probable, on the basis of these facts, that the enzymatic reaction resembles that in the model system in that the hydrogen atoms on the methylene bridge are inert.

The data, although in support of this statement, are on the edge of experimental precision and therefore leave something to be desired. The excellent analyses for tritium, performed at the Argonne National Laboratory, made it possible to carry through this work, but the total amount of radioactivity (*ca.* 10^{-9} curie) cannot be determined accurately. A further difficulty must be considered; this is the possibility of an adverse isotopic fractionation, which would effectively obscure the results here shown. However, the turnover number of the carboxylase used was about 0.7 per second at the *p*H of 4.6. In 30 seconds, the average enzyme molecule should turn over about 20 pyruvate molecules. If the coenzyme is ionized each time a pyruvate molecule reacts, then the coenzyme should have reached equilibrium with the solvent; this is the postulate on which the values in the next to last column of Table I have been calculated. If the enzyme were to ionize the coenzyme only once, and the ionized coenzyme were then converted to its non-ionic form only on acidification, then an adverse isotope effect could obscure the results. This possibility, although it must be considered, appears remote.

Additional experiments were performed to test the hypothesis that one tritium atom is introduced into the thiazole portion of the coenzyme, in accordance with Breslow's mechanism.[2] The experimental conditions were so chosen that the non-enzymatic exchange of hydrogen and tritium occurs too slowly to obscure the exchange. The results of these experiments, unfortunately, were not clear-cut. However, the crude results indicate that the enzyme probably does induce hydrogen–tritium exchange in the coenzyme molecule.

Acknowledgment.—The authors take pleasure in acknowledging the coöperation of Dr. Kenneth Wilzbach, of the Argonne National Laboratories, who carried out the tritium analyses essential to this work. They wish also to acknowledge the help of Mr. Keelin Fry with the deuterium control experiments, and the generous financial support of the National Institutes of Health.

CAMBRIDGE 38, MASS.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Thiation of Nucleosides. II. Synthesis of 5-Methyl-2′-deoxycytidine and Related Pyrimidine Nucleosides[1]

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Uracil or thymine nucleosides were converted into a series of analogs in which the oxygen atom at position 4 has been replaced by other functional groups. This procedure involves thiation of suitably-protected nucleosides and has led to a facile synthesis of the hitherto-rare, naturally-occurring nucleoside, 5-methyl-2′-deoxycytidine, from thymidine. Similarly, 5-methylcytidine and cytidine were prepared from 1-β-D-ribofuranosylthymine and uridine, respectively. The 4-thio intermediates of blocked nucleosides served as excellent chemical precursors for the synthesis of a host of 4-substituted derivatives of 1-β-D-ribofuranosyl-, 2′-deoxyribofuranosyl-, -xylofuranosyl-, and -glucopyranosyl-pyrimidine nucleosides such as the 4-alkylamino, 4-hydrazino, 4-hydroxylamino, 4-thio, 4-azido (or tetrazolo) and other analogs. A comparison of the spectrally-determined pK_a values of nucleosides with and without a 5-methyl substituent is given and the ultraviolet absorption spectra of the more important nucleosides at different *p*H values are described. 1-Methyl-4-thiouracil was synthesized from 1-methyluracil and converted to 1-methylcytosine. 4-Hydrazino-2(1H)-pyrimidinone was prepared from 4-ethoxy-2(1H)-pyrimidinone and 1,5-dimethylcytosine was synthesized from 1,5-dimethyl-4-ethoxy-2(1H)-pyrimidinone.

On the basis of metabolic studies[2] it was demonstrated that in the mammal the naturally-occurring pyrimidine nucleosides (cytidine, and to a lesser extent uridine and thymidine) are extensively incorporated into the nucleic acids. These studies pointed to the desirability of developing methods for the synthesis of pyrimidine nucleosides adaptable for the incorporation of radio-isotopes and for the synthesis of pyrimidine nucleoside analogs for screening as potential chemotherapeutic agents. As a result, relatively facile synthetic routes (*via* the mercuri procedure) to cytidine, 5-methyluridine and uridine[3,4] were developed, and, indeed, doubly-labeled cytidine has since been prepared by these procedures.[5]

Chemical conversion of uracil or thymine moieties of furanosyl nucleosides to other derivatives have been reported.[6] These alterations, however, were effected at position 5 (*i.e.*, 5-halogeno, 5-amino, 5-hydroxy, *etc.*) and at position 3 (*i.e.*, 3-methyluridine) of the pyrimidine residues. Chemical alteration of cytosine moieties of nucleosides to their

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(2) See chapter on the biosynthesis of nucleic acids, G. B. Brown and P. M. Roll, in "The Nucleic Acids," Vol. II (Chargaff and Davidson, eds.), Academic Press, Inc., New York, N. Y., 1955, p. 350.

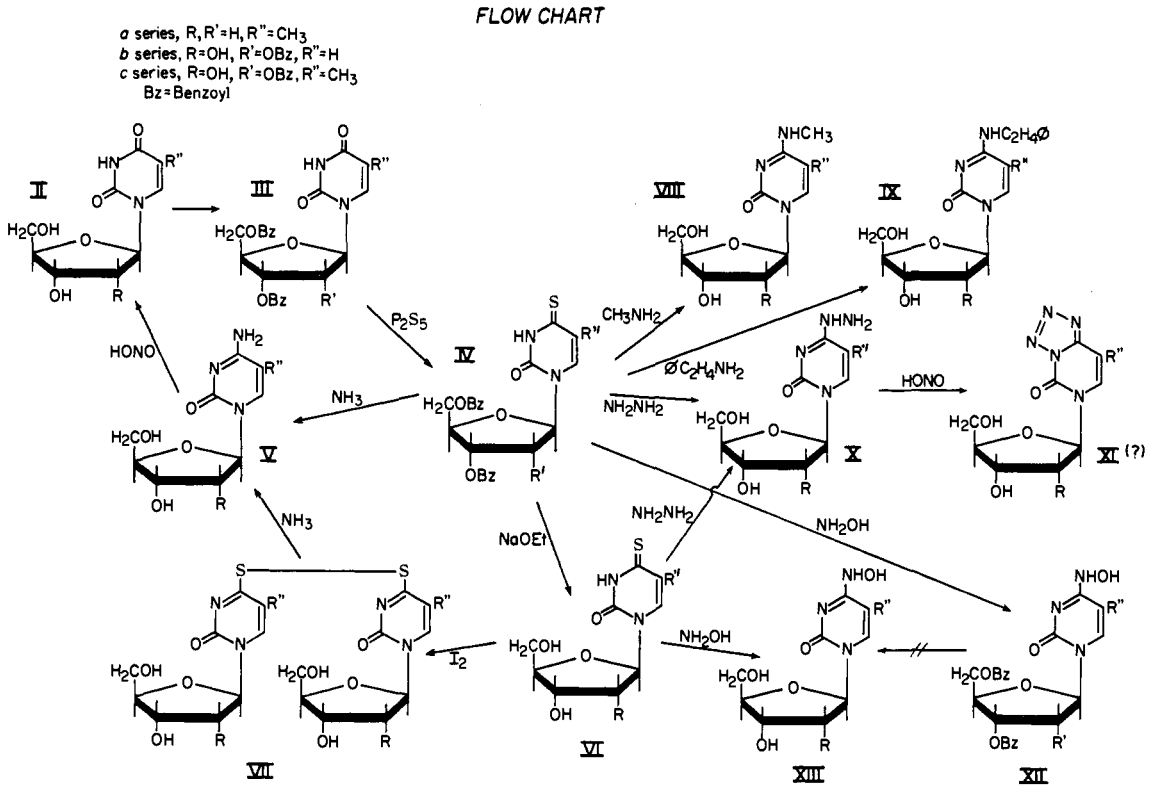
(3) J. J. Fox, N. Yung, J. Davoll and G. B. Brown, THIS JOURNAL, **78**, 2117 (1956).

(4) J. J. Fox, N. Yung, I. Wempen and I. L. Doerr, *ibid.*, **79**, 5060 (1957).

(5) J. F. Codington, R. Fecher, R. Y. Thomson, M. H. Maguire and G. B. Brown, *ibid.*, **80**, 5164 (1958).

(6) (a) P. A. Levene and F. B. LaForge, *Ber.*, **45**, 615, 616 (1912); (b) M. Roberts and D. W. Visser, THIS JOURNAL, **74**, 668 (1952); (c) D. W. Visser, G. Barron and R. Beltz, *ibid.*, **75**, 2017 (1953); (d) T. K. Fukubara and D. W. Visser, *ibid.*, **77**, 2393 (1955); (e) T. K. Fukuhara and D. W. Visser, *J. Biol. Chem.*, **190**, 95 (1951); (f) P. A. Levene and W. A. Jacobs, *Ber.*, **43**, 3150 (1910).

FLOW CHART



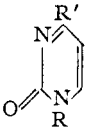
corresponding uracil analogs was accomplished about 50 years ago by Levene and Jacobs[6f] who converted cytidine to uridine with nitrous acid. The reverse of this reaction, that is, the chemical conversion of uridine-type nucleosides to their cytosine counterparts has not been reported.

In the present paper, syntheses are presented for the preparation of analogs of nucleosides with variations at position 4 of the pyrimidine ring *via* direct thiation of suitably-blocked natural or synthetic nucleosides. Thiation studies with naturally-occurring purine nucleosides have been reported recently[7] (part I of this series).

Results and Discussion

Since the first reports[8] of the thiation of barbitals and hydantoins, several studies have appeared dealing with the preparation of thio derivatives of pyrimidines.[9] In these studies, uracil and various 2-thiouracils were treated with phosphorus pentasulfide in inert solvents at elevated temperatures. Application of this type of reaction to pyrimidine nucleosides would require suitable blocking groups for the sugar hydroxyls and milder reaction conditions. The conditions established for the successful thiation of purine nucleosides[7] (*i.e.*, O-benzoyl blocking groups and the use of pyridine as the reaction solvent) were found to be quite satisfactory for thiation in the pyrimidine nucleoside series.

