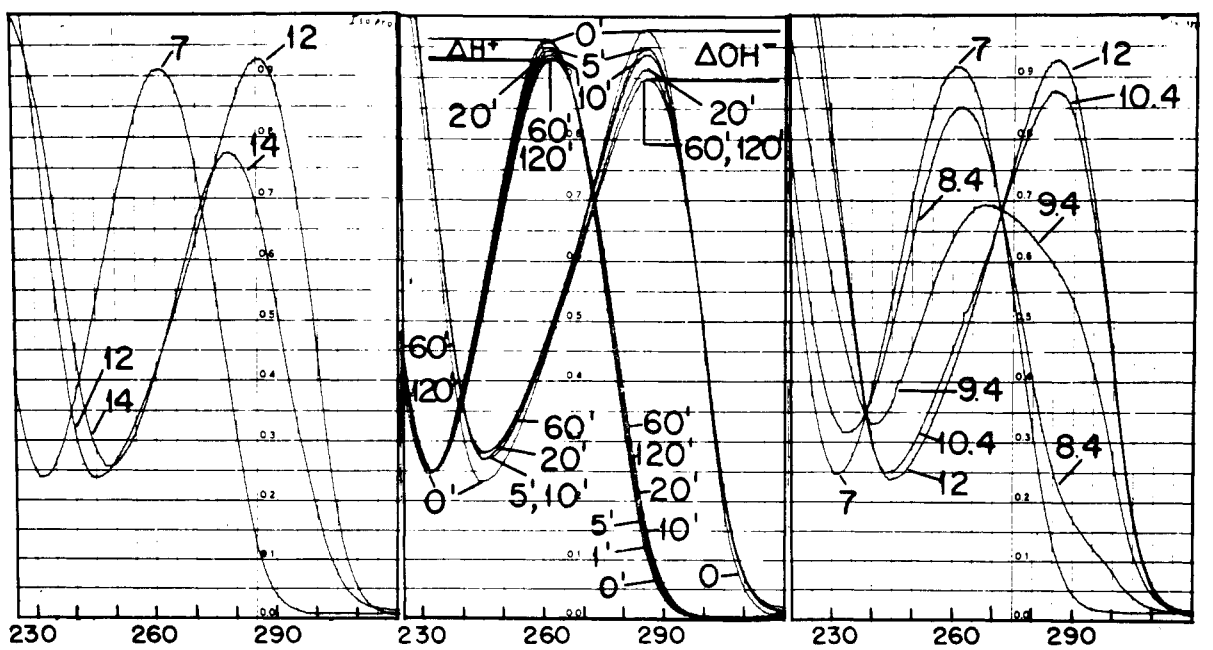


FIG. 2.—Ultraviolet absorption spectra of isopropylidene pseudouridine, automatically recorded. *Left*, spectra at *p*H 7, 12, and 14 (1 N NaOH, involving a 9% dilution), taken on a single sample. *Center*, spectra at *p*H 7 and at *p*H 12 taken at various times during hydrolysis in 0.1 N HCl at 23°. The hydrolysis product is pseudouridine C. *Right*, Spectra at various *p*H values, taken on a single sample. The values indicate a calculated *pK* of 9.3 for the single ionization between *p*H 7 and 12.

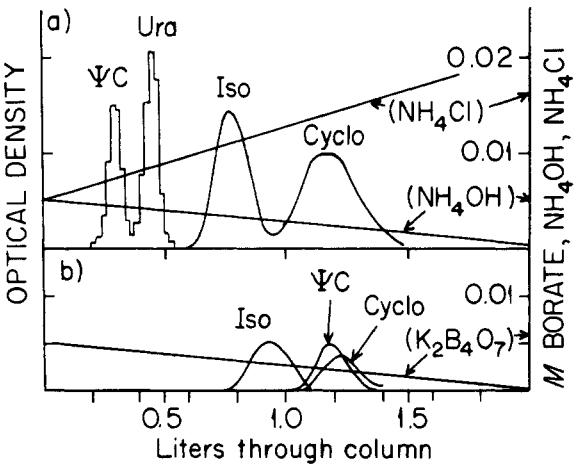


FIG. 3.—Column chromatography of isopropylidene cyclo-pseudouridine and isopropylidene pseudouridine in borate and non-borate gradients. 10 cm × 1 cm² Dowex-1-Cl, 200-400 mesh, 8% divinylbenzene content. Linear gradients of $NH_4OH$ and $NH_4Cl$ (*a*) and $NH_4OH$, $NH_4Cl$, and $K_2B_4O_7$ (*b*). The symbols stand for pseudouridine C (ΨC), uracil (Ura), 2′,3′-*O*-isopropylidene pseudouridine (Iso), O-4:C-5 -cyclo-2′,3′-*O*-isopropylidene pseudouridine (Cyclo).

