[CONTRIBUTION FROM THE DIVISIONS OF NUCLEOPROTEIN CHEMISTRY AND EXPERIMENTAL CHEMOTHERAPY, SLOAN KETTERING INSTITUTE, AND THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Pyrimidine Nucleosides. VI. Nitration of Nucleosides[1]

BY IRIS WEMPEN, IRIS L. DOERR, LOUIS KAPLAN AND JACK J. FOX

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A re-investigation of the experiments of Levene and LaForge on the direct nitration of uridine is described. The product is characterized as 5-nitro-1-(β-D-ribosyluronic acid)-uracil and the preparation of several of its derivatives is reported. The synthesis of 5-nitrouridine by nitration of suitably-protected uridine is given. Reduction of these 5-nitrouracil nucleosides afforded the corresponding 5-amino derivatives. The susceptibility of the uronic acid derivative II, and other 5′-substituted 1-β-D-ribofuranosyl nucleosides (XIII, R′ = CH_3 or CH_2F) to glycosyl cleavage by nucleosidase(s) of *E. coli* B. was examined. Whereas II and XIII (R′ = CH_2F) are not cleaved, some cleavage is obtained with XIII (R′ = CH_3). Preliminary studies on the structural specificity of nucleoside deaminases are also described.

One of the most important series of antimetabolites bearing a structural resemblance to the naturally occurring pyrimidine nucleosides (*i.e.*, uridine, cytidine and thymidine) consists of those nucleosides substituted in the 5-position of the pyrimidine moiety. They may be regarded as nucleoside derivatives of thymine-type bases in which the 5-methyl group has been replaced by other substituents. This group includes such derivatives as the 5-halogeno-, the 5-amino- and 5-hydroxy-uridines.[2] A noteworthy omission from this list is the 5-nitrouridine, an absence, which is rendered more conspicuous in view of the known microbial inhibition exhibited by the base, 5-nitrouracil, in certain biological systems. While 5-nitrouracil is not itself a thymine antagonist, it does appear to exert antifolic action and thereby inhibit growth of certain test organisms.[3] Therefore, it would seem of interest to prepare its nucleoside, 5-nitrouridine, for chemotherapeutic screening.

Introduction of a nitro group into a preformed nucleoside was first performed, in 1912, by Levene and LaForge[4] who reported the synthesis of a nitrated nucleoside derivative, "nitro-uridin-carbonsäure." In the present paper, a reinvestigation of this much-overlooked reaction is undertaken in order to clarify certain structural ambiguities of the original report and to obtain a new type of nucleoside derivative for possible chemotherapeutic screening. Concurrently, the synthesis of the true 5-nitrouridine is also reported.

One of the difficulties encountered by early investigators in their attempts to determine the position of attachment of the sugar moiety in pyrimidine nucleosides was the very considerable stability of the glycosyl bond to acid hydrolysis.[4,5] This stability was demonstrated by the work of Levene and LaForge[4] who evaporated a solution of uridine in nitric acid and obtained a nitrated derivative which still possessed an intact glycosyl bond. The product also contained a free carboxyl group as was demonstrated by titration data, by formation of a silver salt, and by ease of esterification. On the basis of elementary analyses, these investigators[4] concluded that the acidic compound was a *dimer* formed by the loss of a molecule of water between two nucleoside molecules. Simple anhydride formation or lactonization through the carboxyl group were eliminated by the synthesis of the ethyl and butyl esters which also analyzed as dimers. No further elucidation of the exact location of the supposed anhydro linkage was reported, though it may be presumed that the C2 or C3 hydroxyl groups of the sugar moiety were involved.

This original experiment was repeated in our laboratory and the product II displayed the same titratable acid group and salt-forming characteristics as detailed in the original work. Compound II was also easily converted in good yields into the butyl ester IVa and the isopropyl ester IVb. *Both* these esters, as well as the parent acid II, however, gave analytical data consistent with a *monomeric* rather than a dimeric nucleoside. Further, a molecular weight determination, performed on the butyl ester IVa confirmed the *monomer* structure. Additional evidence for the presence of the free carboxyl group was derived from an examination of the molecular model (constructed with Lapine[6] atom models) of II, which shows that the formation of a lactone bridge between the carboxyl group and either the C2 or C3 hydroxyls of the sugar moiety would be sterically most difficult, if not impossible. Therefore, it was concluded further that the furanoid ring originally present in uridine had not been altered and, moreover, that the structure of the product of this nitration–oxidation experiment is the ribosyluronic acid derivative II. Confirmation of the absence of any anhydro-linkage and, indeed, the presence of a vicinal-*cis*-glycol system in this acid and its esters was derived from two sources: the metaperiodate titration data (see below), and the ready formation of the respective 2′,3′-isopropylidene derivatives. Treatment of the isopropyl ester IVb with acetone in the presence of an acid catalyst led to the monoacetone derivative Vb in good yield. Acetonation of the free acid II was also accomplished; as might be expected, however, the inherent acidity of the carboxyl group rendered the ketal extremely susceptible to auto-hydrolysis. Therefore, the isopropylidene derivative was not obtained in an analytically pure state, free from trace amounts of the starting material.

(1) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant No. CY 3190), and from the Ann Dickler League. For a preliminary report see *Federation Proc.*, **18**, 350 (1959).

(2) See chapter on Pyrimidine Nucleosides by J. J. Fox and I. Wempen, *Advances in Carbohydrate Chem.*, **14**, 283 (1959), and J. J. Fox, *Rec. Chem. Prog.*, **19**, 173 (1958), for a general review of these compounds.

