Metabolism of 2-Thiouracil in Pyrimidine Pathways Leading to Nucleotide Synthesis (36350)

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(Introduced by J. A. Pittman)

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Metabolism of the antithyroid drug thiouracil by thymidine phosphorylase has been reported by Strominger and Friedkin (1) and Razzell and Khorana (2). This enzyme converts thiouracil to thiouridine or to thiodeoxyuridine depending on the pentose phosphate provided. The reaction catalyzed by thymidine phosphorylase is similar to that catalyzed by uridine phosphorylase in which uracil is converted to uridine. Both enzymes are present in normal rat liver and are widely distributed in animal tissues (3–5). Although Lindsay *et al.* (6) reported that thiouracil did not appear to be utilized as a substrate by uridine phosphorylase, thiouracil conversion to thiouridine and thiodeoxyuridine by thymidine phosphorylase provides a site, characteristic of animal and many primitive systems, at which thiouracil can enter the pyrimidine pathways. The further metabolism of thiouridine and thiodeoxyuridine has not been studied.

The objects of the present investigation were: (a) to reexamine thiouracil metabolism by uridine phosphorylase using a sensitive isotopic assay; and (b) to determine if thiouridine or thiodeoxyuridine is converted to the corresponding nucleotide by specific enzymes from mammalian tissues.

Materials and Methods. Uridine phosphorylase. Fresh rat liver was homogenized in a Waring blender with 9 vol of 0.02 *M* Tris–maleate buffer (pH 6.5) and centrifuged at 54,000*g* for 40 min. To the supernatant was added 0.2 vol of 5% protamine sulfate in 0.02 *M* Tris–maleate (pH 6.5) to remove uridine kinase. The mixture was allowed to settle in a refrigerator overnight and the precipitate was removed by centrifugation. Solid ammonium sulfate was added to the supernatant to give a 50% saturated solution. The precipitate obtained after centrifugation was extracted with decreasing concentrations of ammonium sulfate (50, 40, 30% saturation). The enzyme protein in each extract was recovered by adding solid ammonium sulfate to 60% saturation. The enzyme protein in the 30% extracts had the highest specific activity (1.0–1.2), representing about a sixfold purification, and was used throughout the studies. The enzyme was assayed by the isotopic method described by Lindsay *et al.* (7).

Uridine kinase. Fresh rat liver was homogenized in a Waring blender with 4 vol of 0.02 *M* Tris–maleate buffer (pH 6.5) and centrifuged at 54,000*g* for 40 min. Solid ammonium sulfate was added to the supernatant to give a final concentration of 25% saturation and the solution centrifuged at 10,000*g* for 10 min. The precipitate was discarded and additional solid ammonium sulfate was added to the supernatant to obtain a 35% saturated ammonium sulfate solution. The precipitate obtained was then extracted with decreasing concentrations of ammonium sulfate (35, 30, 25, 20%) in 0.05 *M* ammonium acetate buffer at pH 8.0. Solid ammonium sulfate was added to the extracts to recover

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the protein. Enzyme protein with the highest specific activity (0.6–0.7) was obtained with the 20% extract and represented about a 12-fold purification. The isotopic method described by Akamatsu *et al.* (8) was used for assaying the enzyme.

Thymidine phosphorylase. Acetone powders were prepared from horse liver and served as the source of the enzyme. Twenty milligrams of acetone powder were usually mixed with 1.0 ml of 0.1 *M* Tris–HCl (pH 7.4) and any insoluble material was removed by centrifuging. The enzyme was assayed according to a slight modification of the isotopic method of Krenitsky (9).

Thymidine kinase. The enzyme from rat liver was prepared according to the method described by Klemperer and Haynes (10) and assayed by a slight modification of the method described by these investigators. Separation of thymidine monophosphate from thymine and thymidine was achieved by descending paper chromatography in ethyl acetate–water–formic acid (60:35:5).

