On the Ionization Constant of 5-Ribosyluracil*

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ABSTRACT: The ionization constant of pseudouridine C has been redetermined; a value of pK = 8.97 rather than the previously accepted value of 9.6 is reached. The pK of pseudouridine B has also been measured and is 9.19. In addition, a simple chromatographic purification of the isomers of pseudouridine is described.

During the course of our studies on the cyanoethylation of 5-ribosyluracil (pseudouridine) by acrylonitrile (Ofengand, 1965; see also Chambers *et al.*, 1963; Chambers, 1965), it became necessary to redetermine the ionization constant of pseudouridine in order to examine more critically a proposed mechanism for the reaction of acrylonitrile with pseudouridine (Ofengand, 1965). The pK value obtained for pseudouridine C was found to be considerably lower than that previously described in the literature (Cohn, 1960; Michelson and Cohn, 1962), and is reported in the present communication. In addition, the pK value for the α anomer of pseudouridine C (the B isomer) has been determined.

Materials and Methods

Chromatography. Purification of commercial pseudouridine was achieved by thin-layer chromatography on 1-mm thick layers of MN 300F (Brinkman Instruments Co.) cellulose in isobutyl alcohol–H_2O (88:12). Seven–ten μmoles of pseudouridine was applied per 20 × 20 cm plate and gave satisfactory separation of the isomers. After the chromatographic run (about 5 hr), solvents were removed by evaporation and the nucleosides were located by viewing under ultraviolet light. Multiple development (usually 10–12 times) was required in order to achieve a suitable separation of the pseudouridine isomers. The nucleosides were eluted with water at 25 to 37°.

Spectral Measurements. Spectra were determined in a Cary Model 14 spectrophotometer in cuvets thermostated at 30°. The reference cell contained the same solution as the sample cell with the exception of the pseudouridine. pH was measured at 30° with a Radiometer PHM25SE pH meter standardized against pH 7.00 and pH 10.00 Beckman standard buffers at 30°. The pH values reported were determined on the pseudouridine solutions immediately after the spectrum was recorded.

The pH 6–7 curves were obtained by dilution of a sample of pseudouridine in water. NaOH (1 N) was then added to the cuvets to 0.01 N, and the alkaline spectrum recorded on the same sample. The spectra at other pH values were obtained by separate dilution of pseudouridine into buffer at the desired pH.

To normalize all curves for purposes of calculation the absorbance at the isosbestic point was determined from the acid–alkali pair of spectra after correction of the alkaline curve for dilution. This value was then compared with the absorbance of the other curves at this wavelength, and correction factors were applied. Figures 2 and 3 show the raw data, however, and it is clear that the corrections involved are only 1–2%.

Calculation of pK. Ionization constants were calculated from the absorbancies at different pH values according to the relation pK = pH + log [A(pH 12) − A(pH)]/[A(pH) − A(pH 7)] (Albert and Serjeant, 1962). For this formula to apply, it is essential that A(pH 7) be a measure of the pure un-ionized species and A(pH 12) be that of the pure ionized species. Since the first pK occurs around pH 9, essentially only the un-ionized species should exist at a pH of 7 or below. This was confirmed by the identity of the spectra at pH 2 to 7 of both isomers. A potentially more serious problem is that of the second ionization of pseudouridine, reported to occur above pH 13 (Cohn, 1960), which might cause the pH 12 spectrum to be a composite of that due to the singly and doubly ionized nucleosides. If this were so, the spectrum should vary with pH in the alkaline region. When the alkaline spectra of pseudouridines C and B were examined at pH values of 11.0, 12.0, and 12.6, the maximum deviation of one spectrum from another was less than 3% for pseudouridine C and less than 2% for pseudouridine B, indicating that at pH 12 each isomer exists almost entirely in the singly ionized form. However, when the pH was raised still further, the second ionization could be detected by the spectral shift to shorter wavelengths as described by Cohn (1960). The isosbestic point for this ionization occurs at 281 mμ for the C isomer and at 286 mμ for the B isomer.

