[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Identification of Cytidylic Acids *a* and *b* by Spectrophotometric Methods[1]

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The study of the variations in the ultraviolet absorption spectra of several pyrimidine nucleosides in the high alkaline range has been continued. These spectral variations which occur at *p*H 12 to 14 are due to the ionization of the 2′-hydroxyl group of the sugar, with a limited contribution from the other hydroxyl groups. On this basis it has been possible to confirm the identity of cytidylic acids *a* and *b* as cytidine-2′-phosphate and cytidine-3′-phosphate, respectively. Since uridylic acid *b* may be obtained by alkaline deamination of cytidylic acid *b*, it is also concluded that uridylic acids *a* and *b* are the 2′- and 3′-phosphates of uridine, respectively. A mechanism whereby the ionization of the sugar moiety affects the chromophore of the pyrimidine ring is suggested.

On the basis of previous spectrophotometric studies[3] it was suggested that isomeric nucleotides might be distinguished by their spectral behavior in the high alkaline range. In the present paper we report results which corroborate the conclusion, reached on entirely different experimental grounds, that cytidylic acids *a* and *b* are, respectively, the 2′- and 3′-phosphates.[4]

Studies of nucleic acid derivatives carried out earlier[3] demonstrated that significant spectrophotometric differences exist in the high alkaline range between pyrimidine nucleosides and the 1-methyl analogs of their free bases. Thus, 1-methylcytosine shows a dissociation spectrophotometrically for the 4-ammonium group in the acid ranges above which no spectral variations are observed, *viz.*, the curves for *p*H 7 through 14 are identical. However, in addition to the ammonium dissociation, cytidine exhibits a new equilibrium, beginning in solutions of 0.01 *N* sodium hydroxide which is essentially complete in 1 *N* base. A similar situation holds for uridine *vs.* 1-methyluracil[5] and, indeed, all the pyrimidine nucleosides investigated show this dissociation in highly alkaline solutions.

It was noted further[3] that consistent differences occurred between ribofuranosyl nucleosides of uracil and cytosine on the one hand and their 2′-deoxy analogs on the other. That is, spectral variations in the high alkaline range in the case of uridine occur at a lower *p*H value than with uracil-2′-deoxyriboside. A similar relationship obtains for cytidine and cytosine-2′-deoxyriboside. Examination of the spectra in Fig. 1 shows that while the curves in 0.01 and 0.10 *N* base are practically identical for cytosine-2′-deoxyriboside, an appreciable spread is evident with cytidine which demonstrates that the sugar of the latter compound is more acidic. Figure 1 also contains the spectra of 1-D-glucopyranosyluracil and its 2′-deoxy analog and here, too, a similar pattern is observed which indicates that this phenomenon holds with pyranosylpyridine nucleosides.

Since it is known that carbohydrates exhibit no

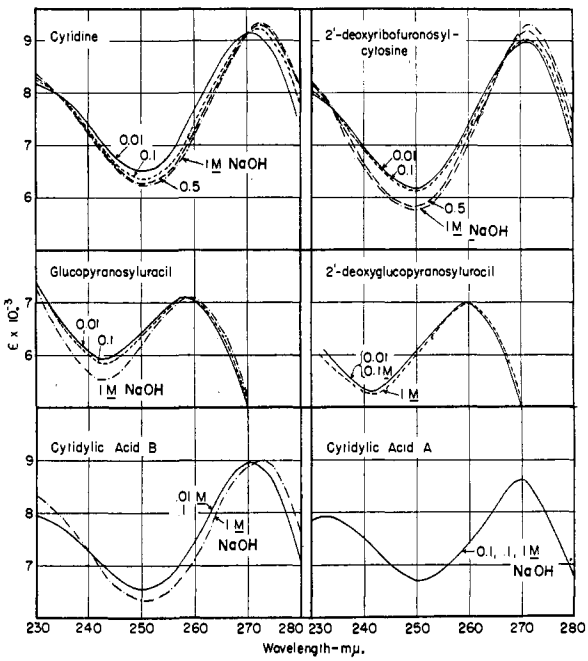

Fig. 1.

specific ultraviolet absorption spectra,[6] the following conclusions can be drawn from these results: (a) In the high alkaline range, where sugars are known to ionize,[7] spectral shifts which have their origin in the pyrimidine ring are a consequence of ionization of the sugar residue, and, (b) in particular, the ionization of the 2′-hydroxyl group shows by far the greatest effect upon the spectra.

