Spectrophotometric Determination of the pK Values for Dissociation of the Sugar Hydroxyls in Pyrimidine Arabinonucleosides

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Arabinonucleosides, Spectrophotometric Titration, Sugar Hydroxyl Dissociation

Spectrophotometric titration in the ultraviolet has been employed to determine the pK values for dissociation of the sugar hydroxyls in pyrimidine arabinonucleosides and some of their O′-methyl and O′-ethyl derivatives.

The order of dissociation of the sugar hydroxyls in the arabinofuranose ring was 2′-OH > 3′-OH > 5′-OH. The higher acidity (lower pK) of the 2′-OH was interpreted in terms of formation of an intramolecular hydrogen bond of the form 5′-OH···2′-O(−) and the accompanying changes in conformation of the arabinose ring.

The various factors affecting the dissociation of specific hydroxyls in some of the O′-alkyl derivatives are discussed in relation to steric, conformational and other effects.

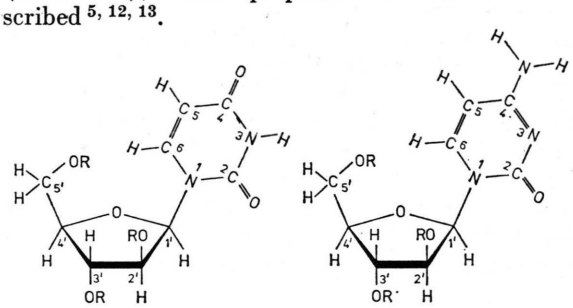
It was shown some years ago that the pyrimidine moiety of pyrimidine nucleosides could be used as a "probe" for following the dissociation of the sugar hydroxyls in strongly alkaline medium by spectrophotometric methods [1]. To date this observation has been profited from principally for identification purposes and, in some instances for qualitatively following the extent of hydroxyl dissociation in various nucleosides [2].

In some instances where the pentose moiety is arabinose or lyxose, so that the 2′-OH is in the "up" position, consequently in the potential vicinity of the pyrimidine ring, the modifications in UV absorption in alkaline medium appeared to us sufficiently large [1, 2] as to permit of quantitative spectral titration and evaluation of pK values. In the present communication we apply this procedure to pyrimidine arabinosyl nucleosides, including also some analogues in which one or more of the hydroxyls are blocked by etherification. Such data are of interest not only in relation to properties of nucleosides such as conformation, affinities to strongly basic ion exchangers [3–5], etc., but, as shown elsewhere [6], are of particular significance in the interpretation of unusual modifications in conformation of the arabinofuranosyl ring accompanying dissociation of the 2′-hydroxyl.

In addition, studies on the properties of the pentose rings of arabinonucleosides are of general interest because of the antimetabolic properties of these compounds, including antiviral and antitumour activities [7–11].

Materials and Methods

AraC, its O′-alkyl derivatives, and 3′-*m*araU (Scheme 1), were prepared as elsewhere described [5, 12, 13].



R = H or CH_3 or C_2H_5

Scheme 1.

Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; araU, 1-β-D-arabinofuranosyluracil; 2′-*m*araC, 2′-O-methyl-araC; 3′-*m*araU, 3′-O-methyl-araU; 2′,3′,5′-$m_3$araC, 2′,3′,5′-tri-O-methyl-araC; 2′,5′-et_2araC, 2′,3′-di-O-ethyl-araC; with similar connotations for other O′-alkyl derivatives; OD, optical density; CPK, Corey-Pauling-Kolhtoun.

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NaOH stock solutions were prepared with fresh, quartz-redistilled water, using analytical grade NaOH pellets which were thoroughly washed with water to remove carbonate. D_2O (99.75 mol % D) was obtained from the Institute of Nuclear Research, Warsaw, and NaOD solutions were prepared with the use of 15 M NaOD (>99.7 mol % D) supplied by Merck (Darmstadt, GFR). Solutions were standardized by titration with HCl, and were all brought to an ionic strength of $\mu = 1$ by addition of 1 M NaCl. All solutions were freshly prepared at least once weekly. A Radiometer PHM4 instrument was used for pH measurements with a G220B glass electrode and a Kl30 calomel electrode.