In order to gain some insight as to which oxygen atom(s) would be replaced when suitably-blocked pyrimidine nucleosides are treated with phosphorus pentasulfide in pyridine, 1-methyluracil was used as a model. Thiation of this substance gave a mono-thio derivative (A) which, when treated with alcoholic ammonia in a sealed tube, yielded the known 1-methylcytosine[10] (I, R = CH_3, R′ = NH_2). Similarly, from 4-thiouracil, cytosine was obtained. The structure of (A) is therefore 1-methyl-4-thiouracil indicating that



I

thiation of 1-substituted pyrimidines (*i.e.*, nucleosides) would proceed selectively to 4-thio analogs.[11]

These results are in accord with the studies of Carrington[8] and Russell, *et al.*,[9b] who showed that with 2,4-dithiobarbiturates, dithiohydantoins and dithiopyrimidines, only the 4-thio group was replaced by treatment with ammonia or amines. It may be concluded, therefore, that the presence of an oxygen atom in place of sulfur at position 2 of 2,4-dithiopyrimidines seems to exert no appreciable effect upon the ease of replacement of the 4-thio function by amines.

(7) J. J. Fox, I. Wempen, A. Hampton and I. L. Doerr, THIS JOURNAL, **80**, 1669 (1958).

(8) H. R. Henze and P. E. Smith, *ibid.*, **65**, 1090 (1943); H. C. Carrington, *J. Chem. Soc.*, 124 (1944); 684 (1947).

(9) (a) G. B. Elion and G. H. Hitchings, THIS JOURNAL, **69**, 2138 (1947); (b) P. B. Russell, G. B. Elion, E. A. Falco and G. H. Hitchings, *ibid.*, **71**, 2279 (1949); (c) E. A. Falco, P. B. Russell and G. H. Hitchings, *ibid.*, **73**, 4466 (1951).

(10) G. E. Hilbert, *ibid.*, **56**, 190 (1934).

(11) Elion and Hitchings[9a] have shown that 1,3-dimethyluracil may be thiated to a mono-thio derivative which, on the basis of spectral comparisons, was assigned the structure of 1,3-dimethyl-4-thiouracil. The spectrum of the neutral species of 1-methyl-4-thiouracil and of 1,3-dimethyl-4-thiouracil are essentially similar. Thiation of uracil, itself, however, gave rise to 2,4-dithiouracil.

Treatment of thymidine (IIa) with two moles of benzoyl chloride in pyridine afforded 3′,5′-di-*O*-benzoylthymidine (IIIa, see flow chart) in 90% yield. This compound had been prepared previously by Weygand and Sigmund[12] from enzymic digests of DNA. When three moles of benzoyl chloride were used, a tribenzoyl derivative of thymidine was obtained. The position of attachment of the third benzoyl group to the pyrimidine ring has not been established. This tribenzoate gave poorer yields than the di-*O*-benzoate in the subsequent reaction. A similar situation obtained in thiation studies on benzoylated guanosine.[7] The di-*O*-benzoate IIIa was thiated with phosphorus pentasulfide in pyridine to the 4-thio analog IVa in about 80% yield. Intermediate IVa served as an excellent precursor for the synthesis of several thymidine analogs.

When allowed to react with alcoholic ammonia in a sealed tube, IVa was debenzoylated and the 4-thio group replaced by an amino function thus giving rise to 1-(2-deoxy-β-*D*-ribofuranosyl)-5-methylcytosine (Va), (also referred to as 5-methyl-2′-deoxycytidine or 5-methylcytosine-2′-deoxyriboside, 5MCDR) which was isolated as the crystalline hydrochloride salt in 60% yield; 5MCDR has been isolated as a constituent of wheat-germ DNA by Dekker and Elmore.[13] Unfortunately, a sample of the naturally-occurring material was not available for comparison. Except for small differences, however, the physical properties of synthetic 5MCDR closely paralleled those reported for the natural substance.

As noted previously,[14] the reported spectrum of 5MCDR[13] did not agree in some details with the spectrum of this substance predicted on the basis of observed spectral relationships among cytosine nucleosides. The ultraviolet absorption spectrum of synthetic 5MCDR (Va, see Fig. 1) as a function of *p*H exhibits a sharp isosbestic point[15] at 271 mμ rather than at 275 mμ. Recently Cohen and Barner[16] have reported that the acid and basic curves for 5MCDR intersect at 272 mμ. An isosbestic point at 272 mμ has been reported for 5-methyl-2′-deoxycytidylic acid by Cohn.[17] Also to be noted is the tangential relationship in the 222 mμ region between the acid and neutral curves for synthetic 5MCDR, a characteristic which also had been predicted on the basis of comparisons to other cytosine nucleosides.[14] Above *p*H 12, the spectrum shifts (see curve for *p*H 14) which denotes a new equilibrium. Similar spectral shifts have been observed with cytidine, 2′-deoxycytidine and, in fact, with all pyrimidine nucleosides hitherto examined[3,14] and are ascribed to the effect of dissociation of the sugar moiety upon the aglycon. A mechanism to explain these spectral shifts in the high alkaline region has been advanced.[18]

Synthetic 5MCDR also gave a crystalline picrate. When treated with nitrous acid followed by chromatography of the reaction mixture with Dowex 1 ion exchange resin, Va yielded crystalline thymidine. This reaction demonstrates that the thiation process does not in any way alter the sugar moiety. A similar conclusion had been reached from thiation studies with purine nucleosides.[7] When Va was hydrolyzed with perchloric acid, 5-methylcytosine was obtained.

Debenzoylation of IVa with sodium methylate in methanol yielded a yellow glass (VIa, 4-thiothymidine) which was converted to the crystalline disulfide (VIIa) by oxidation with iodine. The ultraviolet absorption spectrum of VIa resembled that for 1-methyl-4-thiouracil (see Experimental section). Treatment of IVa with methylamine, β-phenylethylamine or with hydrazine (95%) gave the 4-methylamino (VIIIa), the 4-β-phenylethylamino (IXa) and the 4-hydrazino (Xa) analogs of 5MCDR (Va). All of these reactions were accompanied by removal of the benzoyl blocking groups. "4-Hydrazinothymidine" (Xa) may also be prepared from 4-thiothymidine (VIa) or its disulfide (VIIa) by reaction with hydrazine. The absorption spectra of VIIIa, IXa and Xa were similar to that for 5MCDR as well as other 5-methylcytosine nucleosides.[14] A similar spectral pattern (*p*H region of 1–7) was given by 4-hydrazino-2(1H)-pyrimidinone (I, R = H, R′ = $NHNH_2$) which was prepared by reaction of 4-ethoxy-2-(1H)-pyrimidinone with hydrazine.

Treatment of the 4-hydrazino nucleoside Xa with nitrous acid afforded a crystalline substance XIa which was presumed to be the 4-azido analog. The ultraviolet absorption spectrum of this substance (see Fig. 2) is unchanged between *p*H values 110 in accord with the absence of dissociable groups. Above this *p*H range (*i.e.*, 12–14) the compound decomposes as evidenced by a decrease in absorption over the entire wave length range studied.

Similarly, 4-hydrazino-2(1H)-pyrimidinone also gave a crystalline substance when treated with nitrous acid. The spectrum (see Fig. 2) of this pyrimidine derivative (*p*H 1–4) resembles that for XIa. Above *p*H 4, the maximum of the pyrimidine at 249 mμ undergoes a decrease in extinction and a bathochromic shift accompanied by the appearance of a new maximum at 268 mμ. A similar pattern has been noted for cytosine (*p*H range 10-14) and seems to be characteristic for ionization at the 1:2-positions of 4-amino- or 4-hydroxy-2-pyrimidinones.[19] The spectrally-determined pK_a value for "4-azido-2-pyrimidinone" is 6.95 which makes this substance at least 100,000 times a stronger acid than the corresponding 4-amino analog, cytosine (pK_a 12.2[19]).

Infrared data[20] would indicate, from the absence of absorption in the 2130 $cm.^{-1}$ region, that both XIa and its free base do not possess the azido structure. They could possess the tetrazolo fused

(12) F. Weygand and W. Sigmund, *Z. Naturf.*, **9**, 800 (1954).

(13) C. A. Dekker and D. T. Elmore, *J. Chem. Soc.*, 2864 (1951).

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

(15) For the significance of isosbestic points see ref. 14. See also A. Bendich in "The Nucleic Acids," Vol. 1, Chargaff and Davidson, eds., Academic Press, Inc., New York, N. Y., 1955, p. 81.

(16) S. S. Cohen and H. D. Barner, *J. Biol. Chem.*, **226**, 631 (1957).

(17) W. E. Cohn, THIS JOURNAL, **73**, 1539 (1951).

(18) (a) J. J. Fox, L. F. Cavalieri and N. Chang, *ibid.*, **75**, 4315 (1953); (b) J. J. Fox, J. F. Codington, N. C. Yung, L. Kaplan and J. O. Lampen, *ibid.*, **80**, 5155 (1958).

(19) D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta*, **9**, 199 (1952).

(20) The authors are indebted to Dr. H. J. Schaeffer of the Southern Research Institute for the infrared data.

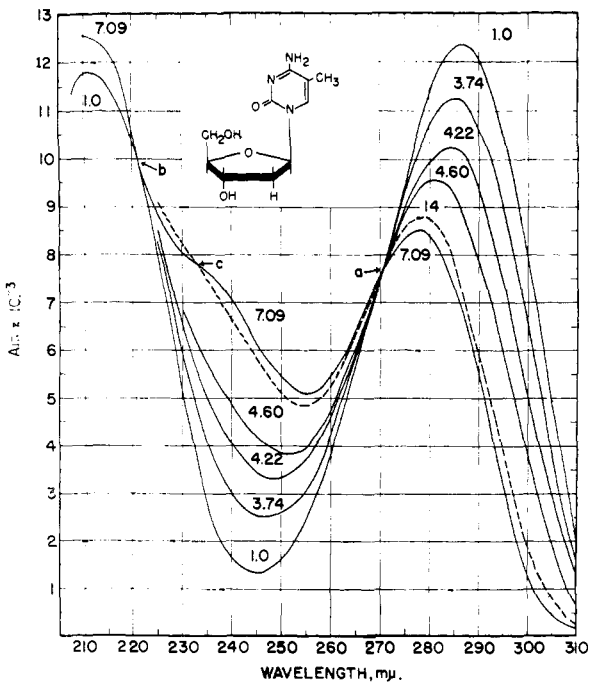


Fig. 1.