We have determined the spectrum of 2′,3′-isopropylidineuridine (Fig. 2) and find a small (but definite) variation in the high alkaline range. (For comparison see the spectra of uridine and uracil-2′-deoxyriboside.[3]) This indicates that the 5′-hydroxyl group plays but a minor role in producing spectral shifts at these *p*H levels. This view is supported by the spectrum of cytosine-2′-deoxyriboside 5′-phosphate (Fig. 2) where the blocking of the 5′-position with phosphate produces no marked changes in the high alkaline range as compared to that of cytosine-2′-deoxyriboside.

It is clear, therefore, that the ionization of the 2′-hydroxyl group may serve as a distinguishing characteristic among nucleoside residues. These conclusions are in conformity with the findings of

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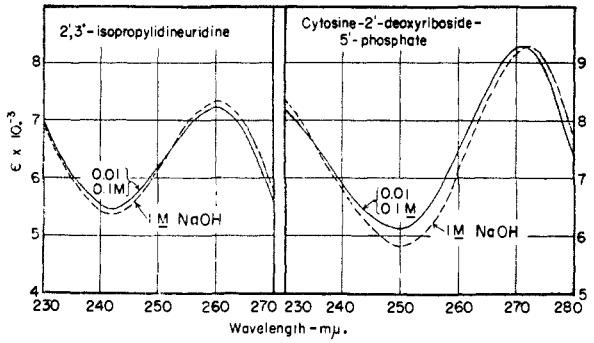

Fig. 2.—The molar extinction values for cytosine-2-deoxyriboside 5'-phosphate are based upon cytosine-2'-deoxyriboside (2'-deoxyribofuranosylcytosine, Fig. 1); ϵ_{250} 6.13×10^3 in 0.010 *M* sodium hydroxide.

Kuhn and Sobotka[7] who showed that the dissociation constants of several O-glucosides differed and indicated that the aglycon influenced primarily the electrochemical character of the 2'-hydroxyl group. It should be expected, therefore, that nucleoside pairs of the same sugar which differ by the presence or absence of a dissociable 2'-hydroxyl group would exhibit differences in the ionization of their sugar components as observed spectrally.

Since the discovery of cytidylic acids *a* and *b*,[8] their identification has been the subject of extensive investigations[9-12] and it is now established that isomerism involves the 2'- and 3'-positions of the sugar.[10,11] From these findings and from the ultraviolet absorption studies cited above, a spectrophotometric basis is provided for the allocation of the position of attachment of the phosphate group to the sugar moiety, since in one of these isomers a free 2'-hydroxyl does not exist. In the high alkaline range the phosphate exists in the completely ionized form ($pK_{a_2} \sim 6$[10,4] for the secondary phosphoryl dissociation) and concurrent dissociation of phosphate and sugar is thereby ruled out. The spectra of these isomers in solutions of 0.01 to 1 *N* base (Fig. 1) show that cytidylic acid *b* exhibits its sugar dissociation at a lower *p*H value, *viz.*, the curves for the *a* isomer are identical in this range. We therefore conclude that the *a* isomer is cytidine-2'-phosphate. These results are in agreement with the findings recently reported[4] on the basis of density and titrimetric measurements. There are, then, three independent lines of evidence all of which lead to the same conclusion.

It has been demonstrated that alkaline deamination of cytidylic acid *b* yields uridylic acid *b*.[13] The identification of the cytidylic acids, therefore, permits the conclusion that the uridylic acids *a* and *b* reported by Cohn[8b] are the 2'- and 3'-phosphates of uridine, respectively. Recently, Khym and coworkers in a preliminary communication[14] have shown that a similar sequential assignment of the phosphate moieties applies to adenylic acids *a* and *b*.[15]

The mechanism whereby the observed spectral shifts arise may involve either one of two effects. First, it is clear that the observed spectral variations in the high alkaline range are associated with the manner in which the ionization of the sugar affects the chromophore in the pyrimidine ring. It would appear the alteration of the electronegativity of the sugar moiety as ionization occurs (thereby changing the inductive effect through the chain) is not a major cause for the spectral shifts observed, since the ionization of a 5'-hydroxyl group (*e.g.*, 2',3'-isopropylidineuridine) results in a small but definite effect, and in the latter the ionizing group is 5 carbon atoms removed from the ring.

The spatial relationship of the sugar hydroxyl groups with respect to the pyrimidine ring is doubtless of greater significance. For example, the fact that the pK_a for the ammonium groups in the 4-positions of cytidine, its 2'-deoxy analog (Table I), and the cytosine pyranosides[3] are different indicates that the *un-ionized* hydroxyl groups interact in some manner with the pyrimidine ring.