Spectral measurements utilized a volume of 2 ml of NaOH solution at the desired concentration in a 10-mm spectral cuvette. To this was added 50 μl of a 1 mg/ml solution of the compound under study, while 50 μl water was added to the control cuvette. Spectral measurements made use of a Zeiss (Jena, GDR) VSU-140 spectrophotometer. Temperature was maintained within the range 22 ± 1 °C during all measurements.

Determination of pK_a values was based on the use of about 15 NaOH solutions covering the concentration range 0.005 – 1 M, so that differences in pH between successive solutions was about 0.2. The pH of a given solution was calculated from the equation:

$$pH = 14 - p[aOH^-] = 14 - \lg[OH^-] - \lg f_{OH^-} \quad (1)$$

where f_{OH^-} is the mean value of the activity coefficient of NaOH – NaCl solutions found at 25 °C [14]. For the lower NaOH concentrations, accessible to direct measurement, the pH values calculated from the above equation agreed to within 0.03 units with those measured potentiometrically. Estimated pK values were based on calculated values of pH, since the measured pH values at high NaOH concentrations are susceptible to appreciable error. In the case of D_2O solutions, in addition to a correction for the "sodium error", the pH meter reading was corrected by addition of the factor 0.45 to obtain the pD in this high alkaline range at 22 ± 1 °C, as determined recently by Force and Carr [15].

Spectral titrations

In general, ionization of the sugar hydroxyl(s) of pyrimidine nucleosides leads to a bathochromic shift of the principal long-wavelength absorption band [1]. In the case of arabinonucleosides, as well as lyxofuranosides [2], where the 2′-OH is in the "up" position, or *cis* to the aglycone and the exocyclic 5′-CH_2OH, this shift is more pronounced (2 – 5 nm) and is accompanied by marked increases in extinction (see various figures, below).

In agreement with the foregoing, the absorption spectrum of 2′,3′,5′-$m_3$araC, where all three sugar hydroxyls are blocked, exhibited no detectable changes in the range pH 7 to 1 N NaOH. The same result was previously reported for 1-methylcytosine [1], thus also excluding any interference from possible dissociation of an amino proton at highly alkaline pH. From this it follows that changes in absorption resulting from ionization of a free sugar hydroxyl reflect the titration of such a hydroxyl.

Spectral titrations made use of two wavelengths, that for the long-wavelength isosbestic point (λ_i), and the wavelength at which the maximal change in absorbance resulted from sugar hydroxyl dissociation (λ_m). The latter wavelength was 285 nm for araC derivatives and 275 nm for 3′-maraU. Measurements were conducted with an accuracy of about 1%, the readings for the OD of the isosbestic points varying by about ± 0.01 OD units. Changes in absorbance at λ_m were only moderate (15 – 30% difference between neutral and dissociated forms for the different derivatives), so that this source of error could not be neglected. Hence for any given derivative, the mean value of the OD for λ_i was taken as the arithmetic mean for solutions measured at different pH values, and the recorded values of OD at λ_m were corrected relative to this from the equation

$$OD(\lambda_m) = \frac{\text{Mean OD}(\lambda_i) \times \text{Recorded OD}(\lambda_m)}{\text{Recorded OD}(\lambda_i)} . \quad (2)$$

These corrected values of $OD(\lambda_m)$ were then plotted *vs* pH.

In several instances the pK_a values were obtained directly from the spectral titration curves (*e. g.* Figs 1 a, b). However, for all the derivatives investigated, including particularly those where the end point (or plateau) was at too high a pH or was not clearly defined (*e. g.* Figs 2 a, b, c), the pK_a was calculated from Eqn (3), as follows:

$$pK_a = pH - \lg \frac{D_i - D_0}{D_\infty - D_i} \quad (3)$$

where D_i is the optical density at λ_i for a given pH; D_∞ is the OD at λ_m for the neutral form; and D_0 is the OD value at λ_m for the neutral form. D_0 was obtained from Eqn (4) [16], as follows:

$$D_i = D_0 - 1/K_a(D_i - D_\infty)\, a\, H^+ . \quad (4)$$

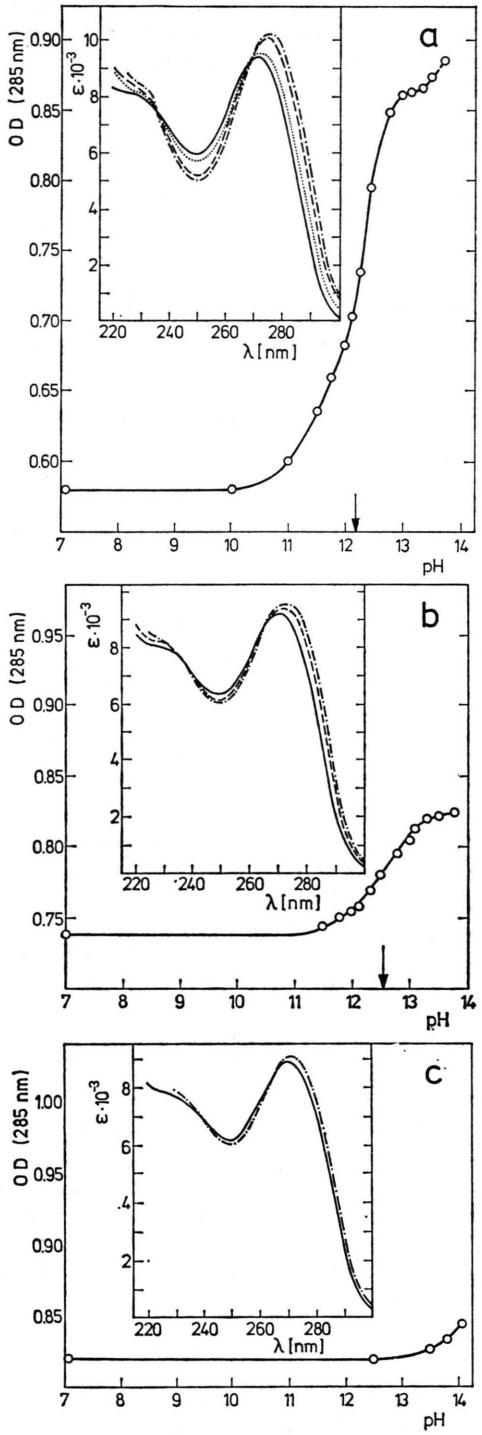


Fig. 1. Spectrophotometric titration curves at 285 nm for: (a) 3′-O-methyl-araC, (b) 2′,5′-di-O-methyl-araC, (c) 2′,3′-di-O-methyl-araC. The inserts show the actual absorption spectra of each derivative at pH 7 (———), in 0.01 N NaOH (·····), in 0.1 N NaOH (— — —), and in 1 N NaOH (— · — · —).