5′-Nucleotidase. Unfractionated *Crotalus adamanteus* venom was used as a source of the enzyme and its substrate specificity was confirmed with isomers of UMP. The 5′-UMP was quantitatively converted to uridine by the enzyme; whereas the 2′- and 3′-isomers were not hydrolyzed at a measurable rate. The enzyme was assayed by a slight modification of the method described by Heppel and Hilmoe (11).

Molar ratios of thiouracil, ribose, and phosphate. Thiouracil in the parent compound was determined by UV absorption of the parent compound using a molar extinction coefficient of 12.4×10^3 for thio-UMP in acid and neutral media. This value was determined with authentic thio-UMP. Ribose was determined by a modification of the orcinol method of Mejbaum (12) using authentic thio-UMP as a standard. Phosphate was determined by the procedure of Fiske and Subbarow (13).

General. The UV absorption spectra were determined with a Beckman DK-2 recording spectrophotometer. Radioactive thiouracil was obtained from Amersham-Searle Corp. and radioactive thymine and thymidine from New England Nuclear Corp. Thiodeoxyuridine and thiodeoxyuridine-2-^{14}C were prepared by a modification of the procedure used by Strominger and Friedkin (1) separating thiouracil and thiodeoxyuridine by ion exchange chromatography on AG 1 × 8 (formate, 200–400 mesh) using distilled water as the only eluent. Authentic thio-UMP was kindly supplied by Dr. R. W. Chambers, New York University Medical Center; and authentic thiouridine was synthesized by a modification of the five-step chemical method of Brown *et al.* (14).

TABLE I. The Reversible Conversion of Thiouracil-2-^{14}C to Thiouridine-2-^{14}C by Rat Liver Uridine Phosphorylase.

Substrate	Total radioactivity ($\times 10^3$ dpm) Initial	Product	Conversion (%)
Thiouracil[a]	2220	3.81	0.172
Thiouridine[b]	55.5	1.69	3.08

[a] The incubation medium contained 2 μmoles of thiouracil-2-^{14}C (2.22×10^6 dpm); 0.5 μmoles of R-1-P; 0.507 mg of enzyme protein in a final volume of 0.2 ml. After 1 hr incubation at 37°, the reaction was stopped by heating in a boiling H_2O bath for 3 min. An aliquot (50 μl) from the supernatant was paper chromatographed (descending) in *n*-butanol–3% boric acid (100:13, v/v) for about 18 hr. Thiouridine (0.1 μmole) was added as a marker. The compounds were located with a UV light. The spots were cut out and eluted with 4 ml of H_2O. Two milliliters of the eluate were placed into liquid scintillation vials and counted. The value for the product was given as the total radioactivity in 0.2 ml of incubation medium.

[b] The incubation medium contained 2 μmoles of thiouridine-2-^{14}C (5.55×10^4 dpm); 25 μmoles of potassium phosphate (pH 8.1); 8 μmoles of Tris–HCl (pH 8.1); and 1.20 mg of enzyme protein in a final volume of 0.315 ml. The incubation period was 2 hr at 37°. The rest of the procedure was similar to the one described for the forward reaction except a 75-μl aliquot was taken for paper chromatography and the paper strips were placed into liquid scintillation vials and radioactivity was counted.

Results. Lindsay *et al.* (6) found that thiouracil was not utilized by rat liver uridine phosphorylase at a measurable rate when the