observing the spectral changes during the hydrolysis (to pseudouridine C) that occurs in dilute acid at room temperature (Fig. 2, *center*). The *pK*, spectrophotometrically determined, is slightly to the acid side of that of pseudouridine C, about 9.3 (Fig. 2, *right*).

Chromatographic properties and spectral constants are given in Tables I and II and Figure 3.

Heating the isopropylidene derivative for 1 hour at 100° in 1 N NaOH gives a mixture of two substances, separable by ion-exchange chromatography (peaks are at 0.36 and 0.84 liters in the system of Figure 3*b*) but both running in butanol-$H_2O$ as elongated spots at the position of the original material (IIA in Table I). The first of these has spectra intermediate between those of pseudouridine C and B (Cohn, 1960), with the ratio $\epsilon_{max}^{pH\ 12}/\epsilon_{max}^{pH\ 7}$ = 0.88. The spectra of the second resemble those of the original material (Fig. 2, *left*, and Table II) but with an augmentation of the same ratio from 1.06 to 1.11 (like pseudouridine $A_s$ in Figure 7 of Cohn, 1960).

*5′-p-Toluenesulfonyl-2′,3′-O-isopropylidene Pseudouridine.*—Toluenesulfonyl chloride (191 mg, 1 mmole) was added to a solution of anhydrous 2′,3′-isopropylidene pseudouridine (142 mg, 0.5

TABLE I

CHROMATOGRAPHIC PROPERTIES OF PSEUDOURIDINE, CYCLO-PSEUDOURIDINE, AND INTERMEDIATES

| Substance | $R_F$ in Solvent A[c] | $R_F$ in Solvent B[d] | $R_F$ in Solvent C[e] | Electrophoresis[a] (cm) | Liters to Peak[b] + Borate | Liters to Peak[b] No Borate |
|---|---|---|---|---|---|---|
| (I) Pseudouridine C (Ψ) | 0.07 | 0.42 | 0.39 | 7.0 | 1.18 | 0.30 |
| (II) 2′,3′-*O*-isopropylidene Ψ | 0.48 | 0.62 | 0.62 | 2.8 | 0.92 | 0.76 |
| (III) 5′-*p*-toluenesulfonyl-II | 0.76 | 0.68 | 0.75 | — | | |
| (IV) O-4:C-5′-cyclo-II | 0.40 | 0.59 | 0.64 | 5.3 | 1.24 | 1.16 |
| (V) III, after alkali[f] (*cf.* II) | 0.48 | 0.62 | 0.62 | 2.8 | | |
| (VI) Toluenesulfonic acid | 0.31 | 0.63 | 0.68 | 10.7 | | |
| (VII) IV + alkali[g] (*cf.* II, V) | 0.48 | 0.62 | 0.62 | 2.8 | | |

[a] On Whatman 1 paper, in 0.02 M sodium borate at 1000 v (30 v/cm) for 105 minutes. [b] In system described in Figure 3. [c] Ascending on Whatman 1 in 1-butanol–water (84:16). [d] Ascending on Whatman 1 in ethanol–M ammonium acetate (5:2). [e] Ascending on Whatman 1 in *tert*-pentyl alcohol–formic acid–water (3:2:1). [f] Conditions noted in text (2 mg in 0.2 cc dioxane, 0.3 cc 0.1 N NaOH, 100°, 1 hour). [g] 0.1 N NaOH, 100°, 1 hour.