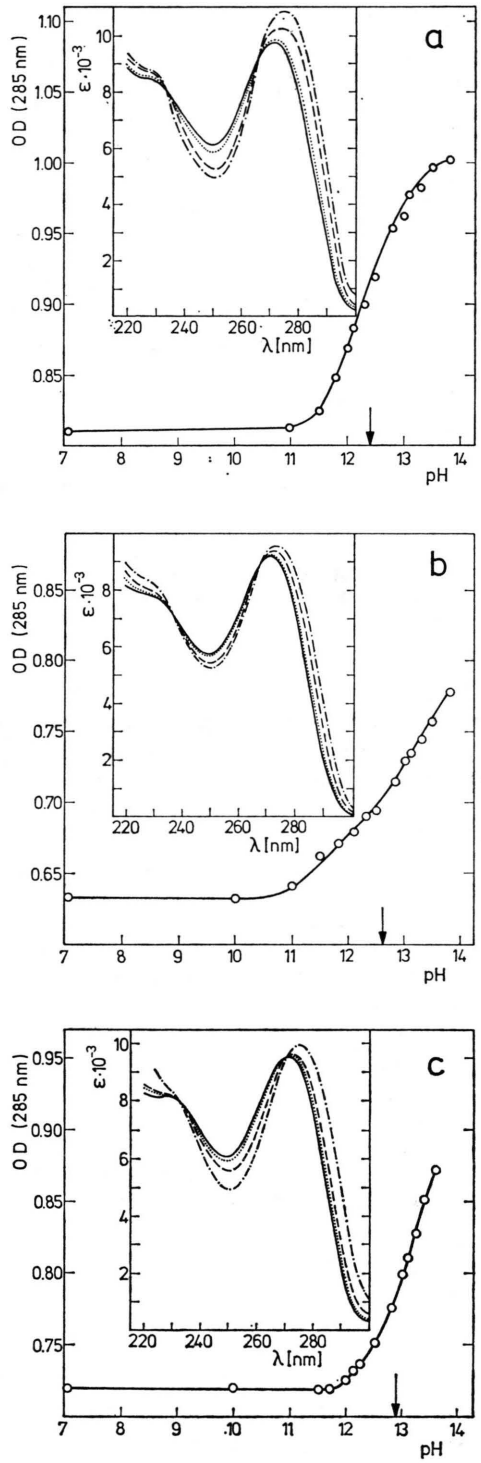


Fig. 2. Spectrophotometric titration curves at 285 nm for: (a) araC, (b) 5′-O-methyl-araC, (c) 3′,5′-di-O-methyl-araC. The inserts show the actual absorption spectra of each derivative at pH 7 (———), in 0.01 N NaOH (·····), in 0.1 N NaOH (— — —), and in 1 N NaOH (— · — · —).

Eqn (4) gives a straight line with a slope $1/K_a$ and intersecting the ordinate axis at the point D_0. Hence a plot of D_i *vs* $(D_i - D_\infty) \cdot a\,H^+$ leads to a graphical determination of D_0 (Fig. 3).

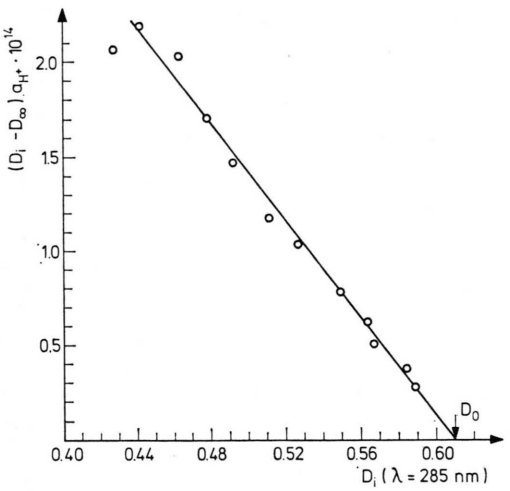


Fig. 3. Graphical determination of D_0 (as described in Materials and Methods) for 3′,5′-di-O-methyl-araC.

Table I and Fig. 3 illustrate the foregoing procedure as applied to determination of the pK_a of the 2′-OH in 3′,5′-$m_2$araC.

Table I. Typical presentation of data employed for calculations of pK_a for hydroxyl ionization from Eqn (3), in this case for 3′,5′-di-O-methyl-araC, at 22 °C; $\mu = 1.0$; D_∞ for phosphate buffer pH 7 = 0.402; D_0 determined graphically = 0.610. Values of D measured at 285 nm.

aH^+ 10^{14}	pH	D_i	$(D_i - D_\infty)$ aH^+ 10^{14}	$pK = pH + \lg\frac{D_i - D_0}{D_\infty - D_i}$	ΔpK
79.4	12.10	0.428	2.06	12.95	+0.06
54.6	12.26	0.442	2.18	12.88	−0.01
32.4	12.49	0.464	2.01	12.86	−0.03
22.4	12.65	0.478	1.70	12.89	0.00
16.2	12.79	0.492	1.46	12.91	+0.02
10.6	12.97	0.512	1.17	12.92	+0.03
8.3	13.08	0.527	1.04	12.90	+0.01
5.3	13.28	0.549	0.78	12.91	+0.02
3.8	13.42	0.564	0.62	12.87	−0.02
3.0	13.52	0.568	0.50	12.92	+0.03
2.1	13.68	0.585	0.38	12.82	−0.07
1.5	13.82	0.589	0.28	12.90	+0.01
				Mean 12.89 ± 0.03 (S.D.)	

The accuracy of the pK_a values determined in this way is estimated at about ± 0.1 unit. Since temperature control was only within the range 22 ± 1 °C, and the temperature coefficient of the pH of NaOH solutions is about 0.03/°C, the source of error from this factor alone is ± 0.06. Possible errors from CO_2 absorption were minimal, the measured pH of solutions stored for several days varying by only about 0.02 units; while absorption spectra were run in teflon-stoppered cuvettes within a few minutes after preparation of the solutions. The advantage of the present method is the minimal quantity of material required, about 1 – 2 mg, but its general application is necessarily limited to those nucleosides where sugar hydroxyl dissociation leads to appreciable changes in absorption, although the use of difference or derivative spectrophotometry could conceivably extend the range of compounds susceptible to such studies.

