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CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS. XXXIX. THE NUCLEOSIDES OF SPONGES. III.[1] SPONGOTHYMIDINE AND SPONGOURIDINE[2]

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A few years ago the senior author and Feeney (1) reported the isolation from the Carribean sponge, *Crypotethia crypta*, of a mixture of nucleosides not previously encountered in nature. Two of these, spongothymidine and spongosine, were obtained in a pure state, and the former, the more abundant of the two, was shown to be a thymine-pentofuranoside. Although it was not possible at that time to characterize the carbohydrate moiety of spongothymidine fully, all available evidence indicated that it was not ribose. This conclusion has found support in the studies by Fox and Shugar (2) on the influence of *p*H on the ultraviolet spectra of nucleosides.

A paper chromatogram[3] of certain crude fractions of nucleosides revealed the presence of relatively small amounts of a third nucleoside which had a similar ultraviolet absorption spectrum to that of uridine but with different R_f values. Preliminary studies showed it to be derived from uracil and it was consequently named spongouridine. A separation of the nucleosides was achieved by fractionation on a column of Dowex-1 resin (OH′ form) and the use of ammonium hydroxide-ammonium formate buffers (3). Spongosine when present was eluted by a buffer of *p*H 9.5, and elution by a buffer of *p*H 8.3 gave four components in the following order: spongothymidine closely followed by thymine, and uracil closely followed by spongouridine. A large scale separation gave gram amounts of pure spongosine and spongothymidine, but the spongouridine fraction also contained some uracil and spongothymidine; these were removed by refractionation. Isolation of the spongouridine was hindered by its great solubility in water and by the presence of ammonium formate, but was eventually accomplished by passing the neutral aqueous solution through a column of Dowex-1 (OH′ form), washing with water, elution with dilute formic acid, evaporation and recrystallization from methanol.

Spongouridine is a nicely crystalline material, m.p. 226–228°, $[\alpha]_D$ +97° in 8% NaOH and +126° in water, *p*K 9.3 (obtained from change of ultraviolet spectrum with *p*H). Its elementary composition, $C_9H_{12}N_2O_6$, corresponds to that of a uracil pentoside. As shown by Figure 1, the nucleoside shows changes of the ultraviolet spectrum at high *p*H values similar to those shown by spongothymidine (2). Formic acid hydrolysis of spongouridine gave uracil as the only heterocyclic

[1] The Nucleosides of Sponges. II. *Angew. Chem.*, **67**, 127 (1955).

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[3] The authors are greatly indebted to Prof. E. Chargaff and his associates for their initial help in this matter.

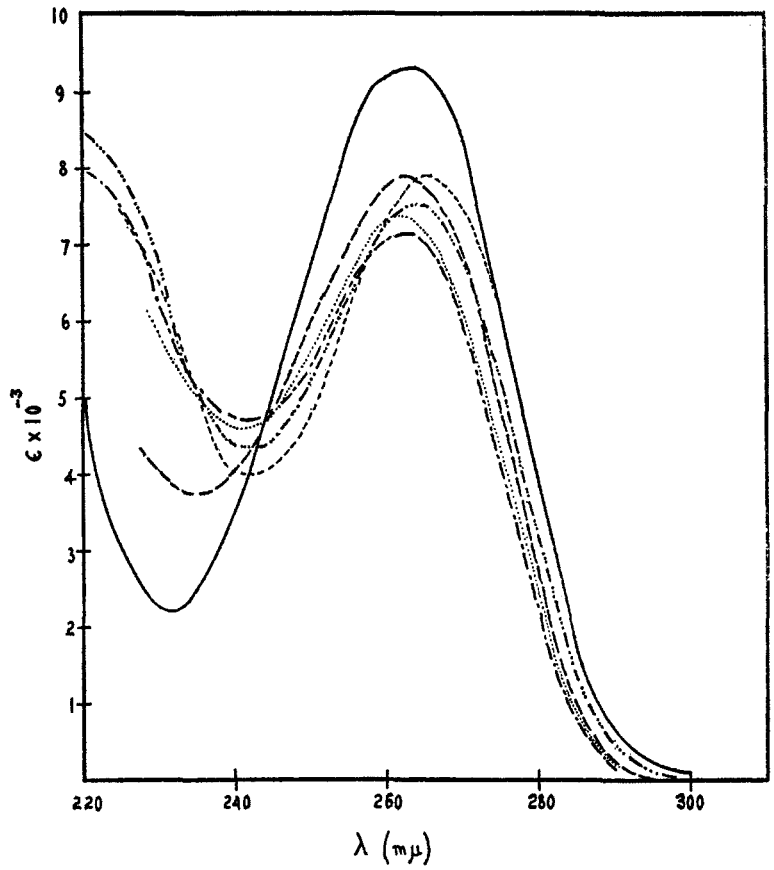

FIG. 1. ULTRAVIOLET SPECTRUM OF SPONGOURIDINE AT *p*H VALUES INDICATED: ——— *p*H 1 and 7.2; - - - *p*H 9.6; ..... *p*H 10.7; - ——— - ——— *p*H 11.5; ••• — ••• *p*H 13; ------- *p*H 14.

component. The nucleoside consumed one mole of sodium periodate without formation of formic acid (4), thus showing it to be a uracilpentofuranoside.