TABLE II

SPECTROPHOTOMETRIC CONSTANTS OF CYCLO-PSEUDOURIDINE AND RELATED SUBSTANCES

| Property | Pseudouridine C (I) | | Isopropylidene Pseudouridine C (II) | | Cyclo-isopropylidene Pseudouridine C (IV) | | Acid hydrolysates[a] of (II) and (IV) | |
|---|---|---|---|---|---|---|---|---|
| | *p*H 7 | *p*H 12 | *p*H 7 | *p*H 12 | *p*H 7 | *p*H 12 | *p*H 7 | *p*H 12 |
| $\lambda_{max}$ | 262, 207 | 286, 217[b] | 261, 207 | 286, 220[b] | 288, 204 | 288, 227 | 262 | 286 |
| $\lambda_{min}$ | 232 | 246 | 231 | 247 | 247 | 250 | 233 | 246 |
| $\epsilon_{max}$[c] | 8.0, 10.8 | 7.8, 10.6 | 8.3, 10.8 | 8.7, ~11 | 3.9, 22 | 5.0, 15 | 8.0 | 7.8 |
| $\epsilon$ 280/$\epsilon$ 260 | 0.40 | 2.0 | 0.34 | 2.1 | 2.9 | 3.1 | 0.40 | 2.0 |
| $\epsilon$ 290/$\epsilon$ 260 | 0.06 | 2.1 | 0.05 | 2.2 | 3.2 | 3.5 | 0.06 | 2.1 |
| $\epsilon_{max}^{pH12}/\epsilon_{max}^{pH7}$ | 1.00[d] | | 1.05[d] | | 1.27[d] | | 0.97[d] | |
| $pK_1$ | 9.6 | | 9.3 | | 8.4 | | | |
| $pK_2$ | >13 | | *ca* 13.7 | | — | | | |

[a] 0.01 N HCl, 23°, 18 hours. [b] Shoulder only. [c] On the basis of $\epsilon_{max}$ = 8.0 (8,000) for pseudouridine C, *p*H 7 (Shapiro and Chambers, 1961). [d] $\epsilon_{max}$ at longer wave lengths only.

mmole) in dry pyridine (4 cc) at 0° and the mixture left at 0° overnight under anhydrous conditions. Water (2 cc) was added and the mixture was left at room temperature for 1 hour when the solvent was removed under reduced pressure. The residue was dissolved in chloroform and extracted twice with water, the aqueous extracts being discarded. The chloroform solution was dried over anhydrous sodium sulfate, then evaporated to dryness under reduced pressure to give a residue that was dissolved in acetone (2 cc). Petroleum ether was added slowly to turbidity, and after 24 hours the crystalline (needles) 5′-*p*-toluenesulfonyl-2′,3′-*O*-isopropylidene pseudouridine (III in Fig. 1) was collected (180 mg), m.p. 172–174° (decomp.). (Found, in material dried at 110° at $10^{-3}$ mm for 24 hours, N, 6.3; $C_{19}H_{22}N_2O_8S$ requires N, 6.4%.)

Whereas triethylamine was without effect on a solution of the 5′-toluenesulfonyl derivative in anhydrous dioxan, the compound was readily hydrolyzed by dilute aqueous sodium hydroxide. A solution of 5′-*p*-tolylsulfonyl-2′,3′-*O*-isopropylidene pseudouridine (III) (approximately 2 mg) in dioxan (0.2 cc) and aqueous sodium hydroxide (0.3 cc of 0.1 N) was kept at 100° for 1 hour. Paper chromatography and paper electrophoresis showed complete conversion into 2′,3′-*O*-isopropylidene pseudouridine (II) and sodium *p*-toluenesulfonate.

*2′,3′-O-Isopropylidene-O-4:C-5′-cyclopseudouridine.*—Sodium *tert*-butoxide (0.4 cc of 0.5 N solution in *tert*-butanol) was added to a solution of 5′-*p*-toluenesulfonyl-2′,3′-isopropylidene pseudouridine (80 mg, 0.18 mmole) in anhydrous *N,N*-dimethylformamide (2 cc), and the solution was kept in a flask with a silica gel drying tube at 100° for 30 minutes. Paper chromatography and paper electrophoresis showed quantitative conversion into the cyclonucleoside (IV in Fig. 1) and *p*-toluenesulfonic acid. Solvent was removed under reduced pressure and acetone added to the residue; sodium toluenesulfonate was removed by filtration and the filtrate evaporated to small volume. Addition of ether then gave crude 2′,3′-*O*-isopropylidene cyclo-pseudouridine (40 mg), which was recrystallized from methanol–acetone–petroleum ether to give rosettes of needles (30 mg), m.p. softens at 255–260°. A second recrystallization from water gave long needles. (Found, in material dried at 125° at $10^{-3}$ mm for 24 hours, N, 10.2; $C_{12}H_{14}N_2O_5$ requires N, 10.5%.)