(3) G. H. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell and H. VanderWerff, *Ann. N. Y. Acad. Sci.*, **52**, 1318 (1950).

(4) P. A. Levene and F. B. LaForge, *Ber.*, **45**, 608 (1912).

(5) P. A. Levene and W. A. Jacobs, *ibid.*, **43**, 3150 (1910).

(6) Arthur S. Lapine and Co., Chicago, Ill.

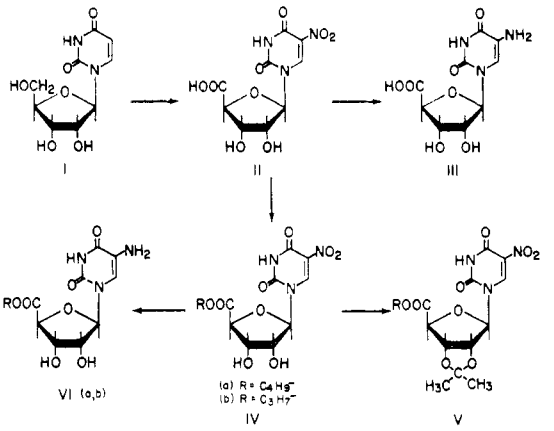


Fig. 1.

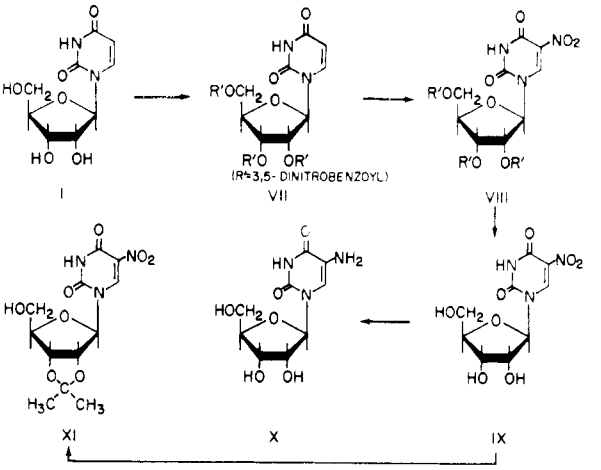


Fig. 2.

The presence of 5-nitrouracil in the mother liquors from which II was isolated was detected spectrophotometrically[7] and by paper chromatography, indicating that some cleavage of the glycosyl linkage had occurred. No uracil or unreacted uridine was detected in the reaction mixture. The nitro group of II and that of its butyl and isopropyl esters (IV) were readily hydrogenated with palladium-on-charcoal catalyst to their 5-amino analogs (III and VI, respectively). The reduced products gave a strongly positive test with alkaline phosphomolybdate reagent[8] in accord with the presence of an hydroxyl function *ortho* to an amino group (and thereby the nitro group of II and IV) on position 5.

From these data, the structure of II is established as 5-nitro-1-(β-D-ribosyluronic acid)-uracil.

It seemed probable that direct nitration of uridine *without* oxidation of the 4′-hydroxymethyl group could be achieved if the hydroxyl groups of the sugar moiety were protected by suitable substituents. The usual agent, benzoyl chloride, was patently unsuitable since the phenyl groups would themselves be subject to nitration.[9] For this reason, 3,5-dinitrobenzoyl chloride was chosen for the preparation of an intermediate (VII) suitable for nitration. Indeed, these protective groups rendered the compound so stable that a quicker, but more vigorous, nitrating system could be used, namely, a mixture of fuming nitric and concentrated sulfuric acids. (The nitration appeared to be only mildly exothermic and no particular precautions were necessary in the rapid addition of VII to the mixture of acids; after a short interval, the nitrated intermediate VIII was isolated in good yield.) The fact that loss of some of the protecting groups, with subsequent cleavage of the glycosyl bond had occurred, was evidenced by the detection of 5-nitrouracil, as well as some 3,5-dinitrobenzoic acid, in the mother liquors. Deacylation of VIII with sodium ethoxide afforded a sodium salt from which the desired product, 5-nitrouridine (IX), was obtained by careful neutralization. Proof that the original 1-β-D-ribofuranosyl configuration of uridine had not been altered by the nitration procedure was afforded by the catalytic hydrogenation of IX to 5-aminouridine (X), which was found to be identical with an authentic sample.[10] The hydrochloride salt of X was also characterized. Further evidence of the retention of configuration by IX was derived from the ready formation of the 2′,3′-isopropylidene derivative XI. A comparison of the molecular rotations of IX and XI with those of uridine and its 2′,3′-isopropylidene derivative (see Table I) indicates the similarity of anomeric configuration in these compounds.

TABLE I

	$[\alpha]_D^a$	$[M]_D$	Difference in $[M]_D$
Uridine (I)	+ 8°	+ 1,890	+9530
1-(2′3′-Isopropylidene-β-D-ribofuranosyl)-uracil	−27	− 7,640	
5-Nitrouridine (IX)	−45	−13,500	+7600
5-Nitro-1-(2′,3′-isopropylidene-β-D-ribofuranosyl)-uracil (XI)	−64	−21,000	

[a] All rotations are reported to the nearest degree and were determined in purified dioxane.