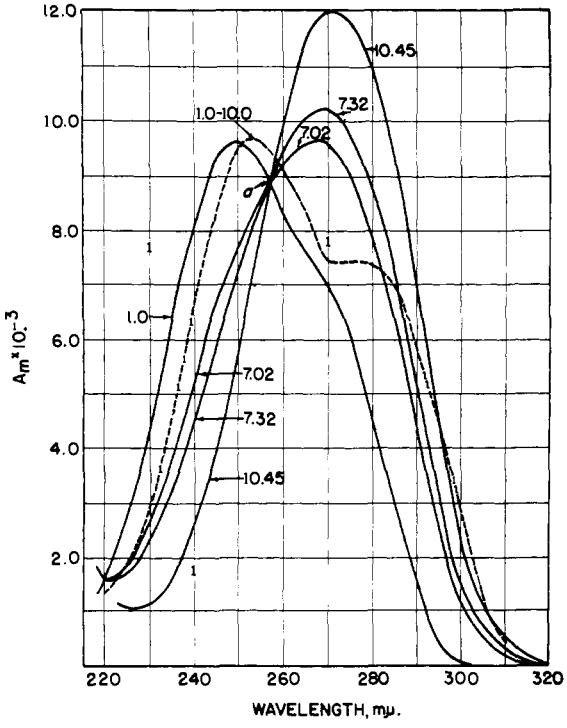


Fig. 2.—Solid lines represent the spectrum of 4-azido(?)-2(1H)-pyrimidinone (probably a tetrazolo structure) in aqueous solutions at *p*H values indicated. Broken-line curve represents the spectrum of XIa in the *p*H range of 1–10.

ring structure (see flow chart) by cyclization of the azido function with nitrogen 3 of the pyrimidine ring. Fargher and Furness[21a] have found that 2-hydrazinopyridine yielded a tetrazolo structure upon treatment with nitrous acid. On the other hand, 2,4-diazidopyrimidine was prepared by Benson, *et al.*,[21b] by nitrous acid treatment of 2,4-dihydrazinopyrimidine and was shown to possess two bands, one at 2160 and the other at 2130 cm.$^{-1}$ by Johnson and co-workers.[22] It is evident that further study will be necessary for the characterization of XIa or its free base with certainty.

By reaction with hydroxylamine in ethanol IVa was converted to the 4-hydroxylamino analog XIIa, but the benzoyl blocking groups were not removed during the process. (It is to be noted that hydroxylamine is a weaker base than ammonia, methylamine, β-phenylethylamine or hydrazine.) Attempts to debenzoylate XIIa to XIIIa with ammonia or alkoxide were unsuccessful, due, most likely, to the instability of the hydroxylamino pyrimidine nucleoside under these conditions. However, XIIIa was obtained by treatment of 4-thiothymidine (VIa) with hydroxylamine.

The spectrum of XIIIa (see Fig. 3) is akin to that for 5MCDR (Fig. 1) in the *p*H range of 0.0

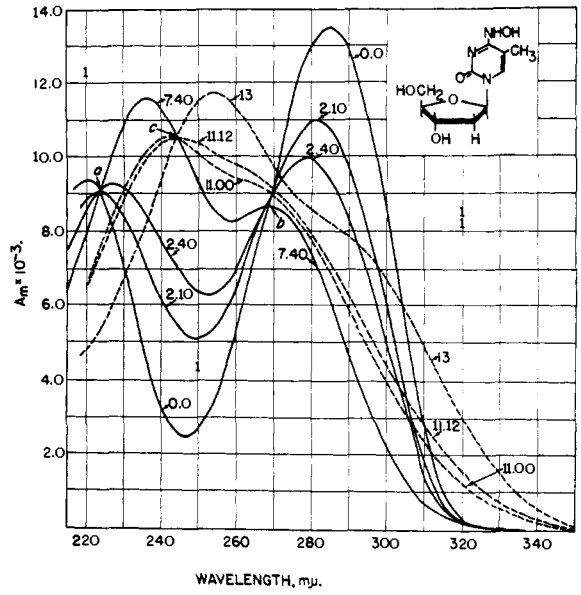


Fig. 3.

to 7.4. Above this *p*H (7.4–13), XIIIa exhibits a second dissociation due to the formation of an anionic species by loss of a proton from the hydroxylamino function. In this regard, the spectrum of XIIIa (and also of XIIIb; see Fig. 4) differ from all other cytosine-type nucleosides hithertofore examined (see Table II). Above *p*H 13 (in the *p*H range where ionization of the sugar would normally occur) XIIIa decomposes. 6-N-Hydroxylaminopurine[23] also has been observed to decompose at high *p*H values. The curve for *p*H 7.40 (neutral species) is the limiting curve for both equilibria and passes, therefore, through all isosbestic points.

A similar sequence of reactions was carried out with uridine (IIb). Benzoylation of this nucleo-

(21) (a) R. C. Fargher and R. Furness, *J. Chem. Soc.*, 688 (1915); (b) F. R. Benson, L. W. Hartzel and E. A. Otten, THIS JOURNAL, **76**, 1858 (1954).

(22) J. A. Johnson, Jr., H. J. Thomas and H. J. Schaeffer, *ibid.*, **80**, 699 (1958).

(23) A. Giner-Sorolla and A. Bendich, *ibid.*, **80**, 3932 (1958).

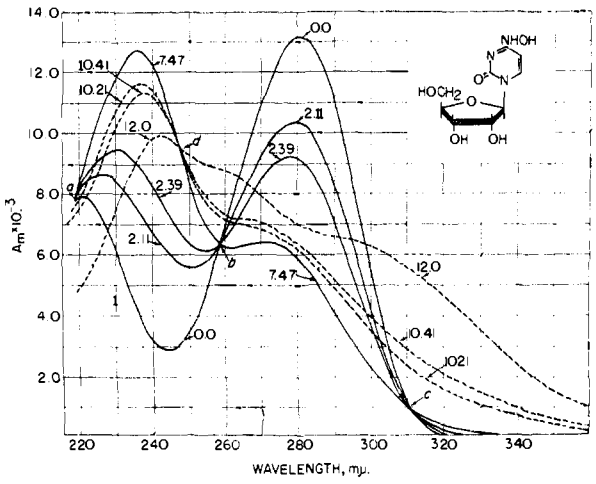


Fig. 4.

side afforded a tri-*O*-benzoyl derivative (IIIb). With a large excess of benzoyl chloride in pyridine, a tetrabenzoylated derivative of IIb was obtained. The tri-*O*-benzoate was thiated with phosphorus pentasulfide in pyridine to the 4-thio intermediate IVb which was used for the preparation of several uridine analogs.

With alcoholic ammonia, IVb was converted to cytidine (Vb, 1-β-D-ribofuranosylcytosine) which was identical with the naturally-occurring nucleoside of RNA as well as synthetic cytidine prepared by another route.[4] With methylamine and β-phenylethylamine, IVb was converted into the corresponding amino analogs (VIIIb and IXb, respectively) of cytidine. 4-Thiouridine (VIb) was obtained as a glass by debenzoylation of IVb and was oxidized to its crystalline disulfide VIIb. The hydroxylamino analog XIIIb of cytidine was obtained by treatment of 4-thiouridine with hydroxylamine in ethanol. It is of interest to note, in this regard, that Lieberman[24] has reported the enzymic conversion of uridine triphosphate to 4-hydroxylamino analogs of a nucleotide (tentatively assigned as the di- and triphosphate analogs of cytidine) by the use of a partially purified enzyme from *E. coli* B. in the presence of hydroxylamine and adenosine triphosphate. The spectral curve reported by Lieberman[24] for the nucleotide is in good agreement with the detailed spectrum of the nucleoside XIIIb (see Fig. 4).

It is obvious that this thiation process may be applied to synthetic nucleosides as well. 1-β-D-Ribofuranosylthymine[3] (IIc) (in the form of its tri-*O*-benzoate IIIc) was thiated to the 4-thio analog IVc and converted to 5-methylcytidine (Vc, 5MCR) with ammonia. When treated with sodium metaperiodate, Vc consumed one mole of oxidant per mole of nucleoside very rapidly, in accord with a furanosyl structure containing α-*cis*-glycols.[25] This nucleoside (Vc) differed in melting point from a "5-methylcytidine" reported previously by Roberts and Visser[6b] and a mixed melting point of the two gave a depression.[26] The absorption spectrum of 5-methylcytidine (see Fig. 5) is closely akin to that for 5-MCDR

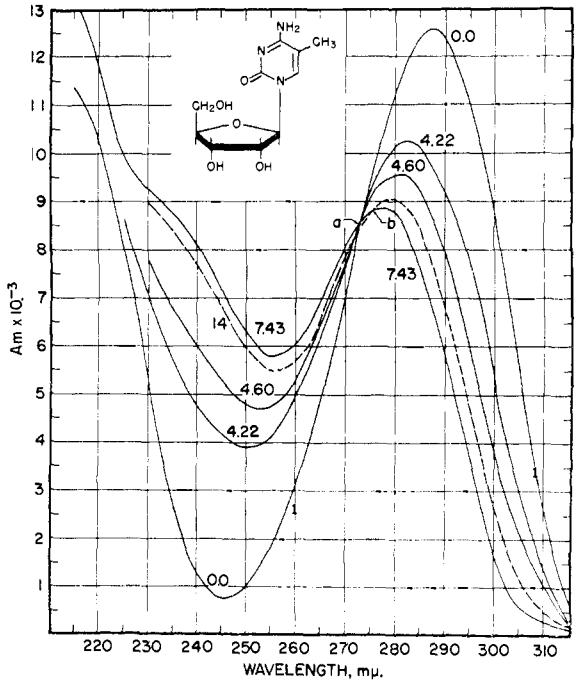


Fig. 5.

