SPECTROPHOTOMETRIC STUDIES OF NUCLEIC ACID DERIVATIVES AND RELATED COMPOUNDS AS A FUNCTION OF pH

II. NATURAL AND SYNTHETIC PYRIMIDINE NUCLEOSIDES

by

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INTRODUCTION

The ultraviolet absorption spectra of nucleic acid derivatives have been used to good advantage for purposes of identification and measurement of concentration[1], particularly in work with nucleic acid hydrolyzates separated by paper chromatography and in enzymic studies[2]. GULLAND and his colleagues[3], in their classic work with methylated xanthines as compared to xanthosine, justified their comparison of compounds in which an alkyl group replaces a glycosyl radical by concluding from the work of GOOS, SCHLUBACH, AND SCHROETER[4] that a simple carbohydrate group would exert no appreciable effect upon the spectra.

It will be shown in the course of this study with pyrimidine nucleosides that significant spectrophotometric differences between alkyl and glycosyl pyrimidines (substituted in the same position of the nitrogenous base) do exist, especially in the high alkaline range. Though these differences do not detract from the validity of the approach used by the GULLAND group[3] for the allocation of the position of attachment of the sugar rest to the base of purine nucleosides, they do, however, suggest that an investigation into the effect of the sugar component upon the spectra of nucleosides is warranted.

The characterization of the sugar moiety of pyrimidine nucleosides—as to the identification of the sugar, the lactol-ring structure, and the configuration at the glycosidic center—has always posed a difficult task. Further, the possibility that the nucleic acids may contain moieties other than those known at present has been brought sharply in focus by the recent work of BERGMANN AND FEENEY[5] who isolated a non-ribose thymine pentoside from sponges. These authors also call attention to the warnings voiced by GULLAND[6], CHARGAFF AND VISCHER[7], and DAVIDSON[8] against the tacit assumption that all polynucleotides are composed of either four D-ribose or four D-2-desoxyribose nucleotides. The recent isolation of 5-methylcytosine[1,9], its nucleoside[10], and the corresponding nucleotide[11] would indicate that these admonitions were not

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* Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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without foundation, and would place even greater emphasis on the need for the development of simplified techniques for the characterization of the sugar components.

This study, which deals primarily with the variation of the spectra of naturally-occurring and synthetic pyrimidine nucleosides as a function of pH and the effect of the sugar moieties in the determination of these spectra, provides a basis—limited at present because of the lack of adequate reference compounds—for the further use and extension of ultraviolet absorption spectroscopy for the identification and characterization of the sugar components of these biologically-important compounds.

EXPERIMENTAL

Methods

All measurements were made with a Beckman Model DU spectrophotometer using 10 mm quartz cells. The preparation of solutions for measurement, the buffers used, and the spectrophotometric measurement techniques have been outlined in Part I of this series[12]. Again, for the sake of simplicity, 1 *N* NaOH is assumed to be pH 14.

It should be mentioned here that while there is nothing new or original about the existence of isosbestic points in the spectra of componnds containing dissociable group(s), a knowledge of their location is of considerable value. Since its wave-length and extinction coefficient are unchanged with pH, reference measurements with respect to this point may be used for calculating concentrations over a wide range of pH values. Since the isosbestic point represents the place of intersection of several curves, it is, in fact, the most precise point in the spectrum and may be used as a good criterion for the purity and identification of a compound as well as for a control on the accuracy of technique.

Materials

Thymidine was obtained from Dr THOMAS G. BRADY of the University College, Dublin; *Cytosine desoxyriboside* from Prof. A. R. TODD, F.R.S., of the University Chemical Laboratory, Cambridge and from Dr O. SCHINDLER of the Pharmazeutische Anstalt der Universität, Basel; and *Spongothymidine* (thymine pentofuranoside) from Dr W. BERGMANN of Yale University, New Haven, Connecticut, to all of whom the authors are deeply indebted. These compounds were in an excellent state of purity as determined both by melting points and spectrophotometric behavior.

1-*Methylcytosine* was prepared according to HILBERT[13] by rearrangement of 2-methoxy-4-aminopyrimidine at 180° and also by treatment with methyl iodide. Both samples were pure, m.p. 303° dec., and gave identical spectra. The 1-D-*glycopyranosyluracil* nucleosides (or uracil glycopyranosides) of glucose, galactose, arabinose, and xylose (see formula I, R = glycopyranosyl, R′ = H) were prepared according to HILBERT[14]. 1-D-*Arabinopyranosylthymine* (I, R = arabinopyranosyl, R′ = CH_3) was synthesized according to VISSER, GOODMAN, AND DITTMER[15]. *Glucopyranosylthymine* and *ribopyranosyluracil* were kindly supplied by Dr I. GOODMAN from previous preparations[15,16]. The former compound was found by spectrophotometric examination to contain traces of thymine which were removed by paper chromatography in butanol: water. The spot containing the nucleoside was eluted with water and the concentration determined from the extinction coefficient previously reported[15].

The 1-D-*glycopyranosylcytosine* nucleosides (II, R = glycopyranosyl, R′ = H) of glucose, galactose, xylose, and arabinose, and the 1-D-*glycopyranosyl-5-methylcytosine* (II, R = glycopyranosyl, R′ = CH_3) nucleosides of arabinose and xylose, and *xylopyranosylthymine* were analytical samples prepared according to FOX AND GOODMAN[17].

Melting points and spectrophotometric behavior indicated that all of these synthetic nucleosides were pure. Many of them were chromatographed (including ribopyranosyluracil) and in all cases, except with glucosylthymine, were shown to be free of their corresponding pyrimidine bases.

*Uracil desoxyriboside** was prepared according to the method of DEKKER AND ELMORE[10] for the nitrous acid deamination of 5-methylcytosine desoxyriboside to thymidine. Five mg of cytosine desoxyriboside in one milliliter of 20% HOAc was treated with a slight excess of sodium nitrite. After several hours at room temperature, the solution was neutralized, the solvents removed *in vacuo*,

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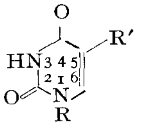
* It should be stated that by their resistance to sodium metaperiodate oxidation BROWN AND LYTHGOE[19] and MANSON AND LAMPEN[20] showed thymidine and cytosine desoxyriboside to be desoxypentose furanosides. Only in the case of the guanine nucleoside of DNA has the sugar been conclusively identified as desoxyribose[21] and confirmation of the identity of the desoxy sugar component of pyrimidine nucleosides is to be desired.

the residue extracted with alcohol and concentrated, and the resulting solution chromatographed on Whatman No. 1 filter paper along with samples of cytosine desoxyriboside, cytosine, and uracil. It has been reported previously[10] that pyrimidine desoxyribosides have the same R_F values in butanol: water as their corresponding free pyrimidine bases.

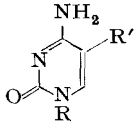
After 20 hours in butanol: water (86:14), three spots were produced on the chromatogram the lower of which, identical with that of cytosine desoxyriboside and with a similar R_F value as cytosine, was starting material. The middle spot, with a similar R_F value as uracil, was uracil desoxyriboside and was shown spectrophotometrically to be uracil-free. The higher component (present in very small amounts), when run spectrophotometrically showed no variation of the spectrum between pH values 3 and 13 and was not characterized further. The overall reaction yield of uracil desoxyriboside was about 30%, which after elution with water was filtered and its spectrum determined.

