ENZYMATIC SYNTHESIS OF THIOURACIL RIBOSIDE
AND THIOURACIL DESOXYRIBOSIDE*

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In a survey of various substrates for pyrimidine nucleoside synthesis, it was noted that inorganic orthophosphate was released when 2-thiouracil and desoxyribose-1-phosphate were incubated together with thymidine phosphorylase (1). This finding, taken as a fairly certain indication of nucleoside synthesis, has now been fully substantiated by isolation and characterization of crystalline thiouracil desoxyriboside.

Initial experiments were carried out with the more readily available ribose-1-phosphate rather than with desoxyribose-1-phosphate. The ribonucleoside of thiouracil was prepared enzymatically by the Kalckar reaction (2) shown in Reaction 1.

\[
\text{Thiouracil} + \text{ribose-1-phosphate} \rightleftharpoons \text{thiouracil riboside} + \text{inorganic orthophosphate}
\]  

(1)

Synthesis of the riboside was demonstrated not only by the release of inorganic orthophosphate but also by the disappearance of pentose. The ribonucleoside was separated from thiouracil by filter paper chromatography. Its ultraviolet spectrum differed markedly from that of thiouracil at pH 9.0. Upon enzymatic arsenolysis thiouracil riboside was cleaved to free thiouracil and free ribose by Reactions 2 and 3.

\[
\text{Thiouracil riboside} + \text{arsenate} \rightarrow \text{thiouracil} + \text{ribose-1-arsenate} \quad (2)
\]

\[
\text{Ribose-1-arsenate} \quad \text{(spontaneous)} \rightarrow \text{ribose} + \text{arsenate} \quad (3)
\]

On the basis of the spectral data obtained with thiouracil riboside, analogous experiments were performed with desoxyribose-1-phosphate and resulted in the isolation of crystalline thiouracil desoxyriboside.

EXPERIMENTAL

Substrates and Enzymes—Ribose-1-phosphate, prepared from guanosine by the method described by Kalckar (2), was fractionated as the calcium salt\(^1\) and freed of contaminating purines with activated charcoal (Norit

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\(^1\) Unpublished procedure of Dr. Graham Webster.
A). Desoxyribose-1-phosphate was prepared as the dicyclohexylammonium salt (1). Thymidine phosphorylase (with an activity of 40 μM of thymidine split per mg. of protein per hour (3)) was prepared from horse liver.

Release of Inorganic Orthophosphate; Measure of Nucleoside Synthesis—When thiouracil (0.18 μM) was incubated with ribose-1-phosphate (0.058 μM) and phosphorylase, inorganic orthophosphate (0.041 μM) was released, whereas in the absence of thiouracil no phosphate was released (Table I). In this experiment, in which an excess of thiouracil was present, 71 per cent of the ribose-1-phosphate initially present entered into nucleoside synthesis.

<table>
<thead>
<tr>
<th>Release of Inorganic Phosphate As Measure of Nucleoside Synthesis</th>
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<tbody>
<tr>
<td>The incubation mixture consisted of 0.18 μM of thiouracil, 0.058 μM of ribose-1-phosphate, and thymidine phosphorylase in a total volume of 62 μl. of 0.074 M Tris-HCl buffer, pH 7.4; temperature, 38°. The reaction was stopped by the addition of 60.6 μl. of magnesia mixture, and inorganic phosphate was determined in the precipitate (4).</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Release of inorganic phosphate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Thioracil absent</td>
</tr>
<tr>
<td>0</td>
<td>0.005</td>
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<tr>
<td>10</td>
<td>0.005</td>
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<td>50</td>
<td>0.005</td>
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<td>60</td>
<td>0.003</td>
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Disappearance of Pentose; Measure of Nucleoside Synthesis—Ribose when bound in nucleosidic linkage with pyrimidine shows a limited response to the orcinol reagent of Mejbaum (5). Paege and Schlenk (6) have taken advantage of this fact in demonstrating the enzymatic synthesis of uridine from uracil and ribose-1-phosphate. Similarly, in the present study when thiouracil (0.68 μM) was incubated with ribose-1-phosphate (0.15 μM) and phosphorylase in a total volume of 148 μl. of 0.03 M Tris-HCl buffer, pH 7.4, for 45 minutes at 38°, 64 per cent of the ribose initially present could not be accounted for by the orcinol method.