Results

Figs 1 and 2 exhibit the pH-dependence of OD (λ_m) for some of the compounds. For a derivative such as 2′,3′-$m_2$araC, dissociation of the 5′-hydroxyl occurs at too high a pH (> 13.5) to permit of the determination of pK_a by the present procedure (Fig. 1 c). For derivatives such as 3′-*m*araC, 3′-*et*araC, and 3′-*m*araU the titration curves are relatively symmetrical and reasonably similar to those for normal titration curves, permitting of the direct evaluation of the pK_a values for the 2′-hydroxyls. The dashed lines in the titration curves for these derivatives, at higher pH values, correspond to initiation of dissociation of the 5′-hydroxyls, as illustrated for 3′-*m*araC (Fig. 1 a).

For the other O′-alkyl derivatives of araC, and free araC, only the second procedure described in the preceding section was employed. When the dissociation of two hydroxyls is involved, with overlapping pK values, such as the 2′-OH and 3′-OH in 5′-O-alkyl-araC and araC, the application of Eqn (3) is somewhat limited, and the estimated value of pK_a is a macroscopic value reflecting the unequal effects of ionization of the 2′-OH and 3′-OH on the spectral absorption of the aglycone.

Table II presents the pK_a values for the 12 derivatives embraced in this study.

Discussion

From Table II it will be seen that the pK_a values for the 2′-hydroxyl in the 3′,5′-di-O-alkyl derivatives of araC are about 0.7 – 0.9 units higher than for the 3′-O-alkyl derivatives. The pronounced acidity of the 2′-OH in the latter derivatives (pK_a = 12.2) is clearly linked to the presence in these of a

Table II. pK_a values at 22 °C and $\mu=1.0$ for sugar hydroxyl(s) dissociation in araC, some of its O′-alkyl derivatives, and 3′-*m*araU, calculated as described in text and Table I, or determined directly from titration curves (see Fig. 2 c). The values in parentheses are for measurements in D_2O.

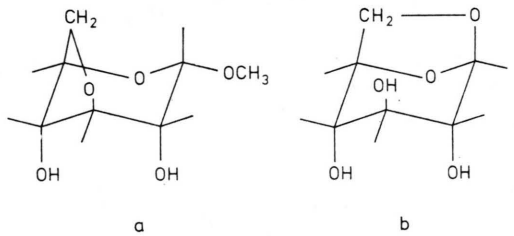
Compound	pK_a from titration curves	pK_a calculated (± 0.10)
araC	—	12.40
2′-*m*araC	12.7	12.65
2′-*et*araC	12.7	12.75
5′-*m*araC	—	12.60
5′-*et*araC	—	12.70
3′-*m*araC	12.2	12.20 (13.00)
3′-*et*araC	12.2	12.25
3′-*m*araU	12.4	12.45 (13.30)
2′,5′-$m_2$araC	12.6	12.50
2′,5′-et_2araC	12.7	12.65
3′,5′-$m_2$araC	—	12.90
3′,5′-et_2araC	—	13.10

free 5′-OH group. Furthermore the macroscopic pK_a for the combined 2′-OH and 3′-OH in araC, and in 5′-O-alkyl derivatives of araC, indicate that the higher pK_a of the latter, about 0.3 units above that for araC, is undoubtedly due to a marked increase in the microscopic pK_a of the 2′-hydroxyl, this being reflected in the resultant measured pK_a.

The foregoing is readily interpreted on the basis of the stabilization of the $2'\text{-}O^{(-)}$ anion through formation of a hydrogen bond with the 5′-OH as donor, *viz.* $5'\text{-}OH\cdots O_{2'}{}^{(-)}$, geometrically feasible only when the conformation of the exocyclic 5′-CH_2OH is *gauche-gauche* and that of the arabinose ring C(2′)*endo*. Formation of such a bond in arabino nucleosides with unsubstituted 2′- and 5′-hydroxyls, in strongly alkaline medium where the 2′-OH is dissociated, has been independently demonstrated by means of proton magnetic resonance spectroscopy, described in detail elsewhere [6]. The reality of such a conformation is further testified to by the fact that, in the crystalline state, the *neutral* forms of both araC and araU are in the conformation C(2′)*endo*, *gauche-gauche*, with an intramolecular hydrogen bond in which the 2′-OH is the donor, *viz.* $2'\text{-}OH\cdots O_{5'}H$ [17–19].