RESULTS AND DISCUSSION

In part I of this series[12] the spectra of several pyrimidine derivatives as a function of pH were shown to give a composite picture of each of these compounds (*viz.*, uracil, cytosine, thymine, 5-methylcytosine, and others) in terms of their potentially-dissociable groups as exhibited by their degrees of dissociation and ionization constants. Thus the spectrum of uracil showed two equilibria, while that of 1-methyluracil (I, R = CH_3, R′ = H) gave only one. Similarly, with two equilibria noted for cytosine for the amino and 2-hydroxy groups respectively, the spectrum of 1-methylcytosine (II, R = CH_3, R′ = H) shows but one equilibrium, that for the 4-amino group. Between pH values 7 and 14 the curves for the latter compound are identical (see Fig. 1).



I



II

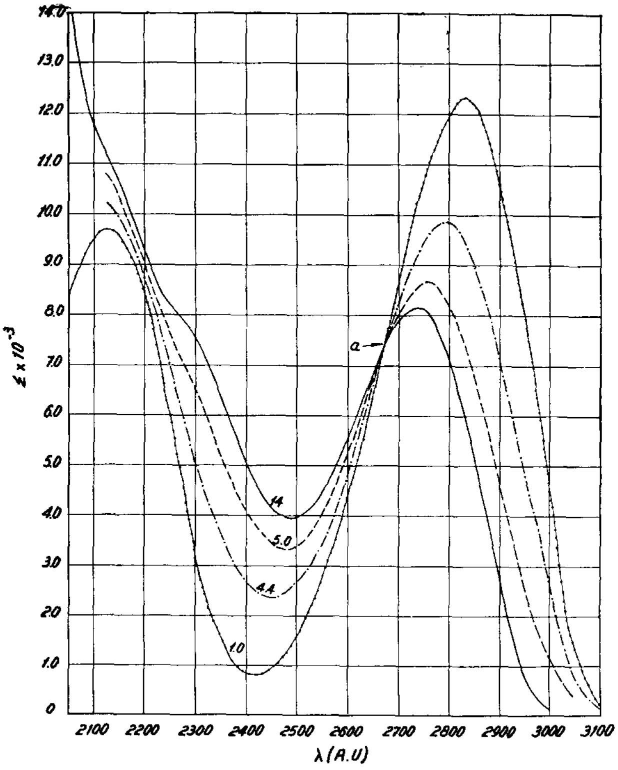


Fig. 1. 1-Methylcytosine at pH values indicated. Curves for pH 7 and 14 are identical as are those for pH 1.0 and 2.0.

No attempt has been made, to our knowledge, to compare the spectra of N-methyl derivatives of uracil and cytosine to that of their corresponding nucleosides in a manner similar to that used by GULLAND and his co-workers with purines[3], presumably because of the wealth of chemical evidence provided by the LEVENE group[22,23] for the allocation of the sugar moiety to the N_1-position of the base. Such a comparison, however, aside from lending further confirmatory evidence, as can be seen from the spectra of 1-methyluracil and 1-methylcytosine as compared to uridine (Fig. 6) and cytidine (Fig. 2), sheds

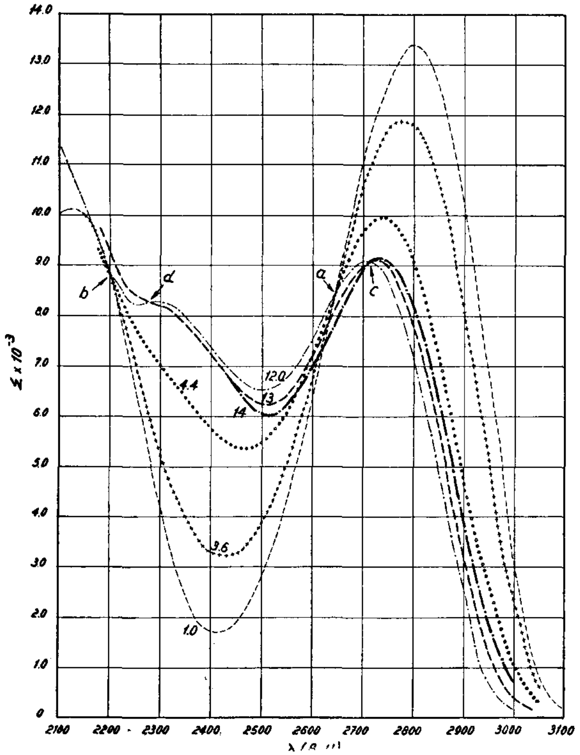


Fig. 2. Cytidine at pH values indicated. Curves for pH 7.2 and 12.0 are identical as are those for pH 1.0 and 2.0. Isosbestic points *a* and *b* represent the amino equilibrium; *c* and *d* correspond to the sugar dissociation.

more light upon the differences in the spectra of the free bases versus their respective nucleosides*.

1-Methyluracil, the spectrum of which as a function of pH has been described previously in detail, has been shown to exist in the diketo form in neutral, aqueous solution and in the 2-keto-4-enol form at pH 12 and above. That a quinoidal structure played no significant role (if any) in the determination of the tautomeric picture was shown by the close similarity of this compound, curve for pH 12.0, to that of 1-methyl-4-ethoxypyrimidone-2.

It is obvious that a similar structural sequence may be assigned to uridine in neutral and alkaline solutions up to pH 12. The fact that 1-methyluracil shows no appreciable change in the position of the maximum with pH is also characteristic of all glycopyranosyl, ribofuranosyl, and desoxyribofuranosyl nucleosides of uracil and thymine thus confirming the suggestion of STIMSON AND REUTER[24] that lactam-lactim tautomerism in alkaline solution involves the 3,4-positions of thymidine. The differences noted by PLOESER AND LORING[25] between the spectra of uracil and uridine are explicable in terms of the number of dissociable groups and the location of substituents in the pyrimidine ring as shown previously for uracil, 1-, and 3-methyluracil[12].

Spectrophotometric evidence of sugar dissociation. Close inspection of the spectra of uridine and cytidine shows that they differ from those of 1-methyluracil and 1-methylcytosine in the high alkaline range. Thus, for uridine, while all curves between pH values 1 and 12.0 pass through isosbestic point *a* to give a pK value for 4-enolization in good agreement with titrimetrically-determined values of LEVENE[27], above this range the curves "break away" from this isosbestic point to produce a new equilibrium, with its corresponding isosbestic points (*b* and probably *c*). A similar equilibrium picture in the high alkaline range is also evidenced by cytidine.