The spectra of the substance are shown in Figure 4, *left*. This spectrum is characteristic of pseudouridine (or of uracil or other pyrimidines with unsubstituted N-1) in alkali and can be

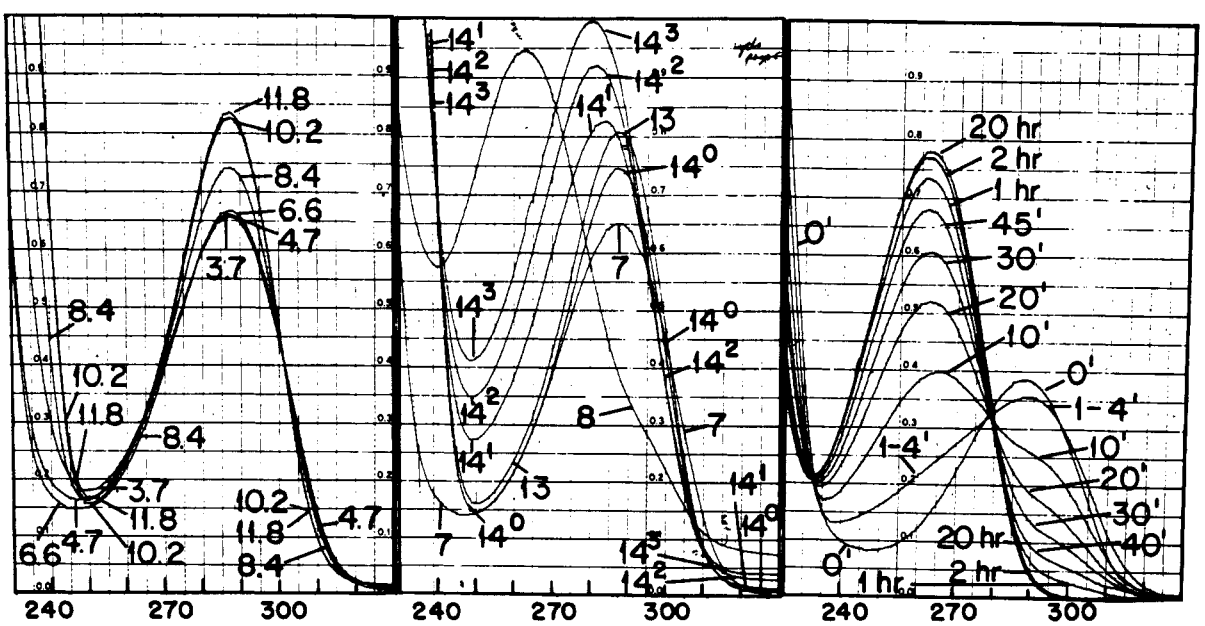


FIG. 4.—Ultraviolet absorption spectra of cyclo-pseudouridine (isopropylidene derivative), automatically recorded. *Left*, spectra at various *p*H values between 3.7 and 11.8 (the spectrum at *p*H 14 is identical with that at 11.8). The absorption at *p*H 3.7 in the region of 230-250 is augmented because of the presence of acetic acid. *Center*, spectra taken during treatment with hot alkali. A single sample was treated as follows: measured at *p*H 7; brought to *p*H 13 (1% dilution) (curve 13); brought to 1 N NaOH (9% dilution) (curve $14^0$); heated 1 hour at 100° (curve $14^1$); heated a second hour at 100° (curve $14^2$); heated a third hour (curve $14^3$); neutralized to *p*H 8 (curve 8). The shift of the maximum after each period of heating and the total shift between the initial curve (*p*H 7) and the final one (*p*H 8) indicate hydrolysis to pseudouridine (isopropylidene derivative). *Right*, spectra taken at various times during hydrolysis in 0.05 N HCl at room temperature (23°). The shift of the maximum and the enhancement of the absorption at the maximum indicate the formation of pseudouridine from the cyclo compound. Note the isosbestic points at 232 and at 278–280.