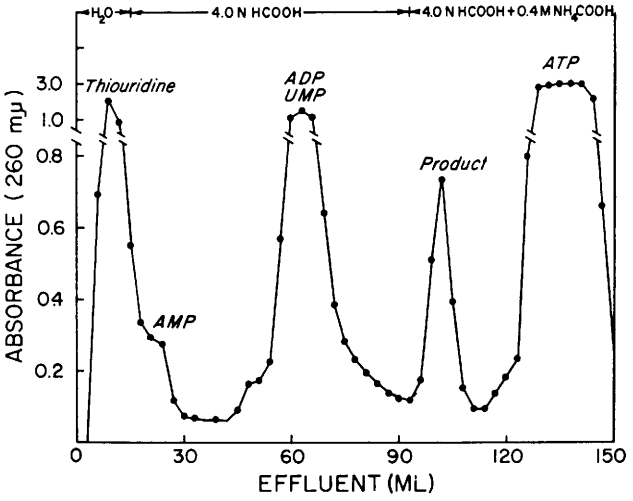

FIG. 1. The product of uridine kinase action on 2-thiouridine: The reaction mixture contained 4 μmoles of thiouridine; 20 μmoles of $MgCl_2$; 30 μmoles of ATP; 20 μmoles of Tris HCl (pH 7.4); and 5 mg of partially purified enzyme protein in a final volume of 0.6 ml. After 2 hr of incubation at 37°, 0.06 ml of 4.0 *N* $HClO_4$ was added to terminate the reaction. The mixture was centrifuged, the precipitate was washed twice with 0.5 ml of 0.4 *N* $HClO_4$, the combined supernatants were neutralized with KOH, then centrifuged to remove the precipitate. The resulting supernatant was applied to a 0.5 × 15 cm column of AG1 × 8 (formate form, 200–400 mesh). Gradient elution was carried out with formic acid and ammonium formate as indicated (top line).

enzyme was assayed by measuring the disappearance of ribose. Consequently, radioactive thiouracil and thiouridine were used in this study to provide a more sensitive assay system. Thiouridine and thiouracil (R_f 0.27 and 0.51, respectively) were separated by paper chromatography in butanol–3% boric acid (100:13) and the amount of radioactivity in each spot was determined. Enzymatic conversion was calculated on the basis of the amount of radioactivity appearing in the thiouracil spot when thiouridine-2-^{14}C was used and in the thiouridine spot when thiouracil-2-^{14}C was the substrate. All values were corrected with zero time controls.

As shown in Table I, uridine phosphorylase converted a very small amount of thiouracil-2-^{14}C (approx 0.2%) to thiouridine-2-^{14}C. When thiouridine-2-^{14}C was utilized as a substrate, approximately 3% was converted to thiouracil. The rate of conversion in the forward and reverse directions may not be compared since the experiments were not performed at the same time and the incubation conditions were necessarily different. Although the amount of conversion is very small, thiouracil and thiouridine are well separated in the chromatographic system used and the conversion appears to be real. The results strongly suggested that rat liver uridine phosphorylase catalyzes the reversible conversion of thiouracil to thiouridine.

Formation of thio-UMP from thiouridine by uridine kinase. Since it appeared that thiouracil could enter the pyrimidine pathways by the action of either thymidine phosphorylase or uridine phosphorylase, the further utilization of thiouridine as a substrate for uridine kinase, which catalyzes the irreversible conversion of uridine to UMP, was investigated. Thiouridine was incubated with partially purified rat liver uridine kinase under conditions optimal for the conversion of uridine to UMP. The reaction was terminated after 2 hr with perchloric acid and the acid-soluble supernatant was chromatographed on an AG 1 anion exchange column with the results presented in Fig. 1. A UV absorbing peak, which did not correspond to any uracil-containing compound, was formed (labeled as "product") and was observed only when thiouridine was incubated with the enzyme.

TABLE II. Summary of Results of Identification Procedures.

	Authentic thio-UMP	Reaction product
UV absorption peaks		
Acid	272–273	272–273
H_2O	272–273	272–273
Alkali	268; 243	268; 243
Cochromatography on AG 1	UV absorbing peak of eluted authentic thio-UMP coincided with radioactive peak of ^{14}C-labeled reaction product.	
Ratio of thiouracil, ribose, and phosphate	1:1:1	1:1:1
Action of 5′-nucleotidase		
Substrate	Yes	Yes
Product	Thiouridine	Thiouridine

This peak was not observed when uridine was the substrate. The maximum UV absorption peak of this unknown was at 273 mμ, which is characteristic of thiouracil-containing compounds, as opposed to 260 mμ for uracil nucleotides; and it appeared to be the product formed by the action of uridine kinase on thiouridine.