The assignment of this spectrophotometric behavior, above pH 12, to the dissociation of the sugar moiety is warranted. It is to be recalled that LEVENE and coworkers[27] conducted extensive electrometric measurements of the dissociation constants of nucleosides and reported pK values of 12.3–12.5 which they assigned to the sugar

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* In this regard, a study of the spectra of 1- and 3-methylorotic acid as a function of pH as compared to that of orotic acid[12] would provide useful information for the allocation of the sugar rest of the nucleoside, orotidine, reported by MICHELSON, DRELL, AND MITCHELL[26].

rests of these pyrimidine nucleosides. The fact that these nucleosides (and, as will be shown, all other pyrimidine nucleosides, whether pyranose, furanose, or desoxyfuranose) exhibit equilibria in this range spectrophotometrically indicates surely that this dissociation is not a manifestation of the 4-amino or of the 4-hydroxyl groups. Indeed, LEVENE demonstrated that adenylic acid, which possesses neither "phenolic" hydroxyls nor keto groups in the molecule, also gives a dissociation constant in this high alkaline range. Lastly, the fact that 1- and 3-methyluracil and 1-methylcytosine fail to exhibit a second dissociation spectrophotometrically[12] or titrimetrically[27] in solutions up to 1 *N* base would confirm this view. It should be added that, for all the nucleosides reported herein at pH 14, acidification of the sample in the quartz cell reproduces the curve for pH 1, showing the reversibility of the equilibrium.

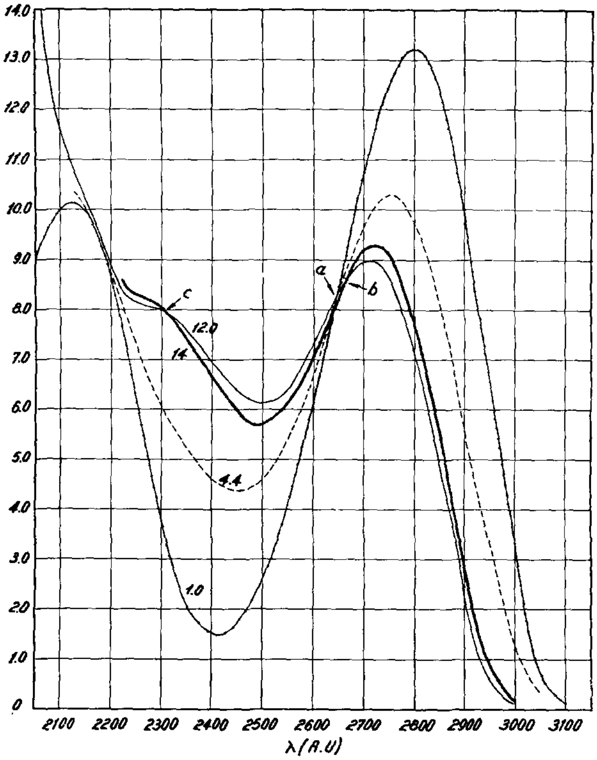

Fig. 3. Cytosine desoxyriboside at pH values indicated. Curves for pH 7.2 and 12.0 are identical as are those for pH 1.0 and 2.0. The curve for pH 13, not represented, is very close to that for pH 12.0. Isosbestic point *a* corresponds to the amino equilibrium, *b* and *c* to the sugar dissociation. Both samples (see discussion under materials) gave identical spectra.

The curve for pH 12, which in the cases of cytosine and 5-methylcytosine nucleosides are identical with that for pH 7, represents, then, the limiting curve for the equilibria involving the 4-substituent and the sugar moiety. Examination of the spectra of the nucleosides shows that differences in this high alkaline range attributable to the sugar components exist amongst them. In the following descriptions, emphasis will be placed mainly on those differences which are independent of concentration.

The cytosine nucleoside series. The absorption spectra of cytidine[25] at pH values 2, 7, and 12 and of cytosine desoxyriboside[20] at pH 7 have been reported previously. The latter authors concluded that the spectra of these two nucleosides were identical. While chemical tests, such as metaperiodate oxidation and the Dische reaction will distinguish between the two, they involve destruction of the compound. In addition, the latter method gave anomalous behavior with cytosine desoxyriboside[20]. Examination of the complete spectra of these nucleosides shows that though they bear resemblance to each other (Figs. 2 and 3), several significant differences do exist. Comparison of the pH 12 curves reveals that cytidine manifests a clear maximum (or "hump") at 2300 A, whereas the desoxyriboside shows an inflection and more nearly resembles 1-methylcytosine in this respect. Even more pronounced is the behavior of both substances in the high alkaline range. As the pH is increased from 12 to 13, the cytidine spectrum breaks away markedly from isosbestic point *a* whereas that for the desoxyriboside shows no perceptible shift. At pH 14 these differences become accentuated. For cytidine, the pH 13 and 14 curves intersect the pH 12 curve at the peak of the latter to produce isosbestic point *c*, 65 A removed from isosbestic point *a*. With cytosine desoxyriboside, the pH 13 (not shown on the figure for the sake of clarity) and 14 curves intersect the pH 12 curve at isosbestic point *b* removed only 25 A from isosbestic point *a*.

That differences exist spectrophotometrically between these two furanosides of cytosine is not surprising in the light of the findings of KUHN AND SOBOTKA[28] who showed that the dissociation constants of several *o*-glucosides (*viz.*, phenyl) differed, and these were dependent upon the *cis* or *trans* relationship of the aglycon to the 2-hydroxyl of the sugar. It would seem reasonable to conclude that these two cytosine furanosides, presumably of the same sugar, D-ribose, but differing in the presence or absence of a 2-hydroxyl group, would show differences in dissociation constants of their sugar components and manifest these differences spectrophotometrically via electronic interaction of the dissociated or non-dissociated forms of the sugar rest on the pyrimidine moeity at a given pH. On this basis it should be expected that the dissociation of the sugar component of cytidine would be manifested at a lower pH value than that of the desoxy analogue, as is actually the case* (see also Table I). A similar situation should also prevail for furanose and desoxyfuranose analogues of uracil and thymine, which, as will be shown, is essentially the case. In the absence of proper reference compounds it is not known whether a similar comparison applies to pyranose and desoxypyranose nucleosides though it is clear that this relationship does not hold when pyranosides are compared with desoxyfuranosides of the same nucleoside series.

TABLE I

SPECTROPHOTOMETRIC DATA OF PYRIMIDINE NUCLEOSIDES**

Compound	*pH curve*	*Maxima*		*Minima*		*Isosbestic points*		
		λ *(A)*	$\varepsilon \cdot 10^{-3}$	λ *(A)*	$\varepsilon \cdot 10^{-3}$	*pH range*	λ *(A)*	$\varepsilon \cdot 10^{-3}$
1-Methylcytosine	1.0–2.0	2130, 2830	9.70, 12.3	2425	0.85	1.0–14	2675	7.45
	4.4	2795	9.82	2450	2.36			
	5.0	2760	8.68	2480	3.35			
	7.2–14	2740	8.15	2495	3.95			
Cytidine	1.0–2.0	2125, 2800	10.1, 13.4	2415	1.70	1.0–12.0	2200	8.85
	3.6	2780	11.8	2430	3.20		2645	8.40
	4.4	2740	9.97	2470	5.35			
	7.2–12.0	2295, 2710	8.30, 9.10	2260, 2505	8.20, 6.50			
	13	2725	9.15	2510	6.20	12.0–14	2280	8.25
	14	2730	9.20	2515	6.03		2710	9.10
Cytosine Desoxyriboside	1.0–2.0	2125, 2800	10.2, 13.2	2410	1.50	1.0–12.0	2645	8.20
	4.4	2755	10.3	2450	4.36			
	7.2–12.0	2710	9.00	2500	6.13			
	13	2715	9.06	2500	6.00	12.0–14	2320	7.90
	14	2725	9.28	2485	5.70		2670	8.65

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* It would be of interest to test the applicability of this method towards the measurement of absorption differences for the sugar dissociation of isomeric nucleotides reported in the literature[29, 30].