Preliminary attempts to obtain 5-nitrouridine (IX) by the selective reduction of the ester group in 5-nitrouracil riburonic acid isopropyl ester (IVb) by means of alkali-metal borohydrides, using either the modification of Paul and Joseph[11] or that of Brown and Subba Rao,[12] failed to yield the desired product. Instead, the compound isolated appeared to be the dihydro derivative of IVb, formed, apparently, by the reduction of the 5,6-double bond in the pyrimidine ring. This conclusion was derived from a study of the spectral behavior in various *p*H ranges. The loss of extinction in the low *p*H range appears to be characteristic of such reduced derivatives.[13,14]

Metaperiodate Studies.—In confirmation of its vicinal-*cis*-glycol system, 5-nitrouridine (IX), on

(7) For a complete spectra (ultraviolet) of 5-nitrouracil, see D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta*, **9**, 210 (1952).

(8) T. B. Johnson and C. O. Johns, THIS JOURNAL, **36**, 545, 970 (1914).

(9) Indeed, attempts to nitrate 2′,3′,5′-tri-*O*-benzoyluridine led to the expected nitration of the blocking groups *with* subsequent deesterification.

(10) M. Roberts and D. W. Visser, THIS JOURNAL, **74**, 668 (1952).

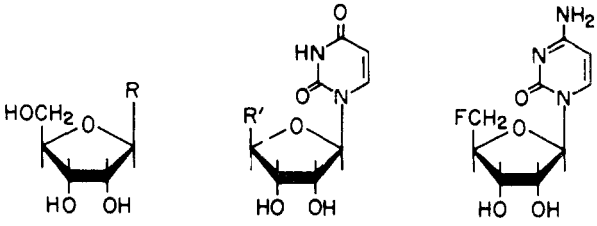
(11) R. Paul and N. Joseph, *Bull. soc. chim. France*, **19**, 550 (1952).

(12) H. C. Brown and B. C. Subba Rao, THIS JOURNAL, **78**, 2582 (1956).

(13) R. D. Batt, J. K. Martin, J. M. Ploeser and J. Murray, *ibid.*, **76**, 3663 (1954).

(14) J. J. Fox and D. Van Praag, *ibid.*, **82**, 486 (1960).

titration with sodium metaperiodate, consumed one mole of oxidant per mole of compound within 2 minutes without the liberation of formic acid. No further consumption of periodate occurred in a 24-hour period. The uronic acid derivative II also consumed periodate on a mole for mole basis within 2 minutes; however, additional increments of oxidant were consumed slowly by the compound over a considerable span of time. The isopropyl ester IVb, on the other hand, gave an anomalous titration value of 1.5 moles of oxidant per mole consumed within 2 minutes, and a continued uptake with time, as in the case of the parent acid. The immediate uptake of metaperiodate in each of these cases is consonant with the presence of vicinal-*cis*-hydroxyls in the molecule.[15,16] The additional uptake in the case of the uronic acid derivatives may be explained by the activating effect of the carboxyl (or ester) group on the residual dialdehyde residues (formed by the initial cleavage of the vicinal-*cis*-glycol system) leading to further oxidation. This hypothesis is at least partially supported by the fact that where the initial reaction with periodate is blocked, as in the isopropylidene derivative Vb, no consumption of metaperiodate occurs. The consumption of additional oxidant, together with certain spectral shifts exhibited by all of these nitro-compounds during the oxidation, are currently under investigation.



XII XIII XIV

Fig. 3.

Spectral Studies and Dissociation Constants.—5-Nitrouridine (IX), 5-nitrouracil riburonic acid (II) and its esters (IV) exhibit curves in *N* HCl almost identical with that for 1-methyl-5-nitrouracil. In the alkaline *p*H range, however, an immediate shift in the initial extinction occurs with the result that neither the pK_a values nor the isosbestic points[17] could be determined with any accuracy. For this reason the extinction values of these compounds are reported for the limiting curve[19] in the acid *p*H range only. In the case of the 5-amino compounds III, VIb and X, only the pK_a for protonation of the amino group was determined. 5-Aminouridine (III) affords a spectrally-determined pK_a value of 3.14. 5-Amino-1-(β-D-ribosyluronic acid isopropyl ester)-uracil (VIb) gives a pK_a value of 2.66; its parent acid II, on the other hand possesses a higher figure, 3.06. The latter value represents a weakening of the acid strength, not displayed by the ester analog, due probably to the formation of a zwitterion between the free 5′-carboxyl and the 5-amino group[20] and should be designated as an "apparent" pK_a value. The true value[21,22] is probably most nearly represented by the pK_a of the isopropyl ester (*i.e.*, 2.66). This effect of zwitterion formation seems to be reflected in the much lower extinction value of II which was found to be about one-half of that determined for either its ester VIb or for 5-aminouridine (X). It has been previously noted[23] that formation of such a dipolar ion tends to lower *both* the acidity and extinction values.

Enzymic Studies.—It has been demonstrated[16] that the specificity, exhibited by nucleosidase(s) present in intact cell suspensions of *Escherichia coli* B. is of a low order with regard to the structure of the pyrimidine moiety. For example, in structure XII, the R-group of the 1-β-D-ribofuranosyl nucleoside may be uracil or thymine, without affecting the susceptibility of these substrates to enzymic cleavage. On the other hand, these enzymic systems demonstrate a high degree of structural specificity with regard to the configuration of the sugar moiety,[16] *viz.*, of the four 1-β-D-aldopentofuranosyl nucleosides of thymine, only the 1-β-D-*ribo*furanosyl isomer is cleaved. A further degree of specificity can now be reported with the availability of 1-β-D-ribofuranosyl nucleosides in which the terminal group (C5) of the sugar moiety has been altered. 5-Nitrouracil riburonic acid (II) proved to be completely resistant to cleavage by this enzyme system (see Table II). On the other hand, 5-nitrouridine (IX) is readily cleaved under the same conditions. In order to demonstrate that the failure of these enzymic systems to act upon II cannot be attributed to a possible inhibitory effect of the terminal carboxyl function, two other nucleosides possessing the 1-β-D-ribofuranosyl structure with modification at C5 of the sugar moiety, namely 5′-deoxy-5′-fluorouridine[24a] and 5′-deoxy-

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

(16) J. J. Fox, J. F. Codington, N. C. Yung, L. Kaplan and J. O. Lampen, *ibid.*, **80**, 5155 (1958).