(see Fig. 1). Further evidence that Vc is indeed 1-β-D-ribofuranosyl-5-methylcytosine is shown (see Table I) by a comparison of the molecular rotations of Vc and Va to cytidine and 2′-deoxycytidine (V, R, R″ = H).

TABLE I

	$[\alpha]_D$[a]	$[M]_D$	Difference in $[M]_D$
Cytidine (Vb)	+12	+ 2,920	−15,690
2′-Deoxycytidine	+82[b]	+18,610	
5-Methylcytidine (Vc)	− 3	− 770	−15,710
5-MCDR (Va)[d]	+62[c]	+14,940	

[a] All rotations are reported to the nearest degree and were determined in 1.00 *N* NaOH. [b] Value from O. Schindler, *Helv. Chim. Acta*, **32**, 979 (1949). [c] Dekker and Elmore[13] report $[\alpha]^{14}D$ +65 ± 4°. [d] The hydrochloric salt of Va was used. The optical rotation is a corrected value for the free nucleoside.

Finally, 1-β-D-glucopyranosylthymine, in the form of its 2′,3′,4′,6′-tetra-*O*-acetate, was thiated to the 4-thio derivative (obtained as a glass) and treated with alcoholic ammonia in a sealed tube. 1-β-D-Glucopyranosyl-5-methylcytosine was obtained. Similarly, thiation of 1-(tri-*O*-benzoyl-β-D-xylofuranosyl)-thymine,[3] followed by treatment

(24) I. Lieberman, *J. Biol. Chem.*, **222**, 765 (1956).

(25) In previous studies[3,4] it has been demonstrated that ribofuranosylpyrimidine nucleosides exhibit a rapid uptake of metaperiodate, being completely oxidized to their dialdehydes within 5 minutes. In a more recent study[18b] a similarly-rapid uptake was noted for 1-β-D-lyxofuranosylthymine which also possesses the α-*cis*-glycol structure.

(26) The fact that Vc and the Roberts-Visser sample were not identical was to be expected since the compound which they designated as "5-methyluridine" (prepared[6b] from the same intermediate which they used for the preparation of a compound termed "5-methylcytidine") differed in melting point, optical rotation and susceptibility to enzymic cleavage from authentic 1-β-D-ribofuranosylthymine synthesized by another route.[3] Authentic 5-methyluridine (1-β-D-ribofuranosylthymine) was cleaved by intact cell suspensions of *E. coli* B. to thymine.[18b] 5-Methylcytidine (Vc) and 5MCDR (Va), when treated similarly with cells of *E. coli* B., also gave rise to thymine (L. Kaplan and J. J. Fox, unpublished data).

of the 4-thio intermediate with alcoholic ammonia, afforded 1-β-D-xylofuranosyl-5-methylcytosine.

Ionization Constants.—The spectrophotometrically-determined pK_a values for cytosine, 5-methylcytosine and their nucleosides are shown in Table II. The presence of a methyl group on either positions of 1 or 5 of cytosine raises the pK_a for the basic dissociation from 0.10 to 0.15 *p*H unit. 1-Methylcytosine and 1,5-dimethylcytosine are, in turn, progressively stronger bases than cytosine indicating that this base-strengthening effect of alkylation at positions 1 and 5 is additive. The pK_a values of 5-methylcytosine nucleosides are approximately 0.15 *p*H unit higher than their corresponding cytosine analogs.

Introduction of a sugar in place of methyl or hydrogen at position 1 of cytosine or 5-methylcytosine lowers the pK_{a1} of the resulting nucleoside. This base-weakening effect is greatest for the 1-D-glycopyranosyl nucleosides and weakest for the 1-β-D-2′-deoxyribofuranosyl derivatives. These consistent differences would emphasize the point made previously[14] that interaction exists between the un-ionized sugar hydroxyls and the aglycon. As expected from previous studies, Va and Vc exhibit spectral shifts in the high alkaline range (*p*H 12–14) due to the effect of dissociation of the sugar moiety upon the aglycon (see Figs. 1 and 5).

TABLE II

SPECTRALLY-DETERMINED "APPARENT" pK_a VALUES FOR CYTOSINE AND 5-METHYLCYTOSINE NUCLEOSIDES AND RELATED COMPOUNDS[a]

	pK_{a1}	pK_{a2}	pK_{a3}
Cytosine[b]	4.45	12.2	None
1-Methylcytosine[c]	4.55	None	None
2′-Deoxycytidine[d]	4.25	None	>13
Cytidine[e]	4.11	None	>13
1-D-Glycopyranosyl-cytosines[e,g]	3.85	None	>13
5-Methylcytosine[b]	4.6	12.4	None
1,5-Dimethylcytosine	4.76	None	None
5-MCDR (Va)	4.40	None	>13
5-Methylcytidine (Vc)	4.28	None	>13
1-D-Glycopyranosyl-5-methylcytosines[e,f]	4.1	None	>13

[a] pK_a values are accurate to 0.05 *p*H unit; pK_{a1} refers to ionization of the 4-ammonium group, pK_{a2} to ionization at the 1:2 position and pK_{a3} to ionization of the sugar moiety. [b] *pK* values taken from ref. 19. [c] *pK* values taken from ref. 14. [d] *pK* values taken from ref. 18; Fox and Shugar[14] report 4.3. [e] These were the D-glucosyl, D-galactosyl, D-arabinosyl and D-xylosyl derivatives. [f] These are the D-arabinosyl and D-xylosyl derivatives.

Experimental[27]

1-(3,5-Di-*O*-benzoyl-2-deoxy-β-D-ribofuranosyl)-thymine (IIIa).—Thymidine (20.0 g., 0.083 mole) in 600 ml. of anhydrous pyridine was treated with exactly 0.166 mole of benzoyl chloride and the reaction flask placed in a constant temperature bath for 65–70 hours at 50–55°. The pale-orange solution was poured in a thin stream into a vigorously-stirred slurry of ice-water. A white somewhat sticky solid precipitated which gradually became granular as the stirring was continued. After complete solidification, the product was filtered off together with unmelted ice and was treated again with stirred ice-water for 15 minutes. After filtration, the precipitate was pressed as dry as possible and recrystallized from 3 liters of boiling ethanol. A white feathery solid was obtained, 31.7 g. (85%), m.p. 192.5–193.5°; Weygand and Sigmund[12] report m.p. 196°.

Anal. Calcd. for $C_{24}H_{22}N_2O_7$: C, 63.99; H, 4.92; N, 6.22; benzoyl no., 2.0. Found: C, 64.38; H, 4.77; N, 5.91; benzoyl no., 2.0.

Tribenzoylthymidine.—A stirred solution of thymidine (4.84 g., 0.02 mole) in 150 ml. of anhydrous pyridine was treated with 8.4 g. (0.06 mole) of benzoyl chloride. After two days at 50–55°, the solution was poured into well-stirred ice-water. The amorphous precipitate was removed and recrystallized from ethanol, 9.1 g. (82%), m.p. 124–125°. A sample was recrystallized for analysis from ethanol, needles, m.p. 125–126°.

Anal. Calcd. for $C_{31}H_{26}N_2O_8$: C, 67.14; H, 4.72; N, 5.05; benzoyl no., 3.0. Found: C, 67.04; H, 4.90; N, 4.88; benzoyl no., 2.8.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-uracil (IIIb).—Uridine (80.5 g., 0.33 mole) in 2300 ml. of anhydrous pyridine was treated dropwise, with stirring, with 128 ml. (1.10 moles) of benzoyl chloride and the reaction mixture allowed to remain 48 hours at 55–60°. About 1500 ml. of pyridine was removed *in vacuo* and the remaining mixture filtered from pyridinium chloride. The filtrate was added slowly in a thin stream to about 5 liters of vigorously-stirred ice-water. After one hour of stirring, some of the oily mass was converted to a fluffy precipitate. This precipitate was removed by filtration and the oily residue remaining in the beaker was treated anew with ice-water. By this procedure, 153 g. of solid was collected. The remaining oily residue was taken up in chloroform, washed with water and dried over sodium sulfate. After removal of the solvent, a residue was obtained. Both the residue and the original solid were combined and recrystallized from about one liter of hot benzene, cooled, filtered and dried; yield 90% (167 g.), m.p. 142–143°. A further recrystallization from benzene did not raise the melting point.

Anal. Calcd. for $C_{30}H_{24}N_2O_9$: C, 64.70; H, 4.34; benzoyl no., 3.0. Found: C, 64.74; H, 4.30; benzoyl no., 3.0.

Tetrabenzoyluridine.—Uridine (4.88 g., 0.02 mole) in 100 ml. of dry pyridine was treated with 30 ml. (0.26 mole) of benzoyl chloride and the mixture stirred or shaken at room temperature for 2–3 hours. The reaction contents were poured into ice-water and stirred for 1 hour. After decantation of the water, the oily residue was dissolved in chloroform and the solution washed successively twice with water, once with cold 2 *N* sulfuric acid, once with cold bicarbonate solution and finally with water. After drying the chloroform solution over sodium sulfate and filtration, the solvent was removed *in vacuo* leaving a slightly pink oily residue. This residue was dissolved in a minimum of warm ethanol and ether was added to turbidity. After several days a precipitate was collected, 2.0 g., m.p. 147–148°. Additional product could be obtained from the mother liquor. A mixed melting point with tri-*O*-benzoyluridine (IIIb) showed a depression, m.p. 134–141°.

Anal. Calcd. for $C_{37}H_{28}N_2O_{10}$: C, 67.27; H, 4.27; N, 4.24; benzoyl no., 4.0. Found: C, 67.12; H, 4.52; N, 4.49; benzoyl no., 3.9.