Chemicals. Pseudouridine, A grade, was obtained from Calbiochem as a mixture of isomers and purified by chromatography (see Results). 1,3-Dimethyluracil was

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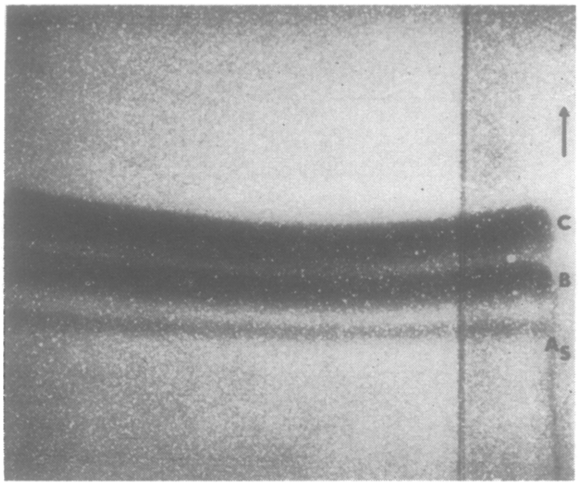

FIGURE 1: Chromatographic separation of isomers of pseudouridine. Pseudouridine (2.5 mg) was applied as a 16-cm band to a 1-mm thin-layer plate of cellulose. Chromatography was in isobutyl alcohol–H_2O (88:12) and was repeated for a total of 12 times. Nucleosides were viewed by ultraviolet light. Final R_F of pseudouridine C = 0.50.

TABLE I: Ionization Constant of Pseudouridine C.[a]

		pK				
Buffer	pH	260 mμ	285 mμ	290 mμ	295 mμ	Average
A	9.68	8.98	9.01	8.99	9.01	
B	9.35	8.89	8.89	8.89	8.90	
A	9.20	8.99	9.00	9.00	9.02	
B	8.87	9.17	8.93	8.94	8.94	
A	8.66	9.00	9.04	9.03	9.03	
B	8.39	8.97	8.89	8.88	8.88	
Average		9.00	8.96	8.96	8.96	8.97

[a] Ionization constants were calculated as pK values according to the procedure described in Methods. Absorbancies at each wavelength and pH were obtained from the spectral curves of Figure 2 and normalized as described in Methods.

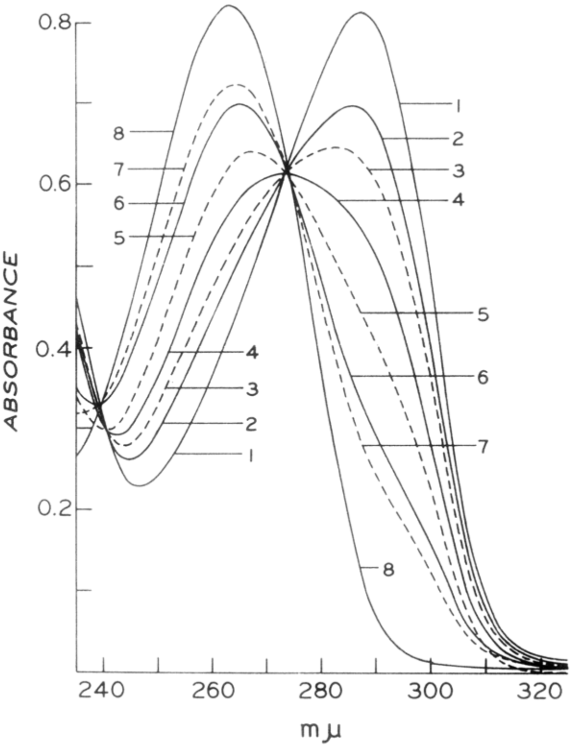

FIGURE 2: Spectrum of pseudouridine C as a function of pH. Spectral and pH measurements were made as described in Methods. Curve 1, 0.01 N NaOH, pH 11.80; curve 2, buffer A, pH 9.68; curve 3, buffer B, pH 9.35; curve 4, buffer A, pH 9.20; curve 5, buffer B, pH 8.87; curve 6, buffer A, pH 8.66; curve 7, buffer B, pH 8.39; curve 8, water, pH 6.52. Dashed line designates buffer B curves.

obtained from Calbiochem, and ribothymidine was purchased from Cyclo Chemical Corp. Buffer A was sodium carbonate buffer (0.05 ionic strength) and buffer B was 2-amino-2-methyl-1,3-propanediol–hydrochloric acid (0.05 ionic strength) prepared according to Long (1961).

Results

Purification of Isomers of Pseudouridine. The four isomers of pseudouridine were first described and designated by Cohn as the C, B, A_S, and A_F isomers (Cohn, 1960). Subsequent structural work has established that the naturally occurring C isomer is 5-β-D-ribofuranosyluracil, the B isomer is 5-α-D-ribofuranosyluracil, the A_S isomer is 5-β-D-ribopyranosyluracil, and the A_F isomer is 5-α-D-ribopyranosyluracil (Michelson and Cohn, 1962; Chambers and Kurkov, 1964).