(17) See refs. 7, 18 for a discussion on the significance of isosbestic points.

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

(19) 5-Nitrouracil exhibits a spectrophotometrically determined pK_a value of 5.3.[7] It would be expected (by comparison of the pK_a values of other bases and their nucleosides) that the pK_a values for the dissociation of the 3:4 position of IX, II and IV would be essentially similar to that of 5-nitrouracil itself. Therefore, the curve for *p*H 0–3 would represent the un-ionized species of IX and IV in solution (limiting curves). With regard to II, the curve for *p*H 3 and that run in 3 *N* HCl are identical which also indicates a limiting curve with respect to the aglycon. The structure of the carboxyl function (neutral or carboxylate form) in this acid region (3 *N* HCl to *p*H 3) is not known since, as expected, no evidence of ionization of this function is exhibited spectrally.

(20) An analogous effect was observed in the case of the isomeric cytidylic acids *a* and *b*, in which the respective change in the pK_{a2} value of each isomer, due to zwitterion formation between the 2′- and 3′-phosphate groups and the 4-amino group, was used to differentiate between the two isomers (L. F. Cavalieri, THIS JOURNAL, **74**, 5804 (1952)).

(21) Measuring the dissociation constants of a zwitterion by the usual method of measuring the *p*H at half neutralization affords only an "apparent" pK_a value. The true magnitude of the basic dissociation constant of a zwitterion can be closely approximated by studying the corresponding ester; see H. B. Bull, "Physical Biochemistry," John Wiley and Sons, Inc., New York, N. Y., 1943, p. 124.

(22) Other analogies may be found in the titration data of amino acids, where similar evidence is derived from a comparison of the base strength of the free amino acids compared with that of their esters (J. T. Edsall and M. H. Blanchard, THIS JOURNAL, **55**, 2337 (1933)).

(23) H. S. Loring, M. L. Hammell, L. W. Levy and H. W. Bortner, *J. Biol. Chem.*, **196**, 821 (1952).

(24) (a) H. M. Kissman and M. J. Weiss, THIS JOURNAL, **80**, 5559 (1958); (b) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

TABLE II

CLEAVAGE OF URIDINE DERIVATIVES BY *E. coli* B.[a]

Nucleoside	Free pyrimidine formed, μmole
Uridine (I)[b]	0.96[c]
5-Nitrouridine (IX)	.87[d]
5-Nitro-1-(β-D-ribosyluronic acid)-uracil (II)	0
5-Fluorouridine[27a]	0.93[c]
5′-Deoxy-5′-fluorouridine (XIII, R′ = CH_2F)	0
5′-Deoxyuridine (XIII, R′ = CH_3)	0.25[c]

uridine, were investigated. The latter compound was synthesized for this enzyme study by hydrogenation of 5′-deoxy-5′-iodo-2′,3′-isopropylideneuridine.[24b] Subsequent hydrolysis of the ketal afforded 5′-deoxyuridine (XIII, R′ = CH_3) in good yield. Of these two compounds, XIII (R′ = CH_2F or CH_3), the former was not cleaved by these resting-cell suspensions, whereas 5′-deoxyuridine was cleaved to the extent of 25%.[25a] Thus it would appear that these nucleosidase(s) permit some variation at C5′ of 1-β-D-ribofuranosyl pyrimidine nucleosides though enzymic action is thereby diminished. (It is to be noted that thymidine is also cleaved by this bacterial system which suggests that either more than one pyrimidine nucleosidase is present, or that perhaps the enzyme is also specific for the 1-(2′-deoxy-β-D-ribofuranosyl) structure. The former seems more probable in view of the findings of Razzell and Khorana[25b] that a purified "pyrimidine deoxyribonucleoside phosphorylase" from *E. coli* shows an absolute specificity for deoxyribose-1-phosphate; ribose-1-phosphate or other pentose-1-phosphates did not serve as substrates. It should be of interest to test the xylo isomer of thymidine as well as other deoxypentofuranosylpyrimidine derivatives as substrates in this whole cell system.)

Cytosine nucleosides, such as cytidine or 2′-deoxycytidine, are cleaved in this system with the liberation of the corresponding free 2,4-dihydroxypyrimidines (*viz.*, uracil, thymine or 5-fluorouracil, see Table III). These findings are not unexpected in view of the studies of Cohen and Barner[26] who showed that *E. coli* B contains an active nucleoside deaminase. They employed a cell-free extract, purified to remove nucleoside phosphorylase activity, and demonstrated the conversion of cytidine and 2′-deoxycytidines to their corresponding uracil nucleosides. However, in the present study (using intact cell suspensions) *both* nucleoside deaminase and phosphorylase(s) are obviously present. Since it has been reported that *E. coli* B. does not normally contain a cytosine deaminase,[26] it is reasonable to expect that deamination precedes glycosyl cleavage. This view finds support in the fact that, (a) no cytosine or 5-methylcytosine was obtained from incubation mixtures containing the corresponding nucleosides, and (b) cytosine, itself, is not deaminated by this system (see Table III).