1-(3,5-Di-*O*-benzoyl-β-D-2-deoxyribofuranosyl)-4-thiothymine (IVa).—Di-*O*-benzoylthymidine (IIIa, 20.0 g., 0.044 mole) in 600 ml. of reagent-grade pyridine was treated with 37.0 g. (0.17 mole) of phosphorus pentasulfide and 1.8 ml. of water.[28] The orange, turbid mixture was refluxed for 4 hours after which it was concentrated to approximately one-half of the volume and poured into stirred, cold water. The precipitate was removed by filtration and dissolved in chloroform. The chloroform solution was filtered from some insoluble material, washed with water and dried over

(27) Melting points were determined by the capillary method and are uncorrected unless stated otherwise. Analyses were performed by the Schwarzkopf Microanalytical Laboratory and by Dr. J. F. Alicino. Paper chromatograms were run by ascending method using Schleicher and Schuell No. 597 paper.

(28) If water is omitted from this reaction, the contents darken considerably within 30 minutes. Isolation of the desired product becomes more laborious and the yields are usually lower. Enough water should be added dropwise so that the reaction takes on an orange-turbid appearance which remains permanent. Attempts to correlate (stoichiometrically) the amount of water needed to produce the turbid-orange appearance with starting materials have been unsuccessful.

sodium sulfate. After filtration, the solvent was removed *in vacuo* and the residue taken up in about one liter of hot ethanol. Yellow needles were obtained, m.p. 156–158°, 15 g. (72%). One recrystallization from ethanol raised the melting point to 159–160°, $[\alpha]^{25}D$ −52° (*c* 1.2 in $CHCl_3$).

Anal. Calcd. for $C_{24}H_{22}N_2O_6S$: C, 61.79; H, 4.75; N, 6.01; S, 6.88. Found: C, 61.67; H, 4.82; N, 5.93; S, 6.84.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-4-thiouracil (IVb).—A mixture containing 5.56 g. of IIIb, 8.88 g. of phosphorus pentasulfide and 150 ml. of reagent grade pyridine was refluxed with stirring for 5 hours. It was not necessary to add water to this reaction. About one-third of the pyridine was removed and the brown-colored mixture was poured into stirred water and the solvent decanted from an oily residue. The residue was dissolved in chloroform and filtered from some insoluble material. The chloroform solution was washed twice with water and dried over sodium sulfate. After filtration, the filtrate was concentrated to dryness *in vacuo* and the residue dissolved in 150 ml. of hot ethanol and cooled slowly to room temperature. Clusters of yellow prisms were obtained, 4.96 g. (87%), m.p. 128–130°. The compound was recrystallized once from ethanol and the precipitate (after filtration) triturated with ether. The melting point was unchanged by recrystallization.

Anal. Calcd. for $C_{30}H_{24}N_2O_8S$: C, 62.93; H, 4.20; N, 4.90; S, 5.59. Found: C, 62.80; H, 4.24; N, 4.92; S, 5.66.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-4-thiothymine (IVc).—A well-stirred mixture of tri-*O*-benzoyl-β-D-ribofuranosylthymine[3] (IIIc) (4.0 g., 0.007 mole) and phosphorus pentasulfide (7.8 g., 0.035 mole) in 125 ml. of reagent grade pyridine was treated dropwise with 0.4 ml. of water[28] and the orange-turbid solution heated at reflux temperature for 7 hours.[29] Approximately two-thirds of the pyridine was removed *in vacuo* and remaining reaction mixture poured into 1 liter of vigorously-stirred water. The cream-colored suspension was stirred for 0.5 hour, filtered, and the precipitate dissolved in methylene chloride. The yellow solution was filtered from some insoluble material. The filtrate was washed twice with an equal volume of water and the separated organic layer dried over sodium sulfate. After removal of the solvent *in vacuo*, a yellow oil was obtained which solidified upon trituration with ethanol. The yellow solid was recrystallized from 300 ml. of boiling ethanol, yield 3.4 g. (83%), m.p. 189–191°. A second recrystallization gave analytically pure material, m.p. 190–191°.

Anal. Calcd. for $C_{31}H_{26}N_2O_8S$: C, 63.47; H, 4.47; N, 4.78; S, 5.47. Found: C, 63.41; H, 4.49; N, 4.47; S, 5.35.

1-(2,3,5-Tri-*O*-benzoyl-β-D-xylofuranosyl)-4-thiothymine.—A well-stirred mixture containing 2.0 g. (0.0035 mole) of 1-(tri-*O*-benzoyl-β-D-xylofuranosyl)-thymine,[3] 3.0 g. of phosphorus pentasulfide and 60 ml. of reagent grade pyridine was treated dropwise with 0.15 ml. of water and the orange-turbid solution refluxed for 7 hours. The reaction mixture was worked up in a manner similar to that used for the preparation of IVc (*vide supra*). The crude product was recrystallized from ethanol, 1.8 g. (87%), m.p. 169–173°. A second recrystallization from ethanol yielded 1.58 g. of pure material, m.p. 166–168° (sinters at 135°).

Anal. Calcd. for $C_{31}H_{26}N_2O_8S$: C, 63.47; H, 4.47; N, 4.78; S, 5.47. Found: C, 63.51; H, 4.41; N, 4.89; S, 5.71.

1-(2-Deoxy-β-D-ribofuranosyl)-5-methylcytosine, 5MCDR (Va).—Di-*O*-benzoyl-4-thiothymidine (IVa, 7.0 g., 0.015 mole) was treated with 80 ml. of alcoholic ammonia (previously saturated at 0°) in a sealed tube at 100° for 24 hours. After the tube was opened, the amber-green solution was concentrated to dryness. Water was added and most of the ethyl benzoate removed by vacuum distillation. The aqueous solution was extracted three times with chloroform to remove benzamide after which it was treated with charcoal, filtered and the filtrate concentrated *in vacuo* to dryness. The residue was taken up in warm ethanol and treated with gaseous hydrogen chloride (with moderate cooling of the reaction flask). Precipitation of the hydrochloride salt of 5MCDR occurred. After cooling overnight, the precipitate was removed (2.85 g., 70%, m.p. 149–151° dec.). Paper chromatography of this product in butanol–water (86:14) showed only one spot. After recrystallization from 125–150 ml. of ethanol, pure material was obtained as glistening prisms which, after drying *in vacuo* for 48 hours at 100°, melted at 154–155° dec., and analyzed for the un-hydrated hydrochloride salt, $[\alpha]^{23}D$ +54° (for the hydrochloride, *c* 1.02 in 1 *N* NaOH) and +62° when calculated for the free nucleoside (Dekker and Elmore report m.p. 156° for the *mono*-hydrated hydrochloride and $[\alpha]^{14}D$ +65 ± 4°). Ultraviolet absorption data: In 0.10 *N* HCl, maxima at 212 and 286.5 mμ, $A_{M(max)}$ of 11,800 and 12,400, respectively; minimum at 245 mμ, $A_{M(min)}$ of 1,310: at *p*H 7–12, maxima at ~208 and 277 mμ $A_{M(max)}$ of ~12,600 and 8,500, respectively; minimum at 255 mμ, $A_{M(min)}$ of 5,020; in 1.00 *N* NaOH, maximum at 279 mμ, $A_{M(max)}$ of 8,770; minimum at 254.5, $A_{M(min)}$ of 4,860: isosbestic point for curves between *p*H 1–12 at 271 mμ, A_M (isosbestic)[16] of 7,600. (These spectral data differ significantly from literature[13] values.) Spectral ratios: at *p*H 1–2, 250/260 = 0.43, 280/260 = 0.30; at *p*H 7–12, 250/260 = 0.99, 280/260 = 1.54.

Anal. Calcd. for $C_{10}H_{16}N_3O_4Cl$: C, 43.24; H, 5.81; N, 15.13; Cl, 12.78. Found: C, 43.43; H, 5.85; N, 15.51, 15.45; Cl, 12.84.

5MCDR Picrate.—Treatment of Va with aqueous picric acid according to Dekker and Elmore[13] afforded the fine, needle-like picrate salt of 5MCDR. After three recrystallizations from ethanol the following melting point properties were observed: begins to darken at 170° and becomes progressively darker as the temperature is raised. At 230° the solid is dark brown (Dekker and Elmore report m.p. 175–178° dec.).

Anal. Calcd. for $C_{16}H_{18}N_6O_{11}$: C, 40.85; H, 3.86; N, 17.87. Found: C, 40.79; H, 3.80; N, 18.20.

Conversion of 5MCDR to Thymidine.—5MCDR (0.7 g.) was treated with 0.4 g. of sodium nitrite in 25 ml. of water. The solution was warmed to 60° for 10 minutes and allowed to sit overnight. Paper chromatography of the reaction contents showed a major spot with an R_f similar to thymidine (1-butanol–water, 86:14) and a minor spot corresponding to starting material. The solution was concentrated to 4 ml. This material was subjected to ion-exchange chromatography according to Anderson, *et al.*[30]

Dowex-1 (chloride) was converted to the formate form (*p*H 10.3) with 0.01 *N* ammonium formate and NH_4OH. The mixed nucleoside solution (4 ml.) was applied and fractions collected by elution with 0.01 *N* ammonium formate (adjusted to *p*H 10.3 with ammonia). The first four 15-ml. fractions contained 5MCDR as determined by ultraviolet absorption spectrum and absorption ratios (see above). Ammonium formate (0.05 *N* adjusted to *p*H 5 with formic acid) then was passed through the column. Fractions 5–10 contained thymidine as shown by spectral characteristics (maximum at 267 mμ, minimum at 235 mμ at *p*H 7). These fractions were concentrated to dryness and heated in a rotary evaporator at 100° for 2 hours to remove ammonium formate. Absolute alcohol was added to the residual gum which crystallized almost instantly; yield 0.3 g., m.p. 183–184°. A mixed melting point with authentic thymidine gave 182.5–183.5°. A small amount of additional thymidine may be obtained from the mother liquor. The ultraviolet absorption properties agreed with the detailed spectrum of thymidine.[14]

Conversion to 5-Methylcytosine.—A sample of 5MCDR (Va) was hydrolyzed for one hour at 100° with 72% perchloric acid (100 mg. of nucleoside per 4 ml. of acid). The black, insoluble residue was centrifuged off and the excess perchloric acid removed as the potassium salt. The filtrate was concentrated to near dryness and after chilling, 5-methylcytosine precipitated (presumably as the perchlorate salt). This product was indistinguishable from an authentic sample of 5-methylcytosine by paper chromatography in two solvent systems (1-butanol–water and water–1-butanol) and by comparison of its ultraviolet absorption spectrum to published data[19] at *p*H 1 and 6. The melting point of the picrate of this product was identical with the picrate obtained from an authentic specimen (m.p. 288–290°[31]). A mixed melting point showed no depression.