TABLE I

SPECTROPHOTOMETRICALLY-DETERMINED "APPARENT" DISSOCIATION CONSTANTS FOR THE 4-AMMONIUM GROUP OF CYTOSINE NUCLEOSIDES AND NUCLEOTIDES

Compound	pKa[a]
Cytidine	4.11
Cytosine-2'-deoxyriboside	4.25
Cytidylic acid *b*[b]	4.16
Cytidylic acid *a*[b]	4.30
Cytosine-2'-deoxyriboside 5'-phosphate	4.44

[a] All values at constant ionic strength (μ 0.05); margin of error 0.03 *p*H unit. [b] The spectra from which these values were calculated[3,5] are given in Fig. 3.

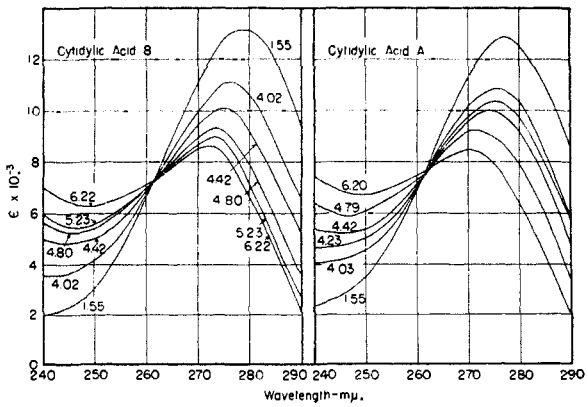

Fig. 3.—Cytidylic acids *b* and *a* in aqueous solutions at *p*H values indicated. The pK_a (4-ammonium group) values for these compounds listed in Table I are determined from these spectra.

A reasonable mechanism for the spectral differences observed in the high alkaline range is therefore the following: The Furberg structure of cyti-

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(14) J. X. Khym, D. G. Doherty, E. Volkin and W. E. Cohn, THIS JOURNAL, **75**, 1262 (1953).

(15) C. E. Carter and W. E. Cohn, *Federation Proc.*, **8**, 190 (1949).

dine based upon X-ray crystallographic analysis[16] would permit hydrogen bonding of the 2′-hydroxyl to the 2-keto group of the pyrimidine ring. Dissociation of the ribofuranosyl residue in the high alkaline range would rupture this hydrogen bridge and give rise to electronic shifts in the pyrimidine component resulting in spectral variations as the alkalinity of the medium is increased. With cytosine-2′-deoxyriboside and cytidylic acid *a* the 2′-position is blocked and hydrogen bonding of the type described above cannot exist.

Though other hydroxyl groups of cytidine may be involved in hydrogen bonding (*viz.*, the 5′—the 3′ is unfavorably located) the 2′-hydroxyl group is the more acidic[7] and rupture of its hydrogen bond in this high alkaline range will be manifest spectrophotometrically at a lower *p*H value than the 2′-deoxy analog.[17]

Evidence for this proposed mechanism exists. We have applied this spectrophotometric procedure to adenylic acids *a* and *b* (which lack a keto group in the purine residue and in which hydrogen bonding of the 2′-hydroxyl group to the 6-amino is structurally impossible) and find no spectral variations whatever for *either* isomer in the high alkaline range, notwithstanding the fact that it was established by Levene and co-workers[18] by electrometric titration that adenylic acid possesses a dissociation for the sugar in this range.

Experimental

Methods.—All measurements were made with a Beckman spectrophotometer, model DU, using 10-mm. quartz cells. Cell corrections were established at frequent wave length intervals with distilled water. Solutions were introduced into buffer solutions with a Scholander precision microburet.[19] Concentrations were generally adjusted so that the important optical density readings were taken in the range of 0.2 to 0.5 at wave length intervals of 2.5 mμ (smaller intervals were used in the vicinity of maxima, minima and isosbestic points). Slit widths were constant for the particular wave length used. Results are expressed as molar extinction coefficients, $\epsilon = d/c$, where d is the optical density in the cell at concentration c expressed in moles per liter. All measurements and dilutions were carried out in duplicate at least and the results were reproducible to within 0.003 optical density unit.