(25) (a) We are indebted to Dr. S. S. Cohen for making available to us the results of his group's study (Dr. M. Nemer and Mr. Govardhan) demonstrating the cleavage of XIII (R′ = CH_3) by a cell-free extract of *E. coli* W_6^- at a slower rate than with uridine. Our data with intact cells confirm their prior observation; (b) W. E. Razzell and H. B. Khorana, *Biochim. et Biophys. Acta*, **28**, 562 (1958).

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

TABLE III

ACTION OF *E. coli* B. ON CYTOSINE NUCLEOSIDES[a]

Substrate	Product of enzymic action
Cytidine[e]	Uracil[c]
5-Methylcytidine[28a]	Thymine[c]
5-Fluorocytidine[27b]	5-Fluorouracil[c,27b]
2′-Deoxycytidine	Uracil[c]
2′-Deoxy-5-methylcytidine[28a]	Thymine[c]
1-β-D-Xylofuranosylcytosine[28b]	Starting material
1-β-D-Xylofuranosyl-5-methylcytosine[28a]	Starting material
5′-Deoxy-5′-fluorocytidine[24a] (XIV)	5′-Deoxy-5′-fluorouridine[f,24a]

[a] The incubation mixture contained 1.0 μmole of nucleoside, 0.8 ml. of 0.067 *M* phosphate buffer (*p*H 7.2) and 0.1 ml. of washed cells of *E. coli* B. in a total volume of 1.0 ml.; incubation carried out at 37° for 60 minutes. [b] Thymidine is cleaved to thymine in this system. [c] Determined by measuring optical density of deproteinized mixture at 300 mμ and *p*H 13. [d] Optical density read at 350 mμ at *p*H 13. [e] Cytosine, itself, is unaffected in this system. [f] Determined spectrally (maximum at 262 mμ, minimum at 242 mμ in 0.1 *N* NaOH); also verified by ascending paper chromatography on Whatman #3 paper in 2-butanol–formic acid–water (7:1:2), R_f 0.51; 5′-deoxy-5′-fluorocytidine gives an R_f of 0.30.

Surprisingly, 5′-deoxy-5′-fluorocytidine[24a] (XIV) is deaminated by these intact cell suspensions to its uridine analog *without* glycosyl cleavage (see Table III). These observations suggest that deaminase(s) present in these cells have a less stringent requirement for the structure of the sugar moiety at C5 than do the nucleosidase(s). The fact that the 1-β-D-xylofuranosyl analogs of cytidine or of 5-methylcytidine are inert to enzymic deamination (as well as to glycosyl cleavage) indicates that, with regard to the C3 hydroxyl of the sugar moiety, deaminases and nucleosidase(s) have similar structural specificity.

Screening Studies.[29]—5-Nitrouridine (IX) and 5-nitro-1-(β-D-ribosyluronic acid)-uracil (II) were tested against Adenocarcinoma 755 in C57 B1 hybrid mice and Sarcoma 180 in the Ha/ICR Swiss mice, respectively. Both compounds were administered by interperitoneal injection for seven successive days; IX at 300 mg./kg./day and II at 500 mg./kg./day. No inhibitory effects upon these tumors were observed.

Acknowledgments.—The authors are indebted to Drs. G. B. Brown and C. C. Stock for helpful discussions and continued interest.

Experimental[30]

5-Nitro-(β-D-ribosyluronic Acid)-uracil (II).—Forty grams (0.165 mole) of uridine was treated with 288 ml. of a nitric acid solution (1 part concentrated acid to 1 part of

(27) (a) R. Duschinsky, E. Pleven, E. Malbica and C. Heidelberger, Abstr. 132nd Meeting, Am. Chem. Soc., 1957, p. 19c; (b) J. J. Fox, I. Wempen and R. Duschinsky, Abstr. Fourth Intl. Congr. of Biochem., Vienna, 1958, p. 6.

(28) (a) J. J. Fox, D. Van Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich and G. B. Brown, THIS JOURNAL, **81**, 178 (1959); (b) J. J. Fox, N. Yung, I. Wempen and I. L. Doerr, *ibid.*, **79**, 5060 (1957).

(29) The authors are indebted to Dr. Donald A. Clarke of the Walker Laboratories of this institute for tumor screening data on these compounds.

(30) All melting points are uncorrected. Elemental analyses were performed by the Schwarzkopf Microanalytical Laboratory.

water) in a shallow dish and evaporated gently on a steam-bath to a hard white solid. After cooling, the solid was triturated thoroughly with 150 ml. of ice-water, filtered, and the damp product recrystallized from 100 ml. of boiling water. On cooling the solution, a white crystalline product was obtained, 29 g. (59%), m.p. 229–231° (eff.) (Levene and LaForge[4] report a m.p. > 200° dec.), $[\alpha]^{26}D$ −31° (*c* 0.92 in water); ultraviolet absorption properties: at *p*H 0 to 3.6, maxima at 238 and 305 mμ, $A_{M(max)}$ 7,970 and 9,780, respectively; minimum at 262 mμ, $A_{M(min)}$ 2,890.

Anal. Calcd. for $C_9H_9N_3O_9$: C, 35.65; H, 2.99; N, 13.86. Found: C, 35.76; H, 3.23; N, 13.94.

Esterification of 5-Nitro-1-(β-D-ribosyluronic Acid)-uracil. A. Butyl Ester (IVa).—A suspension of 1 g. (0.003 mole) of II in 30 ml. of 1-butanol was saturated with HCl gas and the reaction mixture refluxed for 1 hr. Complete solution occurred; on cooling, a copious precipitate of hair-like needles precipitated. After filtration and a thorough wash with cold ether, the product was dried, 1.03 g. (86%). An aliquot was recrystallized twice from water yielding long white needles, m.p. 189–190° (Levene and LaForge[4] report m.p. 190–192°), $[\alpha]^{26}D$ −20° (*c* 0.49 in water); ultraviolet absorption data in *N* HCl: maxima at 238 and 304 mμ, $A_{M(max)}$ 7,800 and 9,680, respectively; minimum at 262 mμ, $A_{M(min)}$ 2,720.