Isolation of Thiouracil Riboside—A mixture consisting of 77 μM of thiouracil, 28.6 μM of ribose-1-phosphate, and phosphorylase, in a total volume

*Tris, tris(hydroxymethyl)aminomethane.
of 16.1 ml. of 0.12 M Tris-HCl buffer, pH 7.4, was incubated for 45 minutes at 38°. The enzymatic reaction was terminated by the addition of 225 ml. of acetone and the protein spun off. The supernatant fluid was evaporated to a small volume and applied to Whatman No. 1 filter paper for ascending chromatographic development with n-butanol saturated with 3 per cent boric acid (7).

Compounds were located on the filter paper with ultraviolet light at \( R_F \) 0.5 (Compound A) and at \( R_F \) 0.25 (Compound B). Compounds A and B were eluted from the filter paper with \( \text{H}_2\text{O} \) and rechromatographed with butanol-borate. Compound A at different pH values had an absorption spectrum identical with that of thiouracil (Fig. 1). Compound B had a spectrum similar to that of thiouracil in acid; however, at pH 9.0 a marked difference was noted (Fig. 2).

When Compound B was incubated with phosphorylase in 0.2 m Tris-0.1 m arsenate buffer, pH 7.4, for 45 minutes at 38°, essentially all of the ribose present was released as measured by the orcinol method (based on the assumption that Compound B was pure thiouracil riboside and that its molar extinction value was the same as that of crystalline thiouracil desoxyriboside (Table II)). When the products of an incomplete arsenolysis from a similar incubation mixture were subjected to paper chromatog-
raphy with the butanol-borate solvent mixture, compounds were again found at \( R_F \) 0.5 and 0.25 and identified by their spectra as thiouracil and Compound B. Since Compound B released thiouracil and free pentose upon enzymatic arsenolysis and since ribose-1-phosphate was the only source of pentose, it is most likely that Compound B is thiouracil riboside.

**Isolation of Crystalline Thiouracil Desoxyriboside**—To 15 mg. of thiouracil dissolved with warming in 5 ml. of \( H_2O \) were added 5 ml. of thymidine phosphorylase (123 mg. of protein; activity, 3.5 \( \mu \)M of thymidine split per mg. of protein per hour (3)) and 79 mg. of dicyclohexylammonium desoxy-

<table>
<thead>
<tr>
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<th>( \varepsilon )</th>
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<th>( \varepsilon )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03 N HCl</td>
<td>274</td>
<td>17,800</td>
<td>270</td>
<td>13,900</td>
</tr>
<tr>
<td>0.02 M Tris-HCl buffer, pH 7.4</td>
<td>272</td>
<td>17,800</td>
<td>268</td>
<td>11,800</td>
</tr>
<tr>
<td>0.2 M ( K_2HPO_4 )</td>
<td>238</td>
<td>23,700</td>
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</tr>
<tr>
<td>0.2 ( \mu ) ( K_2HPO_4 )</td>
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<td>17,300</td>
<td>305</td>
<td>6,150</td>
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*Thiouracil desoxyriboside (0.3 mg.) was dissolved in 1.0 ml. of \( H_2O \) (Solution A). Spectra were determined by diluting 25.5 \( \mu \)l aliquots of Solution A with 1.0 ml. of medium. Desoxyriboside was determined as follows: To 51.0 \( \mu \)l of Solution A were added 51.0 \( \mu \)l of 1 per cent cysteine hydrochloride and 1.0 ml. of \( H_2SO_4 \) (70 ml. of \( H_2SO_4 \) sp. gr. 1.84, plus 30 ml. of \( H_2O \)). After 12 minutes the optical density at \( \lambda = 490 \text{ nm} \) was 0.333. A standard solution of thymidine containing 300 \( \gamma \) per ml. yielded the optical density 0.385. On this basis, Solution A contained 1.07 \( \mu \)M of desoxyriboside per ml. The molecular extinction coefficients were calculated from this value.

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After 6 hours at \( 38^\circ \), the incubation period was terminated by the addition of 150 ml. of \( n \)-butanol and 150 ml. of diethyl ether. The mixture was filtered and evaporated to dryness with an air stream. To the residue, dissolved in 1 ml. of \( H_2O \), were added 15 ml. of \( n \)-butanol and 45 ml. of diethyl ether. The precipitate was spun off and discarded.