Since uridine kinase catalyzes the conversion of uridine to UMP, it was suspected that the product formed by the action of this enzyme on thiouridine was thio-UMP. A number of procedures were carried out to confirm the identity of this product and are summarized in Table II.

The unknown product was isolated and purified by AG 1 anion exchange chromatography, desalted by passing through an AG 50 cation exchange column, lyophilized, and dissolved in either acid or alkali to determine the UV absorption spectra. The product had a maximum absorption peak at 272–273 mμ in 0.1 *N* HCl and H_2O; whereas two broad absorption peaks were observed at 268 and 243 mμ at an alkaline pH. The UV absorption curves of the unknown product were identical to those of authentic thio-UMP.

The purified, unknown radioactive product was mixed with authentic thio-UMP and cochromatographed on an AG 1 column. The principal UV absorbing peak, representing authentic thio-UMP, coincided with the principal radioactive peak produced by the product.

The ratios of the thiouracil, ribose, and phosphate moieties in the unknown product were determined, using thio-UMP as a standard. The unknown molecule contained thiouracil, ribose, and phosphate in a ratio of 1:1:1, a composition which is consistent with its identification as thio-UMP.

After treatment with 5′-nucleotidase, the unknown was quantitatively converted to a compound which was purified by column chromatography on AG 1 and identified as thiouridine since the UV absorption characteristics, elution pattern from AG 1 columns, and the R_f after descending paper chromatography in butanol–3% boric acid (100:13, v/v) were identical to that of an authentic sample of thiouridine. Thus, the unknown was shown to be a substrate for 5′-nucleotidase producing thiouridine.

The product formed by the action of uridine kinase on thiouridine can thus be identified as 2-thiouridine 5′-phosphate (thio-UMP), since the enzyme acting on thiouridine formed 5′-nucleotides, the UV absorption characteristics of the unknown were identical to those of authentic thio-UMP, chromatographic characteristics on AG 1 were identical to authentic thio-UMP, the ratios of the thiouracil, ribose, and phosphate moieties were 1:1:1 and the unknown was a substrate for 5′-nucleotidase forming thiouridine.

Metabolism of 2-thiouracil in the orotic acid pathway. The orotic acid pathway is the primary if not exclusive route for the synthesis of pyrimidines from small molecular weight precursors and the principal pathway for pyrimidine nucleotide production in most biological systems. The possible utilization of thiouracil by enzymes in this pathway was previously investigated (6) to determine if this was a site at which thiouracil might enter the pyrimidine pathways. The results indicated that thiouracil was not utilized as a substrate.

Metabolism of 2-thiouracil in the thymidine phosphorylase–thymidine kinase pathway leading to DNA synthesis. Strominger and Friedkin (1) demonstrated that thymidine

phosphorylase from horse liver was able to convert thiouracil to the corresponding thiouracil deoxyriboside (thiodeoxyuridine), as well as to thiouridine. The riboside and deoxyriboside were produced at approximately the same rate depending on whether ribose-1-phosphate or deoxyribose-1-phosphate was present in the incubation medium. The formation of thiodeoxyuridine from thiouracil provides a site at which thiouracil can enter pathways leading to DNA; consequently, further metabolism of thiouracil by enzymes in this pathway was investigated.

Radioactive thiodeoxyuridine was prepared and used to study the possibility of further metabolism by thymidine kinase which catalyzes the conversion of thymidine to TMP. Thiodeoxyuridine was incubated with rat liver thymidine kinase; and the reaction mixture was subjected to descending paper chromatography in ethyl acetate–H_2O–formic acid (60:35:5, v/v). In this solvent system, all phosphorylated deoxyribonucleosides remained at the origin while the deoxyribonucleosides and free pyrimidines migrated well away from the origin. The radioactivity remaining at the origin when thiodeoxyuridine was used as a substrate was identical to that in controls containing no enzyme (data not shown). This demonstrated that thiodeoxyuridine monophosphate was not produced at a measurable rate. The viability of the enzyme was confirmed in the same experiment since thymidine phosphates were formed from thymidine under the same incubation conditions in quantities amounting to a total conversion of approximately 23% of the substrate.