(29) A shorter reaction time often gives incompletely thiated material.

(30) W. Anderson, C. A. Dekker and A. R. Todd, *J. Chem. Soc.* 2721 (1952).

(31) H. L. Wheeler and T. B. Johnson, *Am. Chem. J.*, **31**, 591 (1904).

1-β-D-**Ribofuranosylcytosine (Cytidine, Vb)**.—Tri-*O*-benzoyl-4-thiouridine (IVb, 3.00 g., 0.0052 mole) was treated with 80 ml. of alcoholic ammonia in a sealed tube at 100° for 18 hours. After treatment of the reaction contents in a manner similar to that employed in the synthesis of 5MCDR (*vide supra*) the alcoholic solution was treated dropwise with concentrated sulfuric acid whereupon a white precipitate of cytidine sulfate was formed. Filtration of the chilled mixture gave 1.35 g. (89%), m.p. 222–223°. A mixed melting point with cytidine sulfate prepared from cytidine gave no depression. The ultraviolet absorption properties of the product agreed with the reported spectrum[14] (*p*H 1 and 7).

1-(β-D-**Ribofuranosyl)-5-methylcytosine (5-Methylcytidine, Vc)**.—Two and one-half grams (0.0043 mole) of IVc was treated with 80 ml. of alcoholic ammonia (previously saturated at 0°) and heated in a sealed tube for 24 hours at 100°. The tube was opened and the greenish solution concentrated *in vacuo* to a sirup. Ethyl benzoate was removed by a brief distillation with water and the aqueous residue extracted with chloroform to remove benzamide. The aqueous phase was decolorized with charcoal and concentrated to dryness. The sirupy residue was dissolved in hot 95% ethanol and allowed to cool slowly, 0.88 g. (80%) of a white solid, m.p. 186–203°. Two more recrystallizations from 90% ethanol gave pure material, m.p. 210–211° (efferv.) (Roberts and Visser[6b] report 238–240°), $[\alpha]^{23}D$ −3° (*c* 2.5 in 1.00 *N* NaOH). A mixed melting point with "5-methylcytidine"[6b] gave a depression, m.p. 189–198° (eff.).

Anal. Calcd. for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.87; N, 16.33. Found: C, 46.58; H, 5.78; N, 16.04.

When treated with sodium metaperiodate according to procedures previously employed,[3,4,32] Vc consumed one mole of periodate per mole of nucleoside within 5 minutes. This consumption of oxidant remained constant for the ensuing 24 hours. Ultraviolet absorption data: In 1.0 *N* HCl, maximum at 287.5 mμ, $A_{M(max)}$ 12,580; minimum at 245 mμ, $A_{M(min)}$ 740; shoulder at 225–240 mμ; *p*H 7–12; maximum at 277.5 mμ, $A_{M(max)}$ 8,880; minimum at 255 mμ, $A_{M(min)}$ 5,790; spectral ratios: in 1.0 *N* HCl, 250/260 = 0.33, 280/260 = 0.35; at *p*H 7–12, 250/260 = 1.03, 280/260 = 1.45.

1-(β-D-**Xylofuranosyl)-5-methylcytosine**.—Four grams of 1-(2,3,5-tri-*O*-benzoyl-β-D-xylofuranosyl)-4-thiothymine was placed in a sealed tube with 100 ml. of ethanol (previously saturated with ammonia at 0°) and heated at 100° for 24 hours. The dark-green solution was removed from the tube and treated in a manner similar to the synthesis of 5-methylcytidine (Vc). The sirup obtained from evaporation of the aqueous phase was dissolved in a minimum of hot ethanol and cooled slowly; yield 0.87 g. (50%) of a white solid, m.p. 205–207° (eff.). This product was found to be hygroscopic and was therefore converted to the hydrochloride salt. An aliquot was dissolved in warm, anhydrous ethanol and hydrogen chloride gas was passed in with occasional cooling. The solution was cooled in an ice-bath for 10 minutes and then was poured into a large volume of anhydrous ether. The white precipitate was filtered, recrystallized from aqueous ethanol, m.p. 207–208° (eff.), $[\alpha]^{23}D$ −2.5° (for the hydrochloride, *c* 1.00 in 1.00 *N* NaOH). The ultraviolet absorption properties were generally similar to those found for Vc: in 1.0 *N* HCl, maximum at 287 mμ, $A_{M(max)}$ 12,630; minimum at 245 mμ, $A_{M(min)}$ 900; at *p*H 7–12, maximum at 277.5 mμ, $A_{M(max)}$ 8,680; minimum at 252.5 mμ, $A_{M(min)}$ 5,000.

Anal. Calcd. for $C_{10}H_{15}N_3O_5 \cdot HCl$: C, 40.89; H, 5.49; N, 14.31; Cl, 12.07. Found: C, 40.87; H, 5.40; N, 14.03; Cl, 11.87.

1-(β-D-**Glucopyranosyl)-5-methylcytosine**.—A vigorously-stirred mixture of 4.0 g. (0.0088 mole) of 1-(tetra-*O*-acetyl-β-D-glucopyranosyl)-thymine,[3] 7.4 g. (0.033 mole) of phosphorus pentasulfide in 125 ml. of reagent-grade pyridine was treated dropwise with 0.3 ml. of water and refluxed for 6 hours. The reaction mixture was worked up in a manner similar to the preparation of IVc. Evaporation of the organic phase yielded a sirup which, upon recrystallization from aqueous methanol, gave 1.7 g. (40%) of an amorphous glass. Efforts to obtain a crystalline solid (presumably the tetraacetate of 1-D-glucosyl-4-thiothymine) were unsuccessful and the glass was used directly for the next step.

The amorphous product was treated with ethanolic ammonia (60 ml. previously saturated at 0°) in a sealed tube for 24 hours at 100°. The tube was opened and the solid removed by filtration; 0.82 g., m.p. 267–272° (eff.). On evaporation of the mother liquor another 0.46 g. of material was obtained (total yield 51%). An analytical sample was obtained after two recrystallizations from 90% ethanol, m.p. 279–280° (eff.), $[\alpha]^{23}D$ −4° (*c* 2.4 in 1.0 *N* NaOH). Spectral properties agreed with those reported for 1-D-glycopyranosyl-5-methylcytosines.[14]

Anal. Calcd. for $C_{11}H_{17}N_3O_6$: C, 45.99; H, 5.97; N, 14.63. Found: C, 46.20; H, 6.16; N, 14.71.

4-Thiothymidine (VIa).—3′,5′-Di-*O*-benzoyl-4-thiothymidine (IVa, 9.32 g., 0.02 mole) was dissolved in 500 ml. of anhydrous methanol and the warmed solution treated dropwise with a total of 25 ml. of 1 *N* sodium methoxide in methanol. The reaction was refluxed for a total of 4 hours (the *p*H of the solution was maintained at 8) after which time it was treated with a few drops of glacial acetic acid (to *p*H 5) and evaporated *in vacuo* to dryness. The residue was taken up in water and extracted several times with chloroform to remove methyl benzoate and any starting material. The aqueous layer was treated with charcoal and concentrated to dryness. The yellow residue was extracted about 10 times with 25-ml. portions of acetone and the residual salts were discarded. The combined acetone extracts were concentrated to dryness. The residue was dried further by azeotroping with benzene. A final treatment with acetone and concentration to dryness gave a fluffy glass, 4.5 g. (87%), of crude product.

Anal. Calcd. for $C_{10}H_{14}N_2O_4S$: C, 46.51; H, 5.40; N, 10.85; S, 12.40. Found: C, 47.29; H, 5.42; N, 10.11; S, 10.72.

Attempts to crystallize the glass from a variety of solvents failed. The analysis of the glass indicated, however, that the product was at least 85% pure. Ultraviolet absorption properties, which were essentially similar to those for 1-methyl-4-thiothymine (*vide infra*), were: *p*H 0–6, maxima at 238 and 335 mμ, $A_{M(max)}$ 4,000 and 22,300, respectively, shoulder at 260 mμ; minimum at 280, $A_{M(min)}$ 1,300; *p*H 12.0, maximum at 320 mμ, $A_{M(max)}$ 20,200; minimum at 257 mμ, $A_{M(min)}$ 1,800; isosbestic point at 325 mμ; spectrophotometrically determined pK_a 8.80.

Thymidine Disulfide (VIIa).—4-Thiothymidine (VIa) was oxidized with iodine (according to the procedure of Miller, *et al.*[33]) to the crystalline disulfide (VIIa); VIa (0.9 g.) was added to 100 ml. of water containing 20 ml. of phosphate buffer (*p*H 6.8) and the cooled solution treated dropwise with 3.0 ml. of 1 *N* solution of iodine. The solution was maintained neutral by additions of a 1 *N* solution of potassium carbonate. The amount of iodine added was less than theoretical amount (3.5 ml. required). (When an exact equivalent was used, subsequent isolation of product was difficult and the yields were much lower.) A white precipitate formed which was chilled and filtered to yield about 0.8 g. of crude disulfide. An aliquot was recrystallized twice from hot water, m.p. 200–203°; ultraviolet absorption spectrum in water at *p*H 7.4, maxima at 257 and 321 mμ, minima at 238 and 282 mμ.

Anal. Calcd. for $C_{20}H_{26}N_4O_8S_2$: C, 46.69; H, 5.06; N, 10.89; S, 12.45. Found: C, 46.56; H, 5.42; N, 10.77; S, 12.20.

4-Thiouridine (VIb) was obtained as a non-crystallized glass in a manner similar to that used for the synthesis of 4-thiothymidine. From 11.44 g. (0.02 mole) of IVb, 3.9 g. (75%) of a fluffy yellow glass was obtained. The compound was not analyzed further but served as an intermediate for the preparation of the disulfide and other crystalline derivatives; spectral characteristics (*p*H 7.4): maxima at 244 and 328 mμ, minima at 225 and 272 mμ.