Since the B, A_S, and A_F isomers are readily produced from the natural C isomer by alkaline or acidic treatment (Cohn, 1960), it was not surprising that the commercial sample of pseudouridine contained more than one isomer. This was first noticed as the frequent appearance of a doublet spot on chromatography in isobutyl alcohol–water.

This observation provided the basis for a very simple chromatographic separation of the isomers of pseudouridine (see Methods). The advantages of this procedure are that it uses neutral, volatile solvents, can be continuously monitored and stopped when the desired separation is achieved, and, while lengthy, requires no trained attention once the plates are prepared. Moreover, the amount of nucleoside that can be separated on a single plate (3 mg) compares favorably with the procedure employed by Cohn, who used a combination

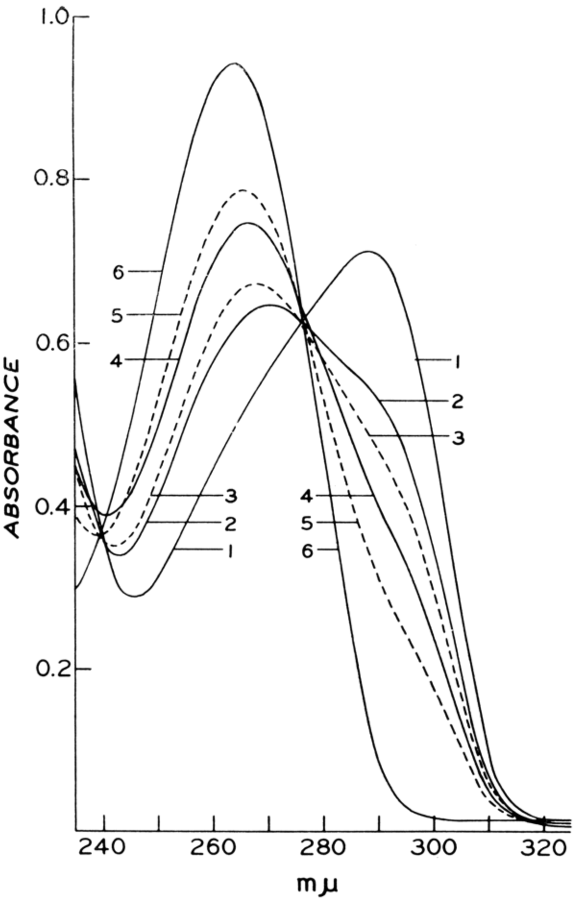

FIGURE 3: Spectrum of pseudouridine B as a function of pH. Spectral and pH measurements were made as described in Methods. Curve 1, 0.01 N NaOH, pH 11.81; curve 2, buffer A, pH 9.65; curve 3, buffer B, pH 9.42; curve 4, buffer A, pH 9.12; curve 5, buffer B, pH 8.92; curve 6, water, pH 5.70. Dashed line designates buffer B curve.

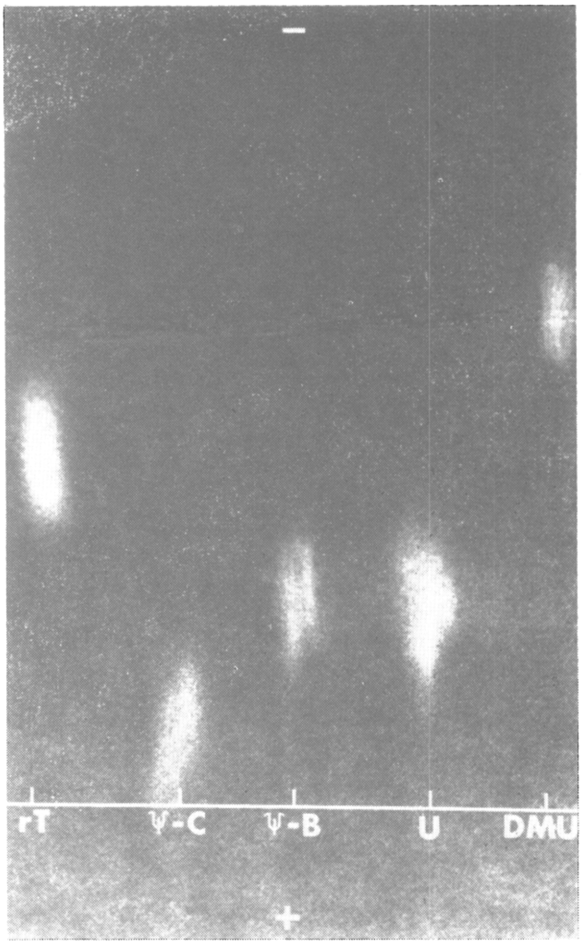

FIGURE 4: Paper electrophoresis of nucleosides. Nucleosides (0.07–0.16 μmole) as indicated were applied to Whatman No. 1 paper. Electrophoresis was in 0.1 M glycine buffer, pH 8.93, for 30 min at 105 v/cm under Varsol. The effect of endosmosis is shown by the migration of 1,3-dimethyluracil (DMU). Ψ-C and Ψ-B indicate pseudouridine C and B, respectively, U is uridine, rT is ribothymidine.

of ion-exchange and paper chromatography to separate the four isomers.