** 1-Methylcytosine is included in this table for comparison purposes. See reference 12 for similar data for 1-methyluracil.

TABLE I (continued)

Compound	*pH curve*	*Maxima*		*Minima*		*Isosbestic points*		
		λ (*A*)	$\varepsilon \cdot 10^{-3}$	λ (*A*)	$\varepsilon \cdot 10^{-3}$	*pH range*	λ (*A*)	$\varepsilon \cdot 10^{-3}$
Cytosine Pyranosides	1.0–2.0	2120, 2760	10.1, 12.7	2405	2.10	1.0–13	2130	10.1
	3.6	2740	10.7	2415	4.45		2210	7.90
	4.4	2705	9.25	2470	6.62		2615	8.15
	7.2–13	2370, 2680	8.22, 8.55	2240, 2510	7.72, 7.50			
	14	2335, 2695	8.30, 8.70	2520	7.13	13–14	2360	8.20
							2640	8.37
5-Methylcytosine pyranosides	1.0–2.0	2125, 2850	11.8, 12.0	2440	1.43	1.0–13	2165	11.5
	3.6	2830	10.5	2470	3.07		2235	8.65
	4.4	2790	8.80	2530	5.10		2670	7.15
	7.2–13	2370, 2755	8.00, 8.05	2310, 2570	7.93, 5.97			
	14	2770	8.15	2570	5.77	13–14	2365	8.00
							2715	7.80
Uridine	1.0–7.2	2620	10.1	2305	2.05	1.0–12.0	2450	5.40
	8.6	2620	9.45	2320	3.20			
	9.5	2620	8.50	2365	4.48			
	12.0	2620	7.45	2425	5.38			
	13	2630	7.41	2430	5.22	12.0–14	2620	7.40
	14	2645	7.50	2430	5.00			
Uracil Desoxyriboside*	1.0–7.2	2620	10.2	2310	2.20	1.0–12.0	2450	5.40
	9.3	2620	8.94	2350	4.22			
	12.0	2620	7.63	2420	5.35			
	13	2620	7.63	2420	5.25			
	14	2635	7.86	2420	5.06	12.0–14	2585	7.4
Uracil Pyranosides	1.0–7.2	2595	10.1	2285	2.35	1.0–13	2435	6.02
	8.4	2595	9.50	2300	3.50			
	9.5	2595	8.45	2335	5.10			
	13.0	2595	7.32	2435	6.00	13.0–14	2290	7.70
	14	2610	7.50	2435	5.77		2555	7.15
Uracil Ribopyranoside	1.0–7.2	2620	11.1	2300	2.25	1.0–12.0	2435	5.17
	8.7	2620	10.1	2330	3.63			
	9.5	2620	9.35	2360	4.46			
	12.0	2620	8.15	2410	5.10			
	13	2620	8.15	2410	5.00			
	14	2630	8.35	2415	4.77	12.0–14	2585	7.95
Thymidine	1.0–7.2	~2075, 2670	9.55, 9.65	2350	2.25	1.0–13	2475	4.65
	9.5	2670	9.03	2375	3.34			
	10.0	2670	8.33	2405	4.08			
	13	2670	7.38	2455	4.58	13–14	2640	7.20
	14	2680	7.51	2455	4.40			
Thymine Pyranosides	1.0–7.2	~2075, 2645	9.8, 9.70	2330	2.40	1.0–13	2460	5.10
	9.5	2645	8.62	2370	4.12			
	13	2645	7.03	2460	5.10			
	14	2660	7.10	2460	4.85	13–14	2640	7.00
"Spongo-thymidine" (Thymine Pentofuranoside)	1.0–7.2	~2075, 2685	9.23, 10.0	2355	2.25	1.0–12.0	2475	4.40
	9.5	2685	9.25	2390	3.33			
	10.1	2685	8.42	2425	4.06			
	12.0	2685	7.87	2450	4.30			
	13	2700	8.16	2455	4.05			
	14	2715	8.70	2455	3.73	12.0–14	2635	7.50

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* Added in proof: After submitting this manuscript for publication, we received from Professor A. R. TODD, F.R.S., a sample of uracil desoxyriboside which gave a spectrum identical to that determined from the eluate of the chromatogram of the nitrous acid deamination product of cytosine desoxyriboside (see page 370 and Fig. 7). Extinction values for uracil desoxyriboside listed in the table are based upon Professor TODD's sample.

Attention is also drawn to the relationship between the pH 12.0 curve (identical with that for pH 7.2) and that for pH 4.4. It is seen that in the desoxyriboside of cytosine the relative displacement of these two curves from each other, at 2400 A for example (though any wave-length removed from the isosbestic points will do), is 1½ times as great as for cytidine. This phenomenon is undoubtedly related to the influence of the sugar components of these compounds on the respective amino pK's (see below).

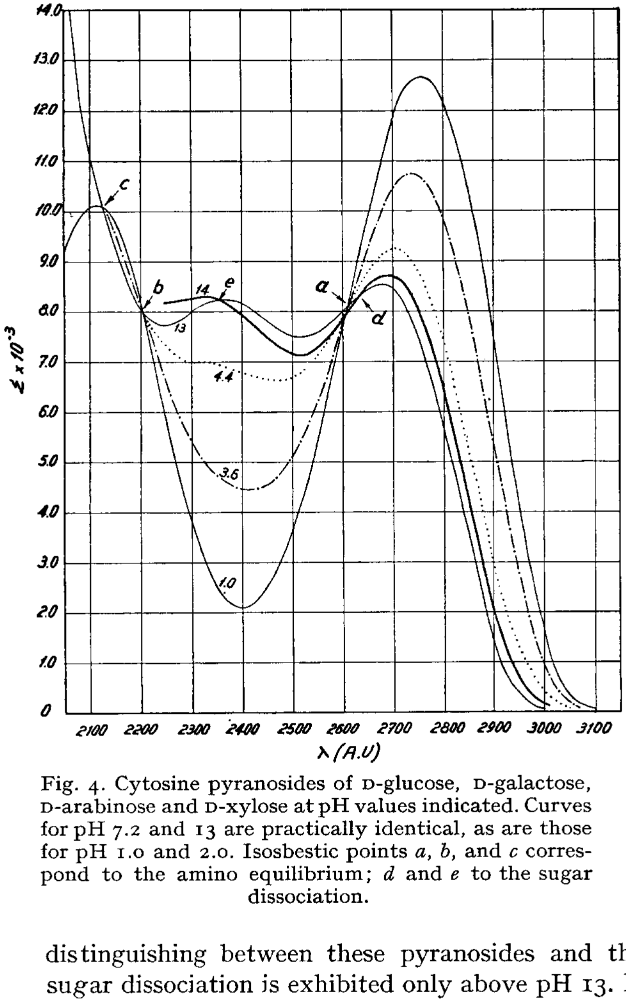

Fig. 4. Cytosine pyranosides of D-glucose, D-galactose, D-arabinose and D-xylose at pH values indicated. Curves for pH 7.2 and 13 are practically identical, as are those for pH 1.0 and 2.0. Isosbestic points *a*, *b*, and *c* correspond to the amino equilibrium; *d* and *e* to the sugar dissociation.