Anal. Calcd. for $C_{13}H_{17}N_3O_9$: C, 43.46; H, 4.77; N, 11.66; mol. wt., 359. Found: C, 43.77; H, 4.76; N, 11.78; mol. wt. (by the isothermal distillation method), 363.

B. Isopropyl Ester (IVb).—Nineteen and one-half grams (0.065 mole) of II was esterified in 250 ml. of isopropyl alcohol following the same procedure outlined for the preparation of the butyl derivative. In this case solution did not occur, although the character of the suspended solid changed to thread-like opalescent needles during the reflux period. After cooling, the blue reaction mixture was filtered and the solid washed with water until neutral and dried, yielding 20.5 g. (93%) of white needles. Recrystallization from boiling water gave a 90% recovery of shining needles, m.p. 240–241° (eff.), s. 238°; $[\alpha]^{24}D$ −31° (*c* 0.57 in dioxane); spectral data in *N* HCl: maxima at 238 and 306 mμ, $A_{M(max)}$ 7,760 and 9,560, respectively; minimum at 262 mμ, $A_{M(min)}$ 2,780.

Anal. Calcd. for $C_{12}H_{15}N_3O_9$: C, 41.74; H, 4.38; N, 12.17. Found: C, 41.76; H, 4.45; N, 12.47.

5-Nitro-1-(2′,3′-isopropylidene-β-D-ribosyluronic Acid Isopropyl Ester)-uracil (Vb).—A suspension of 1 g. (0.0033 mole) of IVb in 30 ml. of dry acetone containing 2 g. of anhydrous copper sulfate and 2 drops of concentrated sulfuric acid was shaken at room temperature for 72 hours. The insoluble material was filtered and the filtrate neutralized with anhydrous sodium carbonate. After removal of the salts, the filtrate was concentrated to dryness. The residue (1 g., 78%) was recrystallized from water, yielding 0.6 g. of white needles, m.p. 188–190°, $[\alpha]^{24}D$ −109° (*c* 0.51 in dioxane).

Anal. Calcd. for $C_{15}H_{19}N_3O_9$: C, 47.14; H, 4.97; N, 10.91. Found: C, 47.09; H, 4.89; N, 11.16.

5-Amino-1-(β-D-ribosyluronic Acid)-uracil (III).—A suspension of 0.5 g. (0.00166 mole) of II and 0.5 g. of 5% palladium–charcoal catalyst in 20 ml. of water was subjected to hydrogenation at room temperature and atmospheric pressure. The calculated amount of hydrogen was taken up in 20 minutes, at which time the reaction was stopped to prevent reduction of the pyrimidine ring. After removal of the charcoal, the colorless filtrate was evaporated and the residual sirup triturated with ether until solidification occurred. The yield of product was 0.37 g. (92%), m.p. 149° (eff.). Recrystallization of the crude solid could not be carried out; however, both paper chromatography and elementary analysis indicated that the product is quite pure. The compound gave a rotation of approximately −1° (*c* 1 in water); ultraviolet properties in *N* HCl: maximum at 294 mμ, $A_{M(max)}$ 4,960; minimum at 234 mμ, $A_{M(min)}$ 1,660; at *p*H 6.67, maximum at 293 mμ, $A_{M(max)}$ 4,060; minimum at 258 mμ, $A_{M(min)}$ 1,660; "apparent" pK_{a1} 3.06.

Anal. Calcd. for $C_9H_{11}N_3O_7 \cdot H_2O$: C, 37.12; H, 4.50; N, 14.43. Found: C, 37.55; H, 4.60; N, 14.49.

5-Amino-1-(β-D-ribosyluronic Acid Isopropyl Ester)-uracil (VIb).—One-half gram (0.00145 mole) of IVa was reduced in aqueous medium under the same conditions described in the preceding experiment. The reduction was complete in 23 minutes. The charcoal was filtered and leached repeatedly with hot water to extract the product. The combined filtrates were evaporated until precipitation began, and then chilled. The white solid was filtered; 0.23 g. (50%), m.p. 199–202°. An additional 0.07 g. (15%) was obtained from the mother liquors. An aliquot was recrystallized twice from water yielding long needles, m.p. 201–202.5°, $[\alpha]^{26}D$ −18° (*c* 1.29 in water); ultraviolet properties in *N* HCl: maximum at 264 mμ, $A_{M(max)}$ 9,190; minimum at 230 mμ, $A_{M(min)}$ 2,050; at *p*H 6.01, maximum at 293 mμ, $A_{M(max)}$ 7,260; minimum at 257 mμ, $A_{M(min)}$ 2,780; pK_{a1} 2.66 (spectrophotometrically calculated).

Anal. Calcd. for $C_{12}H_{17}N_3O_7$: C, 45.72; H, 5.44; N, 13.33. Found: C, 45.87; H, 5.46; N, 13.44.

5-Amino-1-(β-D-ribosyluronic Acid Butyl Ester)-uracil (VIa).—Two grams (0.0056 mole) of IVa were reduced and the reduction mixture treated in the same manner as described for the isopropyl analog. The crude solid was recrystallized from water as long, silky needles, 0.85 g. (56%), m.p. 187–189°, $[\alpha]^{25}D$ −16° (*c* 0.97 in water).