In the first paper (1) it was suggested on the basis of molecular rotation differences that spongothymidine was a xyloside. This suggestion found temporary support in the work of Makino and Satoh (5) who reported the presence of xylose on a paper chromatogram of a sulfuric acid hydrolysis of the nucleoside.[4] We have been unable to repeat this experiment and have never succeeded in subjecting the nucleoside to an acid hydrolysis without destruction of the carbohydrate fragment.

As has been pointed out before (1), spongothymidine is at least as resistant to acid hydrolysis as is uridine. It is not hydrolyzed by refluxing 5%-hydrochloric or 10%-sulfuric acid. When heated with the latter in a sealed tube at 125° a partial hydrolysis occurs during which, however, the pentose is converted to furfural. Equally unsuccessful were attempts to isolate the pentose as a methyl-

[4] Supplied by the senior author.

glucoside by heating the nucleoside with anhydrous 5% hydrochloric acid in methanol at a 100° in a sealed tube. No noticeable methanolysis took place under these conditions.

It has been shown by Levene and LaForge (6) that a hydrolysis of uridine without destruction of the pentose can be achieved by prior catalytic reduction of the pyrimidine ring. Unsuccessful attempts to apply this procedure to spongothymidine have already been reported (1). In continuation of such studies the junior author of the present paper has developed a new and very convenient method for a mild, reductive hydrolysis of pyrimidine nucleosides (7). It consists in the reduction of the pyrimidine ring with sodium and alcohol in liquid ammonia followed by a hydrolysis of the reduced material on a Dowex-50 column (H^+ form). Application of this method to spongothymidine proved the pentose to be D-arabinose, thus refuting the claims mentioned above. Arabinose was also obtained in an analogous manner from spongouridine. The identification of the carbohydrate was accomplished through paper chromatography in various solvents, paper ionophoresis in borate buffer (8), and comparison of the phenylosazone with authentic material.

The presence in both nucleosides of the *trans* C_2-C_3-glycol group of arabinose was confirmed by the rate of periodate oxidation and by ionophoresis in a borate buffer. As is to be expected of *trans* glycols the periodate uptake of the two nucleosides is much slower than that of a ribonucleoside with *cis*-hydroxyls at C_2-C_3. (Figure 2) (9). Similarly the migration rates of the two nucleosides during ionophoresis are not accelerated by borate ions as are those of nucleosides, such as ribosides, with *cis*-oriented glycol groups (10).

The fission products of the periodate oxidations of spongouridine and uridine show the same optical rotatory power, and since uridine is a β-glycoside, the glycosidic link in spongouridine must also be β. Similarly the β-glycosidic structure in spongothymidine was demonstrated through comparison of the rotations

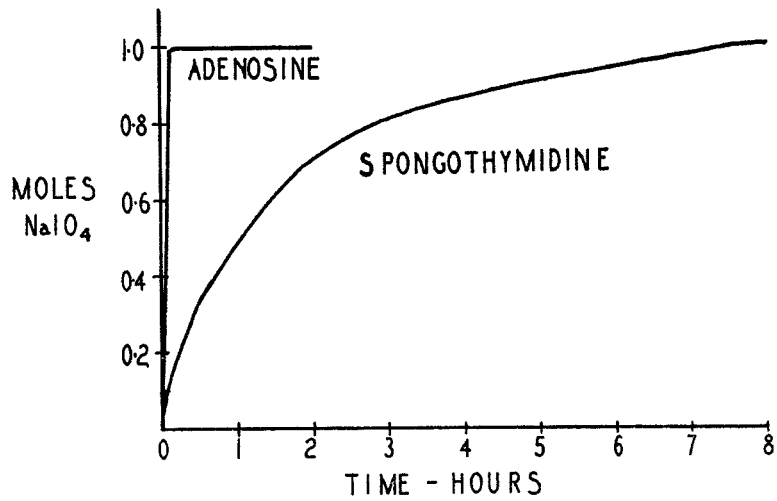

FIG. 2. RATE OF UPTAKE OF PERIODATE BY SPONGOTHYMIDINE

TABLE I

COMPARISON OF MOLECULAR ROTATION SPECTRA

Molecular Rotation of Uracil Nucleoside (A)	Molecular Rotation of Thymine Nucleoside (B)	(A − B)
Deoxyuridine +114[a]	Thymidine +79[a]	+35
Spongouridine +233[b]	Spongothymidine +201[b]	+32

[a] In 1 *N* NaOH. [b] In 2 *N* NaOH.

of its oxidation product with that of 3-β-D-glucopyranosylthymine (11). Spongothymidine has therefore been shown to be 3-β-D-arabofuranosylthymine, and spongouridine to be 3-β-D-arabofuranosyluracil.[5] The close relationship between the two new nucleosides is also evidenced by a comparison of the molecular rotation spectra as shown in Table I.