The differences between the spectra of the cytosine pyranosides and that of their ribofuranose and desoxyribofuranose analogues are striking. Fig. 4 represents the spectra of the 1-D-glycopyranosylcytosine nucleosides of glucose, galactose, xylose and arabinose, all of which were identical. Their curve for pH 13 shows a clearly-resolved maximum at 2730 A. This, coupled with the appearance of two isosbestic points at the shorter wave-lengths, *b* and *c*, for the amino equilibrium, would more than suffice as criteria for distinguishing between these pyranosides and the aforementioned furanosides. The sugar dissociation is exhibited only above pH 13. It is to be noted that while the long-wave isosbestic point for the 4-amino equilibrium of cytidine and cytosine desoxyriboside occurs at the same wave-length, that for the pyranosides is shifted 25 A toward the violet. A similar situation is also encountered in the uracil nucleoside series.

The sugar substituents vary in their effect upon the proton affinity of the amino group of the pyrimidine. Thus, while 1-methylcytosine gives a spectrophotometrically-determined pK value of 4.55, cytosine desoxyriboside, cytidine, and the cytosine glycopyranosides give progressively lower values, 4.3, 4.1, and 3.85 respectively. This decrease in pK is accompanied by a progressive decrease in separation between the neutral (pH 7, 12) and acid (pH 1) curves in the neighborhood of 2100 to 2200 A concomitant with a levelling-out of the neutral curve producing ultimately a clearly-resolved maximum in cytidine which is considerably more pronounced in the cytosine pyranosides. It is to be noted that the short-wave maximum of the acid curves of all these nucleosides of cytosine have the same intensity of absorption, all of which are higher than that for 1-methylcytosine.

5-*methylcytosine nucleosides.* During the course of our investigations, two papers appeared describing the isolation and identification of desoxy-5-methylcytidylic acid[11] and its corresponding desoxyribose nucleoside[10] as components of desoxyribosenucleic acid.

The spectrum of 5-methylcytosine pentosepyranosides of D-arabinose and D-xylose are listed in Fig. 5. In general they bear very close resemblance to their cytosine analogues, as would be expected, allowance given for the 5-methyl shift towards the visible. On the basis of the spectrophotometrically-determined pK values for cytosine and 5-methylcytosine[12] and 1-methylcytosine, the dissociation constant of 1,5-dimethylcytosine would be expected to be in the vicinity of 4.7. The pK value for the 5-methylcytosine pyranosides calculated spectrophotometrically is 4.1, that is, equally lower from assumed value of its 1-methyl analogue as are the cytosine pyranosides from 1-methylcytosine. All other features are essentially similar.

Using this spectrum (Fig. 5) together with the previously described relationships among the cytosine nucleosides (Figs. 2, 3, and 4), one should be able to ascertain spectrophotometric data for 5-methylcytosine desoxyriboside. In the absence of the latter compound, a comparison will be made with the spectrum given by DEKKER AND ELMORE[10] which contained two curves, that for *N*/100 HCl and *N*/90 NaOH. While their alkaline curve agrees well these deductions, their acid curve cuts the alkaline curve almost at its maximum to give an isosbestic point approximately 80–90 A (at approximately 2755 A) to the right of that shown for 5-methylcytosine pyranosides. Further, we would expect the short-wave maximum of the acid curve to give an extinction coefficient of approximately 11,800 instead of the reported 9,780; and lastly the acid and base curves, in the vicinity of 2100–2200 A should be practically tangential. If the acid curve were raised 20–25 %, all these discrepancies are removed, indicating that a dilution error may have occurred. It is to be noted that COHN's spectrum for the corresponding nucleotide[11] lists two curves (one for pH values 6–13, the other for 1–2.5) which intersect at approximately 2720 A. From the curves reported by PLOESER AND LORING[25] for cytidine and cytidylic acid, indications are that the isosbestic points of nucleosides and their corresponding nucleotides have identical wave-lengths for the dissociation involving the 4-substituent. We have confirmed this phenomenon for cytidine and cytidylic acid.

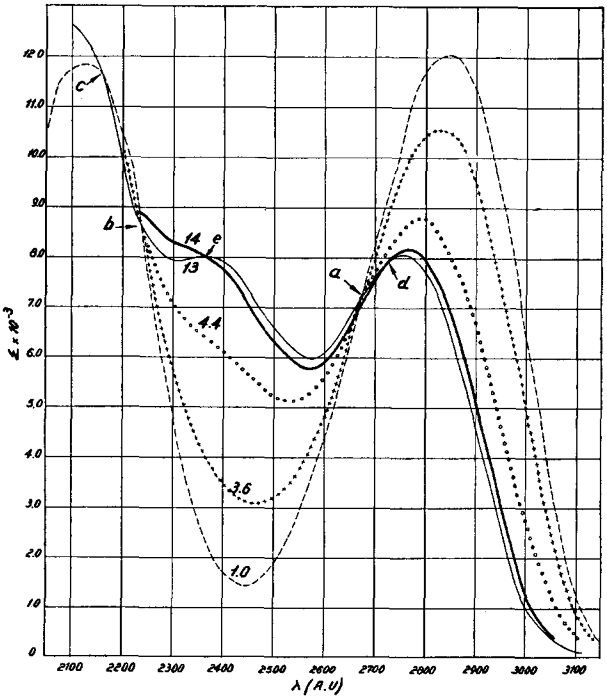

Fig. 5. 5-Methylcytosine pyranosides of D-arabinose and D-xylose at pH values indicated. Curves for pH 7.2 and 13 are practically identical, as are those for pH 1.0 and 2.0. Isosbestic points *a*, *b*, and *c* represent the amino equilibrium; *d* and *e* the sugar dissociation.

TABLE II

SPECTROPHOTOMETRICALLY-DETERMINED "APPARENT" DISSOCIATION CONSTANTS COMPARED WITH VALUES DETERMINED BY TITRATION

Compound	*Dissociation Constant (pK)**	
	*Spectrophotometric***	*Titrimetric*
1-Methylcytosine	4.55	—
Cytidine	4.1	4.22
Cytosine desoxyriboside	4.3	—
Cytosine pyranosides	3.85	—
5-Methylcytosine pyranosides	4.1	—
1-Methyluracil	9.75	9.71
Uridine	9.25	9.17
Uracil desoxyriboside	9.3	—
Uracil pyranosides	9.2	—
Uracil ribopyranoside	9.25	—
Thymidine	9.8	—
Thymine pyranosides	9.7	—
"Spongothymidine"	9.8	—

* These refer to the 4-amino or 4-hydroxy substituent as the case may be. Because of the relatively smaller differences in extinction between the curves involved in the sugar equilibrium and the indications that this dissociation may not be complete at pH 14, the dissociation constants of the glycosyl components were not calculated. It may be said, however, that the pK_{sugar} values of the nucleosides would be in the vicinity of 13.