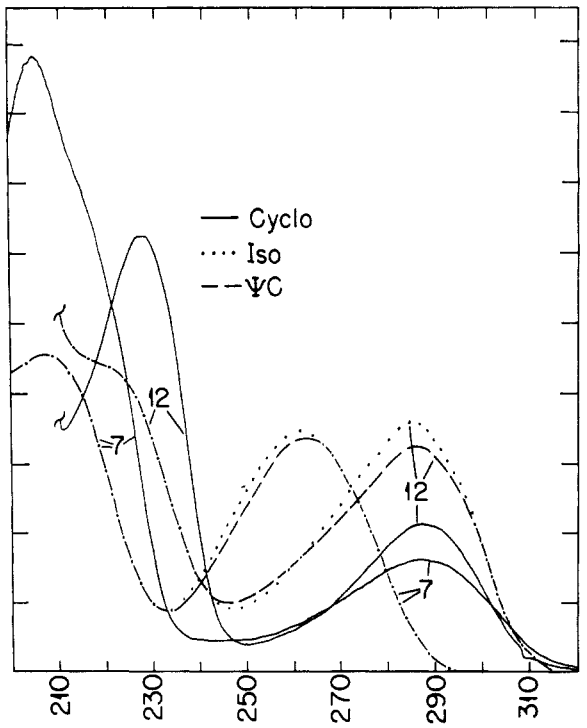


FIG. 5.—Ultraviolet absorption spectra of pseudouridine (ΨC), isopropylidene pseudouridine (Iso), and isopropylidene cyclo-pseudouridine (cyclo), to indicate the unusual peaks found in the cyclo compound at the shorter wave lengths. The molar concentrations of all three substances are identical.

regarded as resulting from the substitution on the O-4 to give a structure similar to 4-ethoxyuracil (Shugar and Fox, 1952). The fact that the *p*H 7 maximum is at 288 mμ, whereas that of 4-ethoxyuracil is at 269 mμ, is qualitatively consistent with the bathochromic effect of the substitution on the 5 carbon. The dissociation in the cyclo compound, with a *pK* of about 8.4 (Fig. 4, *left*), gives a change in spectrum evidenced chiefly by a 27% rise in the $\epsilon_{max}$.

Hydrolysis of the cyclonucleoside (IV) with 0.1 N sodium hydroxide at 100° for 1 hour gives the nucleoside (II) as a result of cleavage of the O-4:C 5′ ether linkage. Standing in 1 N NaOH at room temperature for 2 hours gave no change in spectrum, the maximum remaining at 288 mμ. (Pseudouridine C and its isopropylidene derivative have maxima, in 1 N NaOH, at 278–9 mμ; see Fig. 2, *left*). Heating in 1 N NaOH produces a spectral shift (Fig. 4, *center*) toward the spectra of noncyclic forms at a rate with a $t_{1/2}$ of about 1.1 hour. The principal product of this hydrolysis chromatographs on paper and on anion-exchange columns with the isopropylidene derivative (Table I and Fig. 3) and like it is hydrolyzed by dilute acid to pseudouridine C.

The cyclonucleoside (IV) is rapidly attacked in dilute hydrochloric acid at room temperature, as indicated by the shift in the absorption spectrum to that of psuedouridine C, the maximum moving from 288 mμ to 262 mμ accompanied by a twofold increase in the $\epsilon_{max}$ value (Fig. 4, *right*). Two isosbestic points are observed, consistent with two spectral forms. (However, the difference between the spectra of pseudouridine C and the acetone adduct in neutral or acid solution is insufficient to differentiate these two forms.) The changes observed at 250 mμ and 290 mμ are proportional to each other and logarithmically linear with time, indicating first-order kinetics. The half-times observed at 0.01, 0.05, and 0.2 N HCl (room temperature) were 60, 17½, and 5 minutes, respectively. The final product of acid hydrolysis (0.01 N HCl for 18 hours, room temperature) chromatographs as pseudouridine C (Fig. 3) and has the spectrum of this compound, showing a loss of the acetone group as well as cleavage of the ether link.

The spectra of the cyclo compound in the short ultraviolet (given in Fig. 5) both resemble and differ from those of pseudouridine C and its isopropylidene derivative. Although the maximum of the neutral cyclo form lies at the same wave length (204 mμ), it is nearly as much augmented ($\epsilon_{max}$ = 15) over the corresponding maximum of pseudouridine C as the peak at 288 is depressed ($\epsilon_{max}$ = 4) below it. At *p*H 12 (singly ionized), the secondary maximum shifts to 227 mμ, in contrast to the shoulder at 220 mμ of pseudouridine C and the isopropylidene derivative.