Convincing evidence is still lacking to prove that these new sponge nucleosides are derived from hitherto unknown types of nucleic acids occurring in sponges and to a minor extent possibly also in other animals. It is to be noticed that the most significant difference between spongothymidine and spongouridine on the one hand and the ribonucleosides on the other is the orientation of the C_2-hydroxyl group of the carbohydrate moiety. This hydroxyl group does not appear to be involved in internucleotide linkages (15). On the other hand it seems unlikely that cyclic phosphates could be obtained from locked *trans* (C_2-C_3) glycol groupings such as occur in a furanose ring (16). Experiments aimed at solving these problems are now in progress in this laboratory.

EXPERIMENTAL

Melting points were taken on a Kofler block. Rotations were measured at 20 to 25°.

Materials. The authors are indebted to Dr. J. J. Fox of the Sloan-Kettering Institute, New York, for the gift of D-glucopyranosylthymine. The ribonucleosides were obtained from Schwarz Labs., Mount Vernon, N. Y.

Paper chromatography of nucleoside mixtures. Paper chromatography (descending system) of crude nucleoside mixtures with *n*-butanol: ammonia:water (saturated:1:4) (13) as solvent revealed the following components: Spongouridine (R_f 0.13), spongothymidine (R_f 0.28), spongosine (R_f 0.35), and thymine (R_f 0.41). The ultraviolet absorbing components were detected by viewing with a 'mineralight' lamp with a Corning Filter #9863, and were identified by elution at *p*H 1, 7, and 12 and determination of the ultraviolet spectra.

Ion-exchange separation of nucleoside mixtures. Preliminary experiments using 5–10 mg. of the nucleoside mixtures on a column of Dowex-1 resin (OH′ form, 13 cm. x 2 cm.^{2}) and

[5] Since this work was completed it was learned that Sir Alexander Todd and his group have obtained 3-β-D-arabofuranosyluracil from uridylic acid *a*. Through the courtesy of Sir Alexander Todd it has been possible to make a comparison of this compound with spongouridine. The two compounds have the same melting point and optical rotation and the mixture melting point is undepressed. They behave identically on paper chromatograms: *n*-butanol:water:ethanol (5:4:1) (upper layer) (12); *n*-butanol:ammonia:water (saturated:1:4) (13); and *n*-butanol:diethylene glycol:water (4:1:1) (14); and also on paper ionophoresis in a borate buffer.

ammonium hydroxide-ammonium formate buffers (3) showed that a separation could be obtained: spongosine when present was eluted by buffer on *p*H 9.5, and elution by buffer of *p*H 8.3 gave spongothymidine and spongouridine successively. None of the crude fractions contained more than 15% of spongouridine (as judged by ultraviolet absorption).

Use of a larger column (68 cm. x 20 cm.^{2}) made possible the separation of gram quantities of the nucleoside mixtures. The sequence of elution was the same except that some thymine, identified by paper chromatography and the ultraviolet spectrum, was eluted after the spongothymidine fraction. The spongouridine fraction was shown by paper chromatography to contain some spongothymidine, thymine, and uracil and was refractionated on the large column. Elution at *p*H 8.0 gave a mixture of spongothymidine and thymine followed by some uracil. Further elution gave a spongouridine fraction which gave a single spot on paper chromatography The nucleoside could not be separated from ammonium formate due to its great solubility in water and so an aqueous solution was passed down a column of Dowex-1 resin (OH′ form, 30 cm. x 20 cm.^{2}). No ultraviolet absorbing material was eluted with water but elution with *N*/20 formic acid gave spongouridine. The fraction was evaporated, a little methanol-insoluble material was removed, and the solid was recrystallized from methanol to give spongouridine as white, almost cubic, crystals, m.p. 226–228°, $[\alpha]_D$ +97° (*c*, 0.6, 8% NaOH), +126° (*c*, 1.0, water).

Anal. Calc'd for $C_9H_{12}N_2O_6$: C, 44.26; H, 4.95; N, 11.47.
Found: C, 44.48; H, 5.02; N, 11.51.