The above findings provide a more reasonable interpretation of the enhanced acidity of the 2′-OH of araC relative to the corresponding ribonucleosides than that based on the *cis* relationship of the "up" 2′-OH to the aglycon [2]. The present results, and even more so those derived from analysis of the PMR spectra in strongly alkaline medium [6] (Remin *et al.*, in preparation), clearly demonstrate that it is the *cis* relationship of the "up" 2′-OH to the exocyclic 5′-CH_2OH which is the decisive factor.

In the case of ribonucleosides, formation of a hydrogen bond between the 2′ and 3′ *cis* hydroxyls, *viz.* $3'\text{-}OH\cdots O(2')^- \rightleftharpoons 2'\text{-}OH\cdots O(3')^-$, has been proposed as the source of the increased acidity of these hydroxyls relative to the corresponding 2′(3′)-O-alkyl ribonucleosides or the analogous deoxyribonucleosides [4, 20–22]. This concept has been extended by Totty [23], who pointed out that one of the main factors responsible for the increased acidity of a number of sugars and glycosides is the stabilization of the anionic forms *via* intramolecular hydrogen bonding with a sugar O^- as the acceptor. A striking example of this is methyl 3,6-anhydro-α-D-glucopyranoside, (Scheme 2 a) the conformation



Scheme 2. a. methyl 3,6-anhydro-α-D-glucopyranoside; b. 1,6-anhydro-β-D-glucopyranose.

of which is relatively rigid, and the geometrical parameters of which are such as to favour formation of a hydrogen bond between the 2- and 4-hydroxyls, as actually observed by X-ray diffraction for this compound in the solid state [24]. In aqueous medium it exhibits a pK of 12.2; whereas 1,6-anhydro-β-D-glucopyranose (Scheme 2 b) with an equally rigid conformation, but the geometry of which excludes the possibility of an intramolecular hydrogen bond, exhibits a pK of 13.5 [23].

On the basis of the foregoing considerations, it now becomes possible to provide a reasonable interpretation for the observation of Christensen *et al.* [25] that the pK for sugar hydroxyl dissociation in 9-β-D-xylofuranosyladenine (12.34) is virtually identical with that for adenosine (12.35) [20]. In the latter the *cis* 2′- and 3′-hydroxyls may hydrogen bond, as shown in the previous paragraph, above. No such hydrogen bonding is feasible in the xylofuranosyladenine, where the 2′- and 3′-hydroxyls are *trans*, and Christensen *et al.* [25] consequently invoked en-

hanced hydration to account for the high acidity in this instance. However, no attention was paid to the fact that in xylofuranosyladenine the "up" 3′-OH is *cis* with respect to the 5′-CH_2OH. And, in fact, an examination of a CPK model of this nucleoside demonstrates in an unequivocal manner that formation of an intramolecular hydrogen bond between these two, 5′-OH···O(3′)$^-$, is perfectly feasible, thus accounting for the high acidity of the sugar hydroxyl dissociation in the xylofuranosyladenine. Since this should also be accompanied by changes in conformation of the xylofuranose ring and the exocyclic 5′-CH_2OH, it is planned to check this by means of PMR spectroscopy.

The pK_a values of the 2′-O-alkyl- and 2′,5′-di-O-alkyl analogues of araC, although differing slightly from each other, point to a pK_a for the 3′-OH of 12.6 – 12.7, *i.e.* about 0.5 units higher than for a 2′-OH in the presence of a free 5′-OH. It is not unreasonable to assume that the relative acidities of these two hydroxyls are similar in the parent araC.

The pK_a values for hydroxyl dissociation in O′-ethyl derivatives of araC are consistently slightly higher (by about 0.1 – 0.2 unit) than for the corresponding O′-methyl analogues. This decreased acidity of O′-ethyl derivatives of araC is reflected in a decreased affinity to Dowex OH^- (E. Darżynkiewicz, unpublished). An analogous decrease in affinity for Dowex OH^- has been noted for O′-ethyl derivatives of cytidine relative to the corresponding O′-methyl derivatives (J. T. Kuśmierek, unpublished). The ethyl group, with its higher inductive effect relative to a methyl, leads to slightly stronger binding of the proton to the hydroxyl oxygen. However, it is not feasible to totally exclude some effect due to decreased solvatation by the ethyl substituent, with its more pronounced steric hindrance to removal of a proton from a hydroxyl group.