** Values reported to the nearest half-tenth. Titrimetric values are those reported by LEVENE and co-workers[27].

The uracil nucleoside series. Of special interest in this series would be the examination of the relationships between uridine and uracil desoxyriboside in light of previous information obtained from the corresponding cytosine nucleosides. The presence of uracil desoxyriboside has been indicated by WANG and co-workers[31] who treated cytidine and cytosine desoxyriboside with a bacterial cell-free extract. The rate of deamination of the latter compound was measured spectrophotometrically by assuming that the spectrum of the product (uracil desoxyriboside) would be identical with that for uridine. More recently, DEKKER AND TODD[18] isolated this substance in crystalline form as a by-product of bacterial contamination of a desoxyribosenucleic acid preparation.

The spectrum of uracil desoxyriboside is given in Fig. 7. A visual inspection is sufficient to disclose pronounced differences which distinguish this compound not only from uridine (Fig. 6), but also from the synthetic uracil nucleosides. Striking differ-

TABLE III

SPECTROPHOTOMETRIC DATA OF URACIL AND THYMINE NUCLEOSIDES

Nucleoside	*εmax/min pH 12.0 curve*	*εmax/min pH 7.2 curve*
Uracil pyranosides	1.21	4.30
Uracil ribopyranoside	1.60	4.93
Uridine	1.38	4.92
Uracil desoxyriboside	1.44	4.60
Thymine pyranosides	1.36	4.04
"Spongothymidine"	1.83	4.44
Thymidine	1.61	4.29

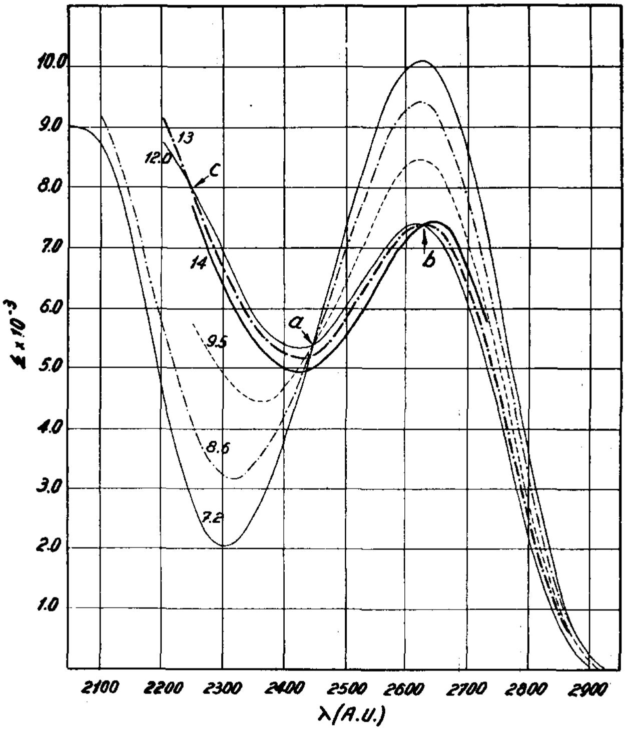

Fig. 6. Uridine at pH values indicated. Curves for pH 1.0 and 7.2 are identical. Isosbestic point *a* corresponds to the equilibrium due to the 4-substituent; *b*, the sugar dissociation.

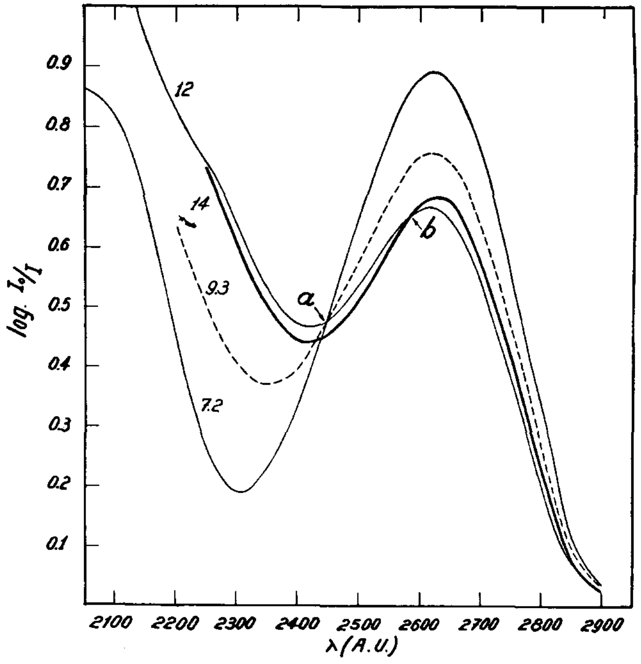

Fig. 7. Uracil desoxyriboside, as obtained from paper chromatogram, at pH values indicated. Curve for pH 13, not listed, is practically identical to that for pH 12.0 and that for pH 7.2 is the same at pH 1.0. Isosbestic point *a* represents the equilibrium due to the 4-substituent; *b*, the sugar dissociation.

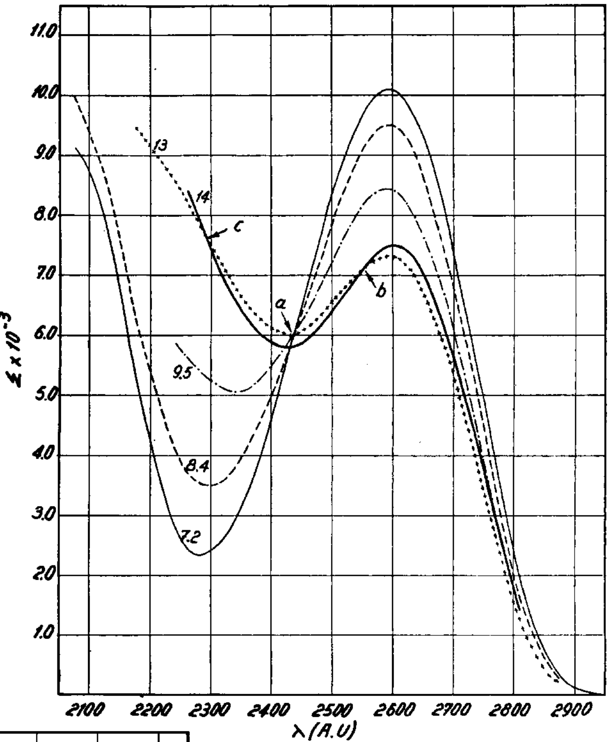

Fig. 8. Uracil pyranosides of D-glucose, D-galactose, D-arabinose and D-xylose at pH values indicated. Curves for pH 12.0 and 13 are identical, as are those for pH 1.0 and 7.2. Isosbestic point *a* represents the 4-substituent equilibrium; *b*, the sugar dissociation.