Buffers.—Buffers of constant ionic strength (μ 0.05) were employed for *p*H values of 1.55 to 7.05. Suitable dilutions of HCl were used between *p*H values 1.55 to 2.50; acetate buffers between 3.85 to 5.80; and phosphate buffers for 5.90 to neutrality. In the high alkaline range, 0.01, 0.10, 0.50 and 1.00 *N* solutions of sodium hydroxide were employed with appropriate additions of sodium chloride to give an ionic strength of 1.00. Since the apparent pK_a values for the sugar dissociation were not quantitatively determined in this range, a knowledge of the *p*H values of these solutions is unnecessary. The apparent pK_a values for the 4-ammonium groups of the nucleosides and nucleotides of cytosine were determined by procedures described previously[3,5] and are given in Table I. Except for the high alkaline range, buffers were checked frequently with the Beckman *p*H meter, the latter calibrated with standard buffer solutions.

Materials.—The authors are indebted to Dr. O. Schindler for a sample of cytosine-2′-deoxyriboside used in this and previous studies[3]; to Dr. G. B. Brown for a recrystallized sample of 2′,3′-isopropylidineuridine, m.p. 159–160° (uncor.); and to Dr. W. E. Cohn for a sample of cytosine-2′-deoxyriboside 5′-phosphate. All of these were spectrophotometrically pure, and gave well-defined isosbestic points.

Cytidylic acids *a* and *b* were pure preparations, fractionated according to the procedure of Loring, *et al.*,[20] previously employed.[4] Adenylic acids *a* and *b* were commercial samples obtained from the Schwarz Laboratories, Inc. N/P ratios indicated greater than 99% purity, salts were absent and both showed but one spot on paper chromatograms. Cytidine was a commercial sample recrystallized three times to constant melting point and spectrophotometric behavior. 1-D-glucopyranosyluracil was prepared according to the procedure of Hilbert and Jansen[21] with modifications[22] and was analytically pure.

The preparation of 1-D-2′-deoxyglucopyranosyluracil (I) has been reported previously by Goodman and Howard[23] from the condensation of the HBr-addition product of triacetyl-D-glucal with 2,4-diethoxypyrimidine to give 1-D-(2-deoxy-3′,4′,6′-triacetylglucopyranosyl)-4-ethoxypyrimidone-2 (II). Subsequent hydrolysis with methanolic hydrogen chloride according to Hilbert[24] gave (I), m.p. 169–170° (uncor.).[25]

We have prepared I from the treatment of 1,3,4,6-tetraacetyl-α-2-deoxy-D-glucose[26] with ethereal hydrogen chloride for 2 days at 5°. Removal of the solvent *in vacuo* gave sirupy 1-chloro-2-deoxy-3,4,6-triacetyl-D-glucose (3 g.) which was treated immediately with 3 ml. of 2,4-diethoxypyrimidine and placed in the oven at 95–100° for 48 hours during which time the solution darkened and a small amount of precipitate (uracil) formed. After cooling, addition of 10 ml. of ether and filtration from the uracil, profuse precipitation occurred in the filtrate. The precipitate was taken up in chloroform, treated with norite, filtered, and the filtrate was evaporated to dryness. The residue was taken up in a minimum of hot ethanol and cooled to 0° to give a white flocculent precipitate which upon recrystallization from ethanol melted at 136–138° (uncor.).

Anal. Calcd. for $C_{18}H_{24}O_9N_2$: N, 6.80. Found: N, 6.72, 6.93.

Its spectrum in water was essentially identical with 1-D-(2′,3′,4′,6′ - tetraacetylglucopyranosyl) - 4 - ethoxypyrimidone-2,[21] but differed from 2-(2′,3′,4′-triacetyl-D-ribopyranosido)-4-ethoxypyrimidine.[27,28] The yield was 0.4 g. of pure material. Hydrolysis of this product in methanolic hydrogen chloride gave I, m.p. 168–169° (uncor.), from methanol–ether. Recrystallization did not raise the melting point.

Anal. Calcd. for $C_{10}H_{14}O_6N_2$: N, 10.85. Found: N, 11.08.

Acknowledgment.—The authors wish to express their gratitude to Dr. George Bosworth Brown for helpful discussions and continued interest.

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(17) Since the 3′-hydroxyl group is presumably less acidic,[7] the same comparative spectrophotometric pattern would obtain for cytidine and cytosine-2′-deoxyriboside regardless of the configuration at the glycosidic center of the latter.

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(28) These compounds bear the same spectrophotometric relationship to each other as do 2,4-diethoxypyrimidine and 1-methyl-4-ethoxypyrimidone-5 (Shugar and Fox, unpublished observations).