Anal. Calcd. for $C_{13}H_{19}N_3O_7$: C, 47.14; H, 5.82; N, 12.76. Found: C, 47.51; H, 5.54; N, 13.00.

1-β-D-(2′,3′,5′-Tri-*O*-(3,5-dinitrobenzoyl))-uridine (VII).—To a well stirred suspension of 10 g. (0.041 mole) of uridine in 400 ml. of anhydrous pyridine was added 31.1 g. (0.0135 mole) of 3,5-dinitrobenzoyl chloride.[31] The temperature rose to 40°. The reaction mixture was stirred until complete solution of the acyl chloride was achieved. The temperature was adjusted to 50° and the amber colored solution allowed to stand for 50 hours. The reaction mixture was then concentrated to a thin sirup and poured into 2 liters of well stirred water. The sirup immediately solidified to a pale yellow solid. The suspension was stirred until completely granular, filtered, and the solid triturated thoroughly with water to break up the lumps and to obtain a finely-ground, homogeneous product. After filtration by suction, the damp product was extracted in a Soxhlet thimble with absolute ethanol until the extraction liquid remained colorless. This process removed such soluble contaminants as 3,5-dinitrobenzoic acid and excess pyridine. The product was finally washed with ether and dried in an oven at 70° for 5 hours; yield of crude solid, 32.5 g. (93%), m.p. 233–239°. An aliquot was purified for analysis by re-extraction in a Soxhlet apparatus for an additional 5 hours. The residue was dissolved in hot acetone, filtered, and the filtrate concentrated *in vacuo* until precipitation began. After chilling, the suspension was filtered, washed with ether and dried *in vacuo* at 100° for 2 hours.

Anal. Calcd. for $C_{30}H_{18}N_8O_{31}$: C, 43.60; H, 2.20; N, 13.55. Found: C, 44.26; H, 2.32; N, 13.35.

Oddly enough, in this case, the analytical figures cannot distinguish among the possible acylated forms, mono-, di-, tri- or tetra-. The only significant value is the "benzoyl" number; however, the values for this determination reported by conventional analytical procedures were found to be widely inconsistent. Therefore, the method used was an indirect one, which utilized the presence of two reducible nitro groups for every acyl group present in the compound. The procedure involved the saponification of an aliquot of VII with methanolic sodium methylate (4 moles of the latter to 1 mole of the compound). After 2.5 hr. of reflux time, the saponification mixture was cooled and acidified with an alcoholic HCl solution. The entire reaction mixture was then subjected to hydrogenation at normal temperatures and pressure using palladium-on-charcoal as catalyst. The uptake of hydrogen was found to be identical with that calculated for the tri-acylated compound.

5-Nitro-1-β-D-(2′,3′,5′-tri-*O*-(3,5-dinitrobenzoyl))-uridine (VIII).—Thirty-one grams (0.0376 mole) of VII was added portionwise to 123 ml. of a mixture (50% by volume) of concentrated sulfuric acid and fuming nitric acid (sp. gr. 1.5) cooled to room temperature. Since the nitration was only mildly exothermic, the addition was made rapidly, the maximum temperature of the reaction mixture rising to *ca.* 50°.

(31) The yield of acylated product was found to depend heavily on the state of purity of the 3,5-dinitrobenzoyl chloride used in the reaction.

The resulting dark-green solution was allowed to stand at room temperature for 0.5 hour. During this time, the color changed to a bright yellow. The reaction mixture was poured slowly into a vigorously stirred slurry of ice and water. The precipitated white solid was filtered immediately and washed repeatedly with water until the *p*H of the wash filtrate became neutral. The solid, washed with alcohol and ether and dried in the oven at 70° for 5 hours, represented a crude yield of 23.1 g. (71%), shr. 150°, starts melting at 155°, eff. at 165°.

During the course of nitration, a displacement of some of the blocking groups occurred; therefore, no analytical data were obtained for this intermediate.

5-Nitrouridine (IX).—Twenty-three grams (0.0265 mole) of VIII was suspended in one liter of anhydrous ethanol and heated to reflux. To the stirred suspension was added, in one portion, a solution of freshly prepared sodium ethylate (1.2 g., 0.053 mole of sodium in 250 ml. of ethanol). The solid dissolved immediately with the formation of a dark red solution followed within 10 minutes by a re-precipitation of solid. The refluxing was continued for a total time of 3 hr., during which time the color gradually changed to orange. The hot reaction mixture was filtered and the precipitate triturated repeatedly with warm ethanol until no more color was extracted into the alcohol. The precipitate was finally washed with ether and dried; yield of crude sodium salt of IX 7 g. (85%). The finely-ground solid was suspended in 150 ml. of ethanol at 70° and treated dropwise with concentrated sulfuric acid (*ca.* 0.8 ml.) until a *p*H of 3 was attained. During addition of the acid, the solid gradually dissolved and simultaneously the precipitation of a fine granular salt occurred. The inorganic salts were filtered off and the bright yellow filtrate concentrated *in vacuo* until precipitation began to occur. After thorough chilling, the off-white solid was filtered, washed with a minimum amount of cold alcohol and a final ether wash; yield of crude product 3.5 g. (46% based on uridine), m.p. 179–182° (eff.). The solid was recrystallized from 200 ml. of ethanol with charcoaling when necessary. After slow cooling, the product was obtained as a white crystalline solid, m.p. 188–190°, $[\alpha]^{24}D$ −45° (*c* 0.53 in dioxane), −18 (*c* 1 in water); ultraviolet absorption properties at *p*H 3: maxima at 238 and 305 mμ, $A_{M(max)}$ 8,470 and 10,100, respectively; minimum at 262 mμ, $A_{M(min)}$ 2,690.