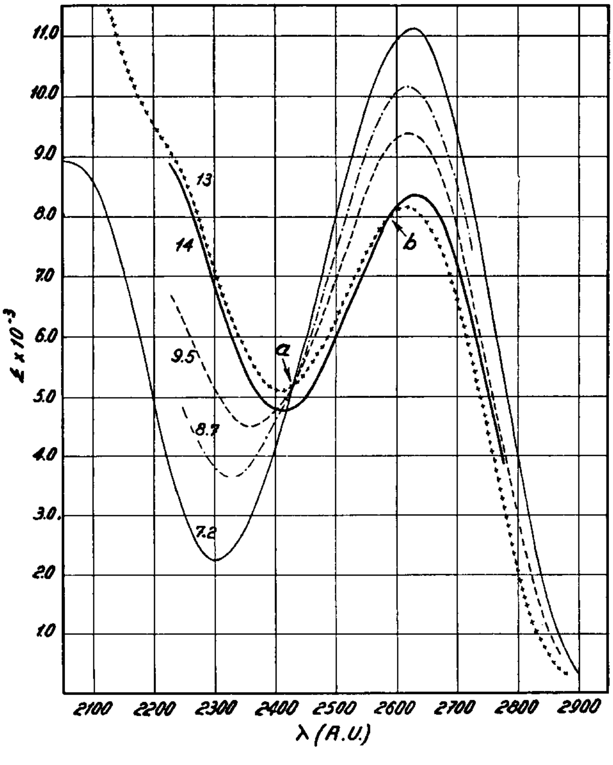

Fig. 9. Uracil ribopyranoside at pH values indicated. Curves for pH 12.0 and 13 are nearly identical and that for pH 7.2 is the same at pH 1.0. Isosbestic point *a* represents the 4-substituent equilibrium; *b*, the sugar dissociation.

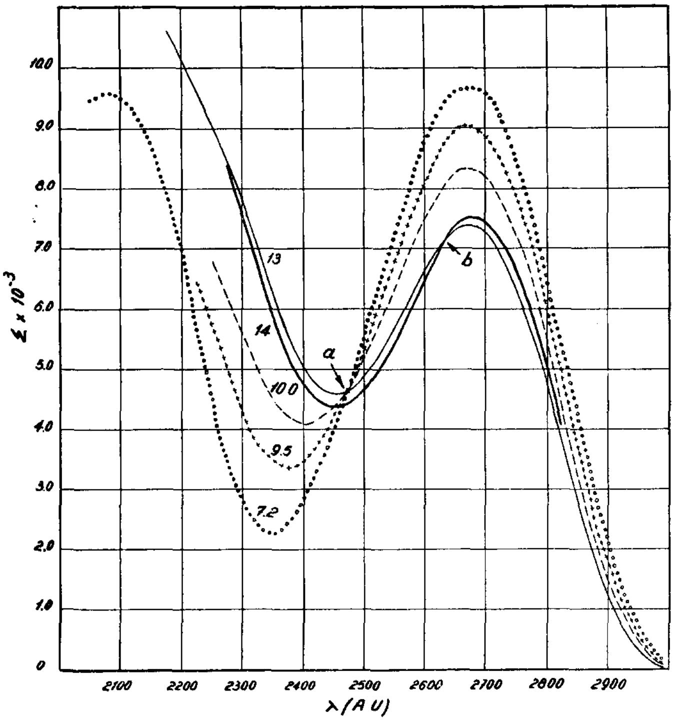

Fig. 10. Thymidine at pH values indicated. Curves for pH 12.0 and 13 are the same, as are those for pH 1.0 and 7.2. Isosbestic point *a* represents the 4-substituent equilibrium; *b*, the sugar dissociation.

ences may be ascertained from the ratios of maximum to minimum for the neutral and pH 12.0 curves (see Table III). Also to be noted with uracil desoxyriboside is, 1) the point of intersection of the pH 12.0 and 14 curves, 2) the slight displacement of the pH 12.0 and 13 curves from each other (see Table I), and 3) the identity of the wave-length of its 4-enolization isosbestic point *a* with that of uridine—all of which gives a similar picture to that encountered previously between cytidine and cytosine desoxyriboside. This suggests strongly that this spectral behavior between a pyrimidine ribofuranoside and its corresponding desoxyribofuranoside for the sugar equilibrium is general.

Coming now to the 1-D-glycopyranosyluracil nucleosides of glucose, galactose, arabinose, and xylose (Fig. 8) we find that all these give identical spectra—as found to be true also in the cytosine and 5-methylcytosine nucleoside series. The spectrum of ribopyranosyluracil, Fig. 9, (this is the only series where a ribose pyranoside was available for measurement), differs from that of the other pyranosides by the position and intensity of the maxima, the position of the minima, and the intensity of absorption of the isosbestic points. Note, however, that the position of the isosbestic points for 4-enolization are identical for *all* pyranosides of uracil, and are displaced toward the shorter wave-lengths relative to those of uridine and uracil desoxyriboside—a phenomenon again reminiscent of the situation found in the cytosine nucleoside series. This would indicate not only that all hexose and pentose pyranosyl nucleosides of a given pyrimidine (*i.e.*, uracil, cytosine and their 5-methyl derivatives) would possess isosbestic points for the first equilibrium at the same wave-length, but would also suggest that the displacement of the ribose and desoxyribose furanoside isosbestic point toward the visibly may be equally true for other furanosides.

Thymine nucleosides. A cursory examination of the spectra of thymine pyranosides of glucose, arabinose, and xylose (Fig. 11) with that of the uracil pyranosides (Fig. 8) and uracil desoxyribofuranoside (Fig. 7) shows that aside from the 5-methyl bathochromic shift their spectral variations are similar. On this basis, the spectrum of a "5-methyluridine" should be equally similar to that of uridine (Fig. 6). More precisely, the spectra of these compounds should show similar sugar equilibria.

Bergmann and Feeney[5] recently isolated a substance from sponges which, on the basis of ultraviolet absorption measurements in acid, neutral and basic media and by elemental analyses, periodic acid titration, formation of a tribenzoate and cleavage to thymine, characterized it as a thymine pentofuranoside. From optical rotational data they suggest it to be a thymine xylofuranoside. Makino and Satoh[32], using Boeseken's boric acid test, ruled out ribose and lyxose as the sugar rest and by sulfuric acid hydrolysis followed by paper chromatography showed that the R_F value of the sugar moiety corresponded to that of xylose.

Aside from this chemical evidence and in the light of previous discussion, examination of the complete spectrum of spongothymidine (thymine pentofuranoside, Fig. 12) as compared to thymine pyranosides (Fig. 11) and thymidine (Fig. 10) shows that it is neither a thymine pentopyranoside nor a desoxyriboside. The pronounced sugar dissociation picture would indicate that it is a furanoside, but surely not of ribose since the spectral variation for its sugar equilibrium differs markedly from uridine and from a hypothetical "5-methyluridine". Examination of Table III provides additional support for the "non-ribose" nature of its sugar component. It is to be noted that the isosbestic point for 4-enolization (Table I) is identical with that of thymidine, both occurring at 2475 A, and both of which are displaced toward the visible from those of the thymine pyranosides (2460 A)—in line with our previous hypothesis that the isosbestic points of all pyrimidine furanosides of a given series will be displaced equally toward the longer wave-legnths from that of the pyranosides.