4-Thiouridine Disulfide (VIIb).—The procedure used for the synthesis of thymidine disulfide was followed. From 1.6 g. of VIb, a total of 1.3 g. of the white disulfide was obtained, which, after recrystallization from hot water, melted at 188–190°; spectral characteristics (*p*H 7.4): maxima at 261 and 309 mμ, minima at 236 and 278 mμ.

(32) E. L. Jackson and C. S. Hudson, THIS JOURNAL, **59**, 994 (1937); B. Lythgoe and A. R. Todd, *J. Chem. Soc.*, 592 (1944).

(33) W. H. Miller, R. O. Roblin and E. B. Astwood, THIS JOURNAL, **67**, 2201 (1945).

Anal. Calcd. for $C_{18}H_{22}N_4O_{10}S_2$: S, 12.34; N, 10.80. Found: S, 12.21; N, 10.94.

Synthesis of Cytidine (Vb) from Uridine Disulfide (VIIb).—A sealed tube containing 0.4 g. of VIIb and 40 ml. of ethanolic ammonia (previously saturated at 0°) was heated at 100° for 15 hours. The tube was worked up in a manner similar to that used in the synthesis of cytidine (see above). Product was isolated (0.36 g.) as the sulfate salt, m.p. 221–222°. A mixed melting point with a sample of authentic cytidine sulfate prepared from natural material was undepressed.

1-(2-Deoxy-β-D-ribofuranosyl)-4-hydrazino-5-methyl-2(1H)-pyrimidinone (Xa).—A solution containing 8.0 g. of IVa in 600 ml. of ethanol containing 28 ml. of hydrazine hydrate (95%+) was refluxed for one hour. After the first 10 minutes the green-colored solution became almost colorless. The solvent was removed *in vacuo* and the ethyl benzoate removed by co-distillation with water. The residue was treated with 40 ml. of warm alcohol. Upon cooling, a white crystalline precipitate of crude product formed; 4.9 g., m.p. 160°. This product is contaminated with benzoic acid hydrazide. Recrystallization from a small volume of hot ethanol afforded pure material, m.p. 178–179° (eff.), 2.8 g. An additional 0.3 g. was obtained from the mother liquor (total yield 72%), $[\alpha]^{25}D$ +30° (*c* 1.1 in water). Light absorption properties: at *p*H 1.0, maxima at 217.5 and 287.5 mμ, $A_{M(max)}$ 10,000 and 13,330, respectively; minimum at 247.5 mμ, $A_{M(min)}$ 2,590; at *p*H 7.0–9.0, maximum at 277.5 mμ, $A_{M(max)}$ 10,850; minimum at 252.5 mμ, $A_{M(min)}$ 7,210; isosbestic points at 223 and 274.5 mμ, $A_{M(isosbestic)}$ 9,380 and 10,780 respectively. In 1 *N* alkali, the spectrum of this substance is altered, with time, as shown by the decrease in the maximum at 277.5 mμ with the appearance of a new maximum of higher extinction (~20,000) at 322 mμ.

Anal. Calcd. for $C_{10}H_{16}N_4O_4$: C, 46.87; H, 6.25; N, 21.87. Found: C, 46.70; H, 6.39; N, 21.63.

Preparation of Xa from Thymidine Disulfide (VIIa).—Three-tenths of a gram of thymidine disulfide was treated with 25 ml. of ethanol and 1.5 ml. of hydrazine and the solution refluxed for 2 hours. After removal of solvent *in vacuo*, the residue was crystallized from ethanol, m.p. 178–180° (eff.). A mixed melting point with material prepared from IVa (see above) gave no depression.

Preparation of Xa from 4-Thiothymidine (VIa).—From 2.0 g. of VIa in 25 ml. of ethanol and 5 ml. of hydrazine, 1.4 g. of product (70%), m.p. 178–180° (eff.), was obtained.

Treatment of Hydrazinothymidine (Xa) with Nitrous Acid (Synthesis of XIa).—A solution containing 3.0 g. of 4-hydrazinothymidine (Xa) in 150 ml. of water and 15 ml. of glacial acetic acid was cooled to 5° and treated with 50 ml. of an aqueous solution of sodium nitrite (3.3 g., 0.048 mole). The temperature rose to 10°. The reaction was left in an ice-bath for one hour, after which it was evaporated to dryness *in vacuo* and the residue triturated with alcohol. After filtration from some salts, the filtrate was concentrated to dryness and again treated with a small volume of ethanol. After filtration from more salt (almost the theoretical amount of sodium acetate was removed), the filtrate was concentrated to dryness and taken up in a minimum of hot ethanol, treated with charcoal and cooled overnight. White rectangular prisms were obtained, m.p. 148–149°, 2.4 g. (76%). This substance is probably the tetrazolo derivative (see text); ultraviolet absorption properties: *p*H 0–7, maximum at 253.5 mμ, $A_{M(max)}$ 9,700, shoulder at 275 mμ (see Fig. 2). The substance decomposes in alkali.

Anal. Calcd. for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 5.24; N, 26.21. Found: C, 45.06; H, 4.90; N, 26.06.

1-(2-Deoxy-β-D-ribofuranosyl)-4-methylamino-5-methyl-2(1H)-pyrimidinone (VIIIa).—A sealed tube containing 4.5 g. of IVa and 100 ml. of 45% methylamine in ethanol was heated overnight at 100°. The tube was opened and the green-colored solution treated in a manner similar to that used in the preparation of 5-methylcytidine (*vide supra*). A residue was obtained which was taken up in hot methanol and cooled; 1.8 g. (74%), m.p. 219–220°. One recrystallization from methanol afforded pure material, m.p. 225–227°, $[\alpha]^{25}D$ +28° (*c* 1.2 in water). Ultraviolet absorption properties: *p*H 1.0, maxima at 218 and 286.5 mμ, $A_{M(max)}$ 11,600 and 13,670, respectively; minimum at 246 mμ, $A_{M(min)}$ 2,370; at *p*H 7–12, maximum at 275 mμ, shoulder at 235 mμ, $A_{M(max)}$ 10,920; isosbestic points at 226 and 285 mμ; pK_a 4.04 (determined spectrophotometrically).

Anal. Calcd. for $C_{11}H_{17}N_3O_4$: C, 51.76; H, 6.66; N, 16.47. Found: C, 51.68; H, 6.43; N, 16.94.

1-(β-D-Ribofuranosyl)-4-methylamino-2(1H)-pyrimidinone (VIIIb).—A sealed tube containing 3.0 g. of IVb and 80 ml. of 45% methylamine in ethanol was heated for 36 hours at 100°. The tube was opened, filtered from a black residue and the filtrate treated as in the preparation of 5-methylcytidine. A residue was obtained which was taken up in hot ethanol and cooled; 1.26 g. (93%), m.p. 202–203°. Recrystallization from ethanol did not elevate the melting point. Spectral properties resembled those for cytosine nucleosides: at *p*H 7, maxima at 237 and 271 mμ, minima at 227 and 248 mμ.

Anal. Calcd. for $C_{10}H_{15}N_3O_5$: C, 46.69; H, 5.84; N, 16.34. Found: C, 47.00; H, 6.04; N, 16.78.

1-(β-D-Ribofuranosyl)-4-methylamino-5-methyl-2(1H)-pyrimidinone (VIIIc).—A sealed tube containing 2.0 g. of IVc in 45% ethanolic methylamine was heated for 24 hours at 100°. The tube was cooled, opened and treated in a manner similar to that employed in the synthesis of VIIIb (*vide supra*). The residue was crystallized from *ca.* 3 ml. of hot ethanol; 0.8 g. (87%), m.p. 190–191°. Spectral properties were similar to those for VIIIb: *p*H 7.4, maximum at 275 and shoulder at 234 mμ; minimum at 252 mμ.

Anal. Calcd. for $C_{11}H_{17}N_3O_5$: C, 48.70; H, 6.27; N, 15.50. Found: C, 48.79; H, 6.33; N, 15.62.

1-(2-Deoxy-β-D-ribofuranosyl)-4-β-phenylethylamino-5-methyl-2(1H)-pyrimidinone (IXa).—A sealed tube containing 7.0 g. of IVa, 70 ml. of ethanol and 30 ml. of β-phenylethylamine was heated for 15 hours at 100°. The tube was cooled and opened and the solvent removed *in vacuo*. Ethyl benzoate was removed by co-distillation with water several times and the solution treated with chloroform. The chloroform layer was washed with water, dried, filtered and concentrated to dryness. The residue was extracted with ether. A crystalline, ether-insoluble residue remained; 0.7 g., m.p. 180–181°. The ether extract was concentrated to dryness, taken up in a minimum of warm ethanol, treated with ether and cooled. After filtration, 2.0 g. of a white, fluffy product was obtained, m.p. 181–182°, which was combined with the 0.7 g. obtained above. The combined precipitates were taken up in a minimum of warm ethanol and treated with ether to incipient precipitation and cooled. Pure product was obtained, 1.3 g., m.p. 183–185°. Additional product (2.5 g.) of lower melting point was obtained from the mother liquors. Ultraviolet absorption properties: in *p*H 1.0, maximum at 289.5 mμ, $A_{M(max)}$ 13,990; minimum at 248 mμ, $A_{M(min)}$ 2,400; at *p*H 7–12, shoulder at 240, minimum at 252.5 mμ, maximum at 277.5 mμ; $A_{M(min)}$ 8,650, $A_{M(max)}$ 11,900; isosbestic points at 280 mμ; pK_a (determined spectrophotometrically) 3.83.

Anal. Calcd. for $C_{18}H_{23}N_3O_4$: C, 62.60; H, 6.66; N, 12.17. Found: C, 62.60; H, 6.59; N, 11.92.