The data presented indicate that thiouracil can enter the thymidine pathway to form thiodeoxyuridine but that subsequent metabolism of this compound, at least by thymidine kinase, does not seem to occur. Consequently, it appears that thiouracil cannot be incorporated into its corresponding deoxyribonucleotides or DNA via this thymidine phosphorylase–thymidine kinase pathway.

Discussion. Many goitrogenic substances such as thiouracil are structurally related to the pyrimidine bases of nucleic acids. Thiouracil has been shown to be incorporated into RNA in several bacterial and viral systems and to be substituted in the positions normally occupied by uracil (15). Although it has been assumed that thiouracil and its derivatives might undergo metabolic conversions characteristic of the pyrimidines in these systems, metabolism at the nucleotide level has received little attention. The conversion of thiouracil to thio-UMP by *E. coli* extracts observed by Amos *et al.* (16) supported the assumption that thiouracil metabolism resembled that of uracil and suggested that a pyrimidine pyrophosphorylase might be involved. Lindsay *et al.* (17) have demonstrated that UMP pyrophosphorylase does utilize thiouracil as a substrate with the subsequent formation of thio-UMP. UMP pyrophosphorylase activity in most animal tissues is barely discernible and is apparently absent in thyroid tissue (4, 18) suggesting that metabolism by this pathway in animal tissues may be relatively unimportant. In contrast, this enzyme is widely distributed in bacterial and, possibly, in viral, as well as plant, systems and may serve as the exclusive pathway for uracil utilization in some of them.

The present results showed that at least two other pathways for thiouracil metabolism to a nucleotide are available and appear to be the important pathways in animal tissues. In one pathway the reversible conversion of thiouracil to thiouridine is catalyzed by liver uridine phosphorylase and further metabolism of thiouridine to thio-UMP is catalyzed by uridine kinase. These enzymes comprise the "salvage" pathway for uracil utilization for UMP synthesis. In the other pathway, thiouracil is converted to thiouridine by thymidine phosphorylase then to thio-UMP by action of uridine kinase. As indicated above, thiouracil metabolism by uridine phosphorylase does not readily occur. Greater conversion is accomplished by thymidine phosphorylase which appears to be the more important pathway for thiouridine synthesis in animal tissues.

The uridine phosphorylase used in this

study utilized thymidine, as well as uridine, as a substrate. Pontis *et al.* (19) suggested that these two enzyme activities may be characteristic of a single protein. However it has become increasingly evident that uridine phosphorylase and thymidine phosphorylase are separate proteins both in bacterial (2, 20) and mammalian systems (21, 22). Mammalian uridine phosphorylase metabolizes both ribonucleosides and deoxyribonucleosides (21, 22); whereas thymidine phosphorylase is specific for deoxyribonucleosides (22, 23). In the present study, thiouridine was converted to thiouracil, a reaction contrary to the known specificity of thymidine phosphorylase. Consequently, the metabolism of thiouridine to thiouracil must have been catalyzed by uridine phosphorylase. In view of the utilization of thiouridine as a substrate by uridine phosphorylase, the reverse reaction was also most probably catalyzed by this enzyme.

Summary. The present results demonstrate that the antithyroid drug 2-thiouracil can be metabolized in animal tissues to the corresponding nucleotide by at least two pathways. In one pathway, uridine phosphorylase catalyzes the reversible conversion of thiouracil to thiouridine and further metabolism of 2-thiouridine to thio-UMP is catalyzed by uridine kinase. In the other pathway, thiouracil is converted to thiouridine by thymidine phosphorylase, then to thio-UMP by uridine kinase. Although thymidine phosphorylase can also form 2-thiodeoxyuridine, further metabolism of this compound by thymidine kinase to a deoxyribonucleotide does not appear to occur.

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Received Nov. 11, 1971. P.S.E.B.M., 1972, Vol. 139.