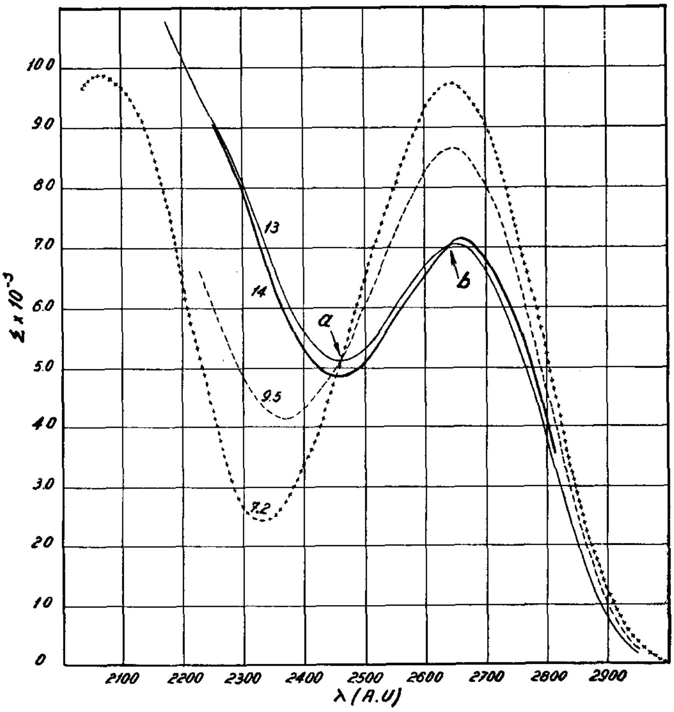

Fig. 11. Thymine pyranosides of D-Arabinose, D-xylose, and D-glucose (the latter from a paper chromatogram) at pH values indicated. Curves for pH 12.0 and 13 are identical as are those for pH 1.0 and 7.2. Isosbestic point *a* corresponds to the 4-substituent equilibrium; *b*, the sugar dissociation.

References p. 384.

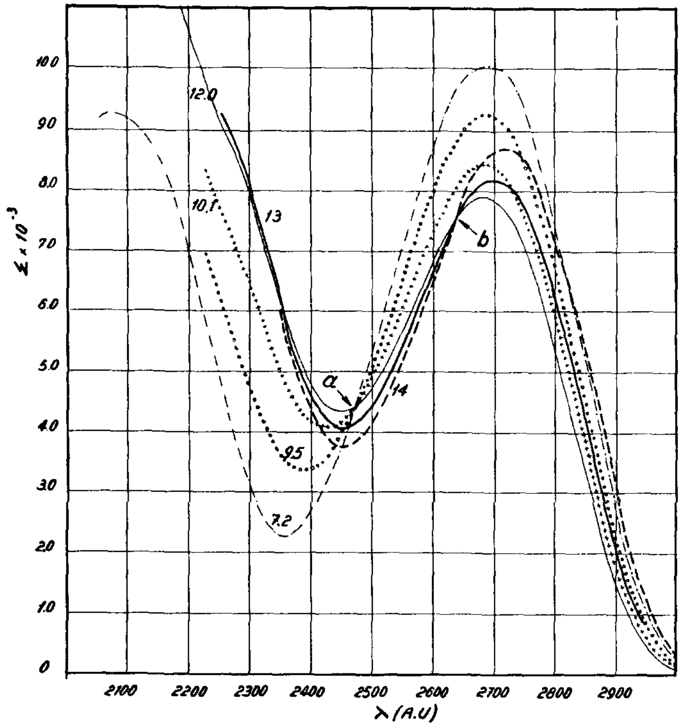

Fig. 12. "Spongothymidine" (Thymine pentofuranoside) at pH values indicated. Curves for pH 1.0 and 7.2 are identical. Isosbestic point *a* represents the 4-substituent equilibrium; *b*, the sugar dissociation.

That spongothymidine gives a different spectrum from that calculated for its corresponding ribofuranoside ("5-methyluridine") is not surprising when one considers that differences were noted between the spectrum of ribopyranosyluracil versus other pyranosides. The spectrum of ribopyranosylthymine in neutral solution (maximum and minimum values) as reported by VISSER and co-workers[15] would indicate that this nucleoside differs in a similar way from the other thymine pyranosides. This suggests that furanosyl nucleosides of thymine and uracil—other than ribose—would give spectra identical to that of spongothymidine or its uracil analogue.

The pK values of the uracil nucleosides are lower than that of 1-methyluracil (see Table II); however, here, unlike the cytosine nucleosides, we find no appreciable differences amongst them. By calculation of the pK for 1,5-dimethyluracil (in a manner similar to that used for 1,5-dimethylcytosine) it can be concluded that similar relationships hold for the thymine nucleoside series.

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SUMMARY

1. The ultraviolet absorption spectra of synthetic and naturally-occurring pyrimidine nucleosides were measured at various pH values and their "apparent" dissociation constants reported.

2. Spectral variation as a function of pH in the high alkaline range is demonstrated and shown to be due to the dissociation of the sugar moieties.

3. General spectrophotometric patterns are presented which enable the distinction between pyrimidine ribofuranosides and their desoxyribofuranoside analogues.

4. Differences between pyrimidine glycopyranosides and glycofuranosides are presented and some implications for the spectra of hitherto unreported pyrimidine nucleosides are suggested.

RÉSUMÉ

1. Les spectres d'absorption dans l'ultra-violet de nucléosides pyrimidiques synthétiques et naturels ont été mesurés à diverses valeurs de pH et leurs constantes de dissociation "apparente" ont été établies.

2. La variation du spectre en fonction du pH dans la zône de haute alcalinité est établie et il est montré qu'elle est due à la dissociation de la fraction sucre.

3. L'allure générale des spectres permet de distinguer entre les ribofuranosides pyrimidiques et leurs analogues désoxyribofuranosides.

4. Des différences entre les glycopyranosides et les glycofuranosides pyrimidiques sont relevées et certaines suggestions relatives aux spectres de nucléosides pyrimidiques encore inconnus sont présentées.

ZUSAMMENFASSUNG

1. Die Ultraviolet Absorptionsspektren synthetischer und natürlich vorkommender Pyrimidinnucleoside wurden gemessen bei verschiedenen pH-Werten und ihre scheinbaren Dissoziationskonstanten festgestellt.

2. Die Variation der Spektren als Funktion des pH in hoher Alkalinitätszone wurde bewiesen, wie auch ihre Abhängigkeit von der Dissoziation der Zuckeranteile.

3. Der allgemeine Verlauf der Spektren gestattet die Unterscheidung zwischen Pyrimidinribofuranosiden und ihren Desoxyribofuranosid-Analogen.

4. Unterschiede zwischen Pyrimidinglycopyranosiden und -glycofuranosiden wurden festgestellt und einige Folgerungen in Beziehung auf die Spektren bisher noch unbekannter Pyrimidinnucleoside werden vorgeschlagen.

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