Thiouracil was removed in several crops by evaporation of the supernatant fluid until the mother liquor exhibited an absorption spectrum in 0.2 M \( K_2HPO_4 \) similar to that of thiouracil riboside (Fig. 2). The mother liquor was applied to Whatman No. 1 filter paper and chromatographed with \( n \)-butanol saturated with \( H_2O \). The water eluate of the band at \( R_F \) 0.5 to 0.6 upon evaporation yielded crystalline thiouracil desoxyriboside (needles).

Approximately 0.3 mg. of the crystalline product was dissolved in 1.0
ml. of H₂O (Solution A). The amount of desoxyribose present was determined by the Dische cysteine-H₂SO₄ method, as modified by Stumpf (8), with recrystallized thymidine as the standard. Solution A contained 1.07 μM of desoxyribose per ml., compared with the value of 1.24 ± 0.17 μM of thiouracil per ml. (based on the amount weighed and a molecular weight of 244).

The molecular extinction values of thiouracil desoxyriboside, based on the more accurately determined amount of desoxyribose present in Solution A rather than on the weight, are compared with those of thiouracil in Table II. Enzymatic arsenolysis of thiouracil desoxyriboside resulted in a change of the ultraviolet absorption spectrum to one identical with thiouracil.

Point of Attachment of Pentose to Thiouracil—In uridine the attachment of the sugar is to nitrogen 1 of the pyrimidine ring (9) and is very stable to acid hydrolysis. On the basis of the close similarities of the absorption spectra of uridine and thymidine at various pH values, Ploeser and Loring (10) concluded that the desoxyribose group of thymidine is attached to nitrogen 1 of thymine. It has been shown that thymidine synthesized enzymatically by the action of thymidine phosphorylase on thymine plus desoxyribose-1-phosphate is identical with natural thymidine (1). Therefore, the phosphorylase definitely acts at nitrogen 1 of the pyrimidine ring of thymine and, quite likely, acts in a similar way on thiouracil. It is concluded that the point of attachment of the sugar in the thiouracil nucleosides is at nitrogen 1. The stability of thiouracil riboside to acid hydrolysis is consistent with this structure.

DISCUSSION

The enzymatic synthesis of the thiouracil nucleosides by reaction of thiouracil with ribose-1-phosphate or desoxyribose-1-phosphate suggests the possibility that nucleoside formation might be the primary event which eventually, if not directly, results in the antimetabolic effects of thiouracil on bacteria (11), tobacco mosaic virus (12), Protozoa (13), seedlings (14), and 2-acetylaminofluorene-induced hepatomas (15). It is conceivable that, although the competition between uracil and thiouracil occurs in the nucleoside phosphorylase reaction, the metabolic damage due to thiouracil riboside formation may occur at some other step.

Jeener and Rosseels (16) have isolated thiouridylic acid from tobacco mosaic virus grown in the presence of S⁴⁰⁻thiouracil and have suggested that the antimetabolic effects of thiouracil may be due to formation of a malfunctioning thiouracil-containing ribonucleic acid. Rutman et al. (17) have reported the incorporation of C⁴⁻thiouracil into the nucleic acid of

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3 The numbering is according to Chemical Abstracts.
rat liver. Kalckar has suggested that thiouracil traps ribose-1-phosphate in the form of an unnatural nucleoside, making it less available for normal pathways at a time when its concentration may be critical.

It is of some interest that thymidine phosphorylase favors the formation of the desoxyriboside rather than the riboside when uracil is the substrate (1); yet with thiouracil as a substrate, both ribo- and desoxyribonucleosides are formed to the same extent.

It is hoped that the thiouracil nucleosides can be prepared in amounts which will make possible tests of their antimetabolic effects on thyroid activity as well as on growth.

SUMMARY

Thiouracil riboside has been synthesized by an enzymatic reaction between thiouracil and ribose-1-phosphate. Similarly, thiouracil desoxyriboside has been synthesized and isolated in crystalline form. The formation of thiouracil nucleosides may be of some significance in explaining the known antimetabolic effects of thiouracil.

Addendum—In a paper which escaped our notice, Shugar and Fox (19) report ultraviolet absorption data for 1-ethyl-2-thiouracil which strongly support our conclusion that the point of attachment of the sugar in the enzymatically synthesized thiouracil nucleosides is at nitrogen 1.

BIBLIOGRAPHY

18. Kalckar, H. M., Biochim. et biophys. acta, 12, 250 (1953).

*Private communication. Since completion of this manuscript a full discussion of Kalckar’s "ribose trap" theory has appeared (18).
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