Anal. Calcd. for $C_9H_{11}N_3O_8$: C, 37.38; H, 3.83; N, 14.54. Found: C, 37.78; H, 3.97; N, 14.59.

5-Aminouridine (X). A. Free Base.—One gram (0.0035 mole) of IX was hydrogenated in ethanol using 5% palladium-on-charcoal catalyst. The hydrogenation was stopped when the calculated amount of hydrogen had been taken up to prevent further reduction of the pyrimidine ring. The catalyst was filtered off and the filtrate concentrated to dryness. The hygroscopic solid was recrystallized from isopropyl alcohol–water (35:1.5), which gave 0.2 g. of a white solid, eff. 202–204°, shr. 198° (Roberts and Visser[10] report a m.p. 214–216°). A mixed m.p. with an authentic[10] sample, however, showed no depression.

B. Hydrochloride Salt of X.—One-half gram of IX was hydrogenated under the same conditions as previously described. The catalyst was filtered and the filtrate concentrated to one-half volume. The solution was cooled to 5°, saturated with dry HCl gas; the resulting white solid was filtered, washed with dry ethanol and ether; yield of the hydrochloride salt, 0.3 g. (59%), m.p. 220° dec. An aliquot was recrystallized twice from 90% ethanol and analyzed for the hemihydrate, $[\alpha]^{24}D$ +2° (*c* 1.74 in water, for the hydrochloride), +2.4° calculated for the free nucleoside; ultraviolet absorption data in *N* HCl: maximum at 266 mμ, $A_{M(max)}$ 9,020; minimum at 231 mμ, $A_{M(min)}$ 1,570; at *p*H 6.67, maximum at 294 mμ, $A_{M(max)}$ 7,200; minimum at 257 mμ, $A_{M(min)}$ 2,600; pK_{a_1} 3.11 (spectrophotometrically determined).

Anal. Calcd. for $C_9H_{13}N_3O_6 \cdot HCl \cdot$hemihydrate: C, 35.48; H, 4.96; N, 13.79; Cl, 11.64. Found: C, 35.83; H, 5.24; N, 13.79; Cl, 11.49.

5-Nitro-1-(2′,3′-isopropylidene-β-D-ribofuranosyl)-uracil (XI).—A suspension of one gram (0.0035 mole) of IX, 1.3 g. of anhydrous copper sulfate in 30 ml. of dry acetone containing 3 drops of concentrated sulfuric acid was shaken at room temperature for 10 hours and allowed to stand overnight. After removal of the insolubles, the filtrate was neutralized with anhydrous sodium carbonate to a *p*H of 5, the salt filtered, and the filtrate concentrated to dryness. The yield of crude product was 1.1 g., m.p. 203–205°. Recrystallization from 70 ml. of water gave 0.64 g. (56%) of needles, m.p. 207–208°, $[\alpha]^{24}D$ −64° (*c* 0.71 in dioxane).

Anal. Calcd. for $C_{12}H_{15}N_3O_8$: C, 43.77; H, 4.59; N, 12.76. Found: C, 43.40; H, 4.62; N, 13.08.

5-Deoxyuridine (XIII, R′ = CH_3).—5′-Deoxy-5′-iodo-2′,3′-isopropylideneuridine[25] (0.94 g., 0.0024 mole) was dissolved in 25 ml. of warm methanol. The solution was cooled, 0.5 g. of a 5% palladium-on-charcoal catalyst and 1 ml. of *n*-butylamine were added, and the reaction hydrogenated at atmospheric pressure and room temperature. The uptake of hydrogen was quite rapid, the theoretical amount being consumed within 5 minutes. The catalyst was removed and the filtrate concentrated *in vacuo* to a colorless sirup, which was then refluxed for 10 minutes with 10 ml. of *N* HCl. The solution was neutralized with ammonium hydroxide and evaporated *in vacuo* to dryness. The residue was extracted several times with portions of hot acetone, and the combined acetone filtrates concentrated to dryness. The residual sirup was reconcentrated with ethanol until solidification occurred. The yield of crude product was 0.41 g. (75%), m.p. 174–177°. Recrystallization from a minimum amount of ethanol gave clusters of silky white needles, m.p. 174–176°, $[\alpha]^{26}D$ +11° (*c* 0.57 in water); ultraviolet absorption data at *p*H 0–6.67: maximum at 262 mμ, $A_{M(max)}$ 9,540; minimum at 230 mμ, $A_{M(min)}$ 2,185; at *p*H 12: maximum at 262 mμ, $A_{M(max)}$ 7,340; minimum at 242 mμ, $A_{M(min)}$ 5,410; pK_a 9.22 (spectrophotometrically calculated).

Anal. Calcd. for $C_9H_{12}N_2O_5$: C, 47.37; H, 5.30; N, 12.28. Found: C, 47.25; H, 5.46; N, 12.55.

Spectrophotometric Studies.—Ultraviolet absorption data were determined with a Cary recording spectrophotometer, model 11, using buffers and techniques previously described.[7,18] Phosphate buffers were employed for the *p*H range of 6 to 7, while the curves in the strongly acid *p*H range of 0 to 3 were measured in HCl solutions. The pK_a values were determined spectrophotometrically by methods previously employed.[7,32]

Polarimetric Determinations.—Optical rotations were determined using equipment and techniques previously described.[15]

Metaperiodate Titrations.—The metaperiodate titrations were carried out according to the procedures described in a previous publication.[15]

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