Formic acid hydrolysis of spongouridine. The nucleoside (5 mg.) was heated in a sealed tube with 90% formic acid (2 cc.) for two hours at 150°. Paper chromatography of the product with *n*-butanol:water:ethanol (5:4:1) (upper layer) (12) and *n*-butanol:ammonia:water (saturated:1:4) (13) as solvents showed two ultraviolet-absorbing spots. These were identified by R_f values and by elution and determination of ultraviolet spectrum, as unchanged spongouridine and uracil.

Acid hydrolysis of spongothymidine. (a). Spongothymidine (3.5 mg.) was refluxed in 2 cc. of 5% hydrochloric acid for three hours. Paper chromatography showed that no hydrolysis had occurred.

(b). Spongothymidine (1.2 mg.) was refluxed in 2 cc. of 10% sulfuric acid for five hours. Paper chromatography showed only the presence of unchanged spongothymidine.

(c). Spongothymidine (3 mg.) was heated in 2 cc. of 10% sulfuric acid in a sealed tube for 2 hours at 125°. Paper chromatography showed that some thymine had been produced but no pentose could be detected.

(d). Spongothymidine (3 mg.) was heated in a sealed tube with 15 cc. of 5% methanolic hydrochloric acid at 100° for five hours. Paper chromatography showed that no hydrolysis had occurred.

Sodium and liquid ammonia reduction of nucleosides. Spongothymidine (467 mg.) in dry liquid ammonia (100 cc.) and dry ethanol (5 cc.) was reduced with sodium (0.4 g.) as described by Burke (7). The resulting solution showed only end absorption in the ultraviolet and was passed down a column of Dowex (H^+ form, 25 cm. x 3 cm.^{2}). Evaporation gave 400 mg. of a yellow gum $[\alpha]_D$ −51° which was shown by paper chromatography with *n*-butanol:ethanol:water and *n*-butanol saturated with water (17) as solvents and by paper ionophoresis in a borate buffer (8) to contain arabinose as the only carbohydrate constituent. Treatment with phenylhydrazine in the usual way gave a phenylosazone; the latter, when recrystallized from aqueous methanol, gave material, the melting point of which, 154–155°, was not depressed by admixture with the phenylosazone from ribose. The infrared spectrum was identical with that of the phenylosazone from ribose (in dioxane), but different from that of the phenylosazone from xylose (in the 9.75 region). The material had $[\alpha]_D$ −29° (in methanol: pyridine (2:1) after one hour). D-Ribose phenylosazone under identical conditions had $[\alpha]_D$ −32° and D-xylose phenylosazone $[\alpha]_D$ −19°.

Similar reduction of spongouridine (5.3 mg.) followed by paper chromatography with

n-butanol:ethanol:water and *n*-butanol saturated with water as solvents showed the sugar to be arabinose.

Periodate oxidation of nucleosides. A weighed quantity of the nucleoside (20–50 mg., dried at 150° and 0.1 mm.) was dissolved in water, 5 cc. of 0.2808 *N* sodium periodate was added, and the volume was made up to 50 cc. At intervals aliquots were removed and the uptake of periodate was measured by the method of Lythgoe and Todd (4). The following results were obtained:

Time (hours)	No. of Moles of Periodate Consumed					
	Adenosine	Guanosine	Cytidine	Uridine	Spongothymidine	Spongouridine
0.1	0.99	0.97	0.93	0.98	0.10	
.25	1.00	.97	.95	1.00	.21	0.19
.50	1.00	.97	1.00	1.00	.33	.34
1.0	1.00	.97	1.00	1.00	.48	.48
2.0					.74	.73
3.0					.81	.81
5.0					.91	1.04
8.0					1.00	1.15
24.0					1.00	1.20

A 14.1 mg. sample was used in the titration of spongouridine and these figures are less accurate than the others; no formic acid was produced.

Paper ionophoresis of nucleosides. The following figures were obtained: spongothymidine Mg 0.50, spongouridine Mg 0.68.

Stereochemistry of glycosidic link. Oxidation of spongothymidine (23 mg.) with 1 cc. of 0.26 *N* sodium periodate (total volume 3.09 cc.) gave after 24 hours a solution $[\alpha]_D$ +16.3° (calculated for anhydrous fission product). Similar oxidation of D-glucopyranosylthymine gave a solution $[\alpha]_D$ +17°.

Similar oxidation of spongouridine (21 mg.) gave after 24 hours a solution $[\alpha]_D$ +15°. Oxidation of uridine (25 mg.) gave a solution $[\alpha]_D$ +15.2°. Davoll, Lythgoe, and Todd (18) record $[\alpha]_D$ +16° for the uridine oxidation product.

SUMMARY

1. Spongothymidine has been shown to be 3-β-D-arabofuranosylthymine.

2. A new nucleoside named spongouridine, obtained from the Carribean sponge *Crypotethia crypta*, has been shown to be 3-β-D-arabofuranosyluracil.

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