Treatment of the isopropylidene cyclo-pseudouridine with ammonia yielded isopropylidene "pseudocytidine" (5-ribosylcytosine) with $\lambda_{max}$ = 286 mμ, $\lambda_{min}$ = 242 mμ in 0.01 N HCl, 287 mμ and 251 mμ in 0.01 N NaOH. Deamination of this compound with sodium nitrite and acetic acid gave material with the UV absorption characteristics of isopropylidene pseudouridine.

Chromatographic and spectral properties are given in Tables I and II and Figures 3, 4, and 5.

## DISCUSSION

A study of molecular models shows that cyclization from C-5′ to O-4 of the pyrimidine ring is possible only when the D-ribosyl linkage is β, stereochemical considerations precluding ring formation in the α compound. It may be noted that O-4 in pseudouridine is analogous to O-2 of the other pyrimidine nucleosides, which forms cyclonucleosides with the 2′, 3′, and 5′ carbons of the β ribosyl moiety of all of the latter. Of interest also is the fact that, unlike tosylates of simple primary alcohols in general, 5′-toluenesulfonyl-2′,3′-isopropylidene pseudouridine is readily hydrolyzed to toluenesulfonic acid and isopropylidene pseudouridine by mild alkali as a result of the "neighboring group effect" of the C-4 carbonyl group in the pyrimidine ring. Again this indicates a β-D-ribosyl linkage.

It is clear from these results that pseudouridine C is 5-β-D-ribofuranosyluracil, and hence that

pseudouridine B (Cohn, 1960; Shapiro and Chambers, 1961) is probably the $\alpha$ anomer.

The spectrophotometric behavior of the cyclo-pseudouridine is not what would be predicted from the published observations on related substances. The closest analogue to O-4:C-5′-cyclo-pseudouridine would be 4-ethoxy-5-hydroxymethyluracil, not hitherto reported. However, the spectra and *pK* values of 5-hydroxymethyluracil differ only slightly from those of uracil ($\lambda_{max}$ values are 3 m$\mu$ greater at *p*H 7, 12, and 14 and $\epsilon_{max}^{pH12}/\epsilon_{max}^{pH7}$ is 0.89 vs. 0.74; Cohn, 1960) so that the spectra and ionization constants of 4 - ethoxyuracil (4 - ethoxy - 2(IH) - pyrimidinone) might be expected to approximate those of our cyclo-pseudouridine. 4-Ethoxyuracil is reported (Shugar and Fox, 1952) to have $\lambda_{max}^{neutral}$ = 270 and $\lambda_{max}^{alk}$ = 278 (and 220), with a *pK* of 10.7, whereas the cyclo compound has the values 288, 288 (and 227), and 8.4 respectively. (2-Ethoxyuracil has a *pK* of 8.2, but its maxima are at 259 and 264.) Indeed, only uracil, thymine, and 5-hydroxymethyluracil, in the singly ionized form (*p*H 12), have maxima (283, 291, 286) (Cohn, 1960) approximating those of cyclo-pseudouridine in both its forms, implying a common structure that is not significantly altered by the ionization (presumably giving a 1,2 double bond) of the cyclo compound. This is consistent with the existence of the lactim form at position 2 in neutral solution. Yet this is inconsistent with the established mode of ionization of the uracils (Shugar and Fox, 1952)—forming the 1,2 double bond at the *p*H 8–10 with a large bathochromic shift and the 3,4 double bond at higher *p*H with a reverse shift—forcing us to the conclusion that the nature of the substituting residue has a greater influence on the spectrum than is usually assumed, and also that—as indicated by a comparison of the three ethoxyuracils at alkaline *p*H—there is a considerable difference in ultraviolet absorption between ionization and substitution at the 2 and 4 positions. Additionally, we have to assume that the specific nature of the substituents in cyclo-pseudouridine, besides introducing a bathochromic shift of unexpected magnitude, also increases markedly the acidity of the remaining ionizable proton. (It must be remembered that the spectra under discussion [Fig. 4, *left*, and 5] are for the 2′,3′-*O*-isopropylidene derivative.)

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