The result of a typical experimental run is shown in Figure 1. After elution of the separated nucleosides they were identified by their characteristic spectra in acid and alkali (Cohn, 1960) (see also Figures 2 and 3). The average of two such analyses showed the commercial sample to contain 66% C isomer, 31% B isomer, and 2.5% A_S isomer. No A_F isomer could be detected, nor did the spectrum of the A_S isomer show any contamination with A_F isomer.

Determination of the Ionization Constants of Pseudouridines C and B. Because pseudouridine shows a pronounced bathochromic shift at alkaline pH due to the ionization of a proton from the N_1 of the uracil ring, spectrophotometric titration is particularly easy. Spectra were recorded on the purified isomers as described in Methods. The results are shown in Figures 2 and 3 and the calculated p*K* values tabulated at several wavelengths in Tables I and II. For each isomer, the p*K* values obtained at different wavelengths on either side of the isosbestic point and in different buffers are in good agreement with each other. Note that buffer A is an anionic buffer, while buffer B is a cationic buffer.

We conclude that the true p*K* value of pseudouridine C, the natural isomer, is 8.97 and that of its α anomer, pseudouridine B, is 9.19.

Confirmatory evidence for these findings was obtained by electrophoresis of the nucleosides together with standards at a pH chosen to display the maximum difference in net charge. The result of such an experiment (Figure 4) shows that at pH 8.9 (a) pseudouridine C has a greater net charge than both pseudouridine B and uridine and thus a lower p*K*, and (b) pseudouridine B has about the same net charge as uridine (p*K* = 9.25; Fox and Shugar, 1952), and thus a very similar p*K* value.

TABLE II: Ionization Constant of Pseudouridine B.[a]

Buffer	pH	p*K* 260 mμ	p*K* 290 mμ	p*K* 295 mμ	Average
A	9.65	9.25	9.24	9.24	
B	9.42	9.18	9.23	9.22	
A	9.12	9.09	9.16	9.16	
B	8.92	9.16	9.18	9.19	
Average		9.17	9.20	9.20	9.19

[a] Ionization constants were calculated as p*K* values as described in Table I, using the spectral curves of Figure 3.

Discussion

The reason for the discrepancy between the above values and the previously determined p*K* of 9.6 are not clear. Nevertheless, the close agreement among the p*K* values determined at different wavelengths and in different buffers, both anionic and cationic, encourage us to believe that these values are correct. One may speculate that the slightly lower p*K* value for the C isomer as compared to the B isomer may be due to the ability of the C isomer to form a hydrogen-bonded structure between the 5′-hydroxyl group and the 4-carbonyl of the uracil ring. Chambers *et al.* (1963) have suggested such a structure to explain the spectral differences at pH 12 between the two isomers since the B isomer is unable to hydrogen bond in this way because of its α-glycosidic linkage. Similarly, one might expect that such internal hydrogen-bond formation would favor the tendency to lose a proton and hence lower the p*K* of the C isomer as compared to the B isomer.

References

Albert, A., and Serjeant, E. P. (1962), Ionization Constants of Acids and Bases, New York, Wiley.

Chambers, R. W. (1965), *Biochemistry 4*, 219.

Chambers, R. W., and Kurkov, V. (1964), *Biochemistry 3*, 326.

Chambers, R. W., Kurkov, V., and Shapiro, R. (1963), *Biochemistry 2*, 1192.

Cohn, W. E. (1960), *J. Biol. Chem. 235*, 1488.

Fox, J. J., and Shugar, D. (1952), *Biochim. Biophys. Acta 9*, 369.

Long, C., ed. (1961), Biochemists Handbook, London, Spon, E., and F., Ltd., pp. 35, 36.

Michelson, A. M., and Cohn, W. E. (1962), *Biochemistry 1*, 490.

Ofengand, J. (1965), *Biochem. Biophys. Res. Commun., 18*, 192.