The higher pK_a value for 3′-*m*araU (~0.2 units) relative to 3′-*m*araC is probably related to the fact that in the former the N_3 proton of the uracil ring is dissociated (pK ~9), the resultant negative charge being partially localized on the O [2]. A similar effect has been observed in the ribonucleosides, where entropy titration gave a macroscopic pK_a for uridine hydroxyls about 0.3 units higher than that for cytidine [21].

Finally, it should be noted that the pK_D value determined for the 2′-OD in 3′-*m*araC in D_2O medium (Table II) is in good agreement with the value obtained by PMR titration in D_2O (Remin *et al.*, in preparation). The application of PMR spectroscopy to the measurement of sugar hydroxyl macroscopic pK values is now being extended.

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[1] J. J. Fox and D. Shugar, Biochim. Biophys. Acta **9**, 369–384 [1952].
[2] J. J. Fox, J. F. Codington, N. C. Yung, L. Kaplan, and J. O. Lampen, J. Amer. Chem. Soc. **80**, 5155–5160 [1958].
[3] C. A. Dekker, J. Amer. Chem. Soc. **87**, 4027–4029 [1965].
[4] J. B. Gin and C. A. Dekker, Biochemistry **7**, 1413–1420 [1968].
[5] E. Darżynkiewicz and D. Shugar, Acta Biochim. Pol. **21**, 305–322 [1974].
[6] E. Darżynkiewicz, M. Remin, A. Dworak, and D. Shugar, Cancer Biochem. Biophys. **1**, 85–88 [1975].
[7] B. Goz and W. H. Prusoff, Annual Rev. Pharmacol. **10**, 143–170 [1970].
[8] S. S. Cohen, Prog. Nucleic Acid Res. **5**, 1–88 [1966].
[9] P. Roy-Burman, Recent Results Cancer Res. **25**, 1–111 [1970].
[10] R. J. Suhadolnik, Nucleoside Antibiotics, Wiley-Interscience, New York 1970.
[11] D. Shugar, FEBS Letters **40**, (Supplement) S 48–S 62 [1974].
[12] E. Darżynkiewicz, J. T. Kuśmierek, and D. Shugar, Biochem. Biophys. Res. Commun. **46**, 1734–1741 [1972].
[13] J. Giziewicz and D. Shugar, Acta Biochim. Pol. **20**, 73–81 [1973].
[14] H. S. Harned and B. B. Owen, The Physical Chemistry of Electrolyte Solutions, 3rd ed., Reinhold, New York 1958.
[15] R. K. Forcé and J. D. Carr, Anal. Chem. **46**, 2049–2052 [1974].
[16] A. Albert and E. P. Serjeant, Ionization Constants, London and New York 1962.
[17] A. K. Chwang and M. Sundaralingam, Nature, New Biol. **243**, 78–80 [1973].
[18] P. Tollin, H. R. Wilson, and D. W. Young, Acta Crystallogr. **B 29**, 1641–1647 [1973].
[19] P. P. Tougard and O. Lefebvre-Soubeyran, Acta Crystallogr. **B 30**, 86–89 [1974].
[20] R. M. Izatt, L. D. Hansen, J. H. Rytting, and J. J. Christensen, J. Amer. Chem. Soc. **87**, 2760–2761 [1965].
[21] J. J. Christensen, J. H. Rytting, and R. M. Izatt, J. Phys. Chem. **71**, 2700–2705 [1967].
[22] J. T. Kuśmierek, J. Giziewicz, and D. Shugar, Biochemistry **12**, 194–200 [1973].
[23] R. Totty, Ph. D. Thesis, University of Edinburgh 1968.
[24] B. Lindberg, B. Lindberg, and S. Svensson, Acta Chem. Scand. **27**, 373–374 [1973].
[25] J. J. Christensen, J. H. Rytting, and R. M. Izatt, J. Amer. Chem. Soc. **88**, 5105–5106 [1966].