1-(β-D-Ribofuranosyl)-4-β-phenylethylamino-2(1H)-pyrimidinone (IXb).—A sealed tube containing 5.56 g. of IVb, 70 ml. of ethanol and 30 ml. of β-phenylethylamine was heated at 100° for 24 hours. The contents were treated in a manner similar to the preparation of the thymidine analog (IXa, see above). In this case, the water layer contained the product. A residue was obtained from the aqueous layer which was taken up in ethanol and treated with gaseous hydrogen chloride to yield 2.25 g. (59%) of crude hydrochloride. One recrystallization from hot ethanol afforded pure material, m.p. 205–206°; spectral properties: *p*H 7, maxima at 241 and 272.5 mμ, minima at 229 and 247 mμ.

Anal. Calcd. for $C_{17}H_{21}N_3O_5 \cdot HCl$: C, 53.20; H, 5.75; N, 10.93; Cl, 9.25. Found: C, 52.64; H, 5.75; N, 10.73; Cl, 8.96.

1-(2-Deoxy-3,5-di-*O*-benzoyl-β-D-ribofuranosyl)-4-hydroxylamino-5-methyl-2(1H)-pyrimidinone (XIIa).—A solution of hydroxylamine in methanol was prepared by neutralization[34] of 10 g. of hydroxylamine hydrochloride with sodium methylate in methanol and filtration from precipitated sodium chloride. This solution was added to a solution of 2.3 g. of IVa in methanol and refluxed for 4 hours. The pale-yellow solution was concentrated to dryness and

(34) In the preparation of the hydroxylamine solution from the hydrochloride it is important to avoid "over-neutralization" with alkoxide since the product XIIIa is rather unstable to warm alkali. A similar situation obtains with XIIIb.

the white residue taken up in about 100 ml. of ethanol and cooled. The precipitate was filtered (1.75 g., 74%) and recrystallized by redissolving in 50 ml. of hot ethanol, treating with charcoal, and filtration. Upon cooling, 1.40 g. of pure product was obtained, m.p. 169–170°, as white needles.

Anal. Calcd. for $C_{24}H_{22}N_2O_7$: C, 61.93; H, 4.95; N, 9.03. Found: C, 61.51; H, 5.13; N, 8.88.

1-(2-Deoxy-β-D-ribofuranosyl)-4-hydroxylamino-5-methyl-2(1H)-pyrimidinone (XIIIa).—A solution containing 1 g. of VIa in ethanol was treated with about 0.2 mole of hydroxylamine[34] (prepared as in the synthesis of XIIa, see above) and refluxed for 4 hours. The faint-yellow solution was concentrated to dryness *in vacuo*. Upon the addition of a small amount of ethanol, a small amount of white crystalline material formed, m.p. *ca.* 80–90°, which possessed no ultraviolet absorption and was discarded. Concentration of the filtrate to dryness and trituration with ethanol yielded another 100 mg. of material with similar characteristics which also was discarded. The mother liquor was concentrated to dryness and the residue dried by azeotropic distillation with benzene. The residue was dissolved in 1.5 ml. of methanol and stored in the refrigerator for two weeks, after which large prismatic crystals formed, m.p. 114° (sinters at 107°), for a total yield of 0.10 g. The product was recrystallized from a minimal amount of hot methanol, and analyzed best for the hemihydrate of XIIIa, m.p. 114°. This substance gave a spectral pattern between *p*H 0 and 13 which resembled that for its uridine analog (see Figs. 3 and 4). The spectrally-determined pK_a values are 2.3 and 11.1 (±0.1). In strong alkali, the compound decomposes.

Anal. Calcd. for $C_{10}H_{15}N_3O_6 \cdot {}^1/_2H_2O$: C, 45.11; H, 6.02; N, 15.79. Found: C, 44.50; H, 6.47; N, 15.48.

1-(β-D-Ribofuranosyl)-4-hydroxylamino-2(1H)-pyrimidinone (XIIIb).—A solution containing 520 mg. of VIb in ethanol was treated with hydroxylamine[34] in a manner similar to that employed in the preparation of XIIIa (*vide supra*). After 3 hours at reflux temperature, the clear, colorless solution was concentrated *in vacuo* to dryness. Upon the addition of ethanol, a white precipitate (inorganic salts) formed. After separation from the inorganic material, the filtrate was concentrated to dryness, treated with warm ethanol and again separated from salts. (In other preparations of this substance it was necessary to repeat this step several times.) The filtrate was concentrated to dryness and taken up in a minimum amount of hot ethanol, treated with charcoal, and the filtrate stored in the refrigerator. After several days, product separated as large prisms, 250 mg. (48%), m.p. 169–172°. Recrystallization from methanol did not alter the melting point. Spectral properties (see Fig. 4): *p*H 0, maxima at ~223 and 280.5 mμ, $A_{M(max)}$ 7,770 and 13,160, minimum at 245 mμ, $A_{M(min)}$ 2,900; *p*H 7.0, maxima at 236 and 272 mμ, $A_{M(max)}$ 12,800 and 6,500; minimum at 262 mμ, $A_{M(min)}$ 6,300; *p*H 12, maxima at 242.5 mμ, shoulders at ~258 and ~295 mμ, $A_{M(max)}$ 9,900. Isosbestic points for curves between *p*H 0 to 7 are at 259 and 312 mμ; for curves between *p*H 7–12 at 248 mμ. pK_a values determined spectrophotometrically are 2.26 and 10.5 for ionization involving the cationic and anionic species, respectively.

Anal. Calcd. for $C_9H_{13}N_3O_6$: C, 41.69; H, 5.02; N, 16.22. Found: C, 41.95; H, 5.18; N, 16.51.

1-Methyl-4-thiouracil.—A mixture containing 12.6 g. of 1-methyluracil[35] 6.6 g. of phosphorus pentasulfide and 400 ml. of pyridine was stirred and refluxed for three hours. The deep-green mixture was concentrated *in vacuo* to approximately 250 ml. and filtered. The precipitate was discarded and the filtrate concentrated to dryness. The residue was taken up in hot ethanol, charcoaled, filtered and cooled, 8.8 g. (62%) m.p. 191–193°. Recrystallization from ethanol followed by one recrystallization from water gave yellow needles, m.p. 193–194° (sinters at 183°); spectral data: at *p*H 7, maxima at 244 and 333 mμ, minimum at 277 mμ.

Anal. Calcd. for $C_5H_6N_2OS$: N, 19.71; S, 22.53. Found: N, 19.43; S, 22.05.

1-Methylcytosine.—A sealed tube containing 500 mg. of 1-methyl-4-thiouracil in 30 ml. of alcoholic ammonia was heated for 24 hours at 120°. Upon cooling, product precipitated, 300 mg., m.p. 285–286°. One recrystallization from 95% ethanol gave m.p. 294–296° dec. (Hilbert[36] reported 303° dec.). The spectrum of the product was identical with that previously reported.[14]

4-Hydrazino-2(1H)-pyrimidinone.—4-Ethoxy-2(1H)-pyrimidinone[37] (14 g.) in 500 ml. of ethanol was treated with 50 ml. of hydrazine and refluxed for 2 hours. The solvent was removed *in vacuo* and the residue taken up into hot 95% ethanol from which it crystallized. One recrystallization from ethanol and a few drops of water gave prisms 12.0 g., m.p. 305–310° dec. Spectral properties: in 1.0 *N* HCl, maximum at 276 mμ, $A_{M(max)}$ 11,000, minimum at 239.5 mμ, $A_{M(min)}$ 1,930; *p*H 7.0, maximum at 268 mμ, $A_{M(max)}$ 7,650; minimum at 247 mμ, $A_{M(min)}$ 5,450. The substance decomposes in alkali.

Anal. Calcd. for $C_4H_6N_4O$: C, 38.09; H, 4.76; N, 44.44. Found: C, 38.28; H, 4.80; N, 44.09.

Reaction of 4-Hydrazino-2(1H)-pyrimidinone with Nitrous Acid.—A solution containing 1.26 g. of I (R = H, R′ = $NHNH_2$) and 15 ml. of glacial acetic acid in 200 ml. of water was cooled to 5° and treated with 2.8 g. of sodium nitrite in 50 ml. of water. The temperature of the stirred solution rose to approximately 10° after which it was allowed to remain in the ice-bath for about 2 hours. Product precipitated, 0.89 g., which was recrystallized from water containing a few drops of ethanol. Very fine, white needles were obtained, m.p. 241–242° (with vigorous decomposition and fuming). The spectral characteristics of this "azido" pyrimidine (probably a tetrazolo structure akin to the pyrimidine moiety in XIa) resembled that for XIa in acid and near-neutral solution, but manifested spectral shifts in media above *p*H 5.9 (see Fig. 2). The spectrally-determined pK_a is 6.95.

Anal. Calcd. for $C_4H_5N_5O$: C, 35.04; H, 2.19; N, 51.09. Found: C, 35.33; H, 2.38; N, 50.90.

1,5-Dimethylcytosine (I, R, R′ = CH_3).—A sealed tube containing 0.4 g. of 1,5-dimethyl-4-ethoxy-2(1H)-pyrimidinone[38] was treated with 30 ml. of ammonia in ethanol (saturated at 0°) and heated for 24 hours at 150°. The tube was opened, and the contents evaporated to dryness. The residue was taken up in a minimum of hot ethanol, cooled and filtered; 250 mg., m.p. 308–309°. Spectral data: 1.0 *N* HCl, maximum at 291 mμ, $A_{M(max)}$ 11,450, minimum at 244 mμ, $A_{M(min)}$ 590; *p*H 7.0 maximum at 280 mμ, $A_{M(max)}$ 7,770; minimum at 253 mμ, $A_{M(min)}$ 3,210; isosbestic point at 275 mμ, $A_{M(isos)}$ 7,400; spectrophotometrically-calculated pK_a 4.76.

Anal. Calcd. for $C_6H_9N_3O$: N, 30.20. Found: N, 30.19.

Spectrophotometric Studies.—Measurements were made with a Cary recording spectrophotometer, model 11, using techniques and buffers previously described.[18,19] The apparent pK_a values are accurate to within 0.05 *p*H unit unless specified otherwise and were determined spectrophotometrically by methods previously employed.[19,39]

Key to Figures.—All the spectra listed were run in aqueous solutions at *p*H values indicated on the curves. The italicized letters refer to isosbestic points.[15]

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