Metabolism of the Nucleotide (2-Thio-UMP) of the Antithyroid Drug 2-Thiouracil (36617)

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(Introduced by J. A. Pittman)

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The metabolism of the antithyroid drug 2-thiouracil to the thiouracil nucleotide (thio-UMP) can occur by several pathways. In the two pathways characteristic of animal tissues, 2-thiouracil is first converted to 2-thiouridine by the action of uridine phosphorylase (1) or by thymidine phosphorylase (2, 3). Further metabolism of 2-thiouridine to thio-UMP is catalyzed by uridine kinase which uses thiouridine as a substrate almost as readily as uridine (1). Thiouracil can also be converted to thio-UMP by direct phosphoribosylation with PRPP (4). This reaction is catalyzed by UMP pyrophosphorylase, an enzyme not found in measurable amounts in most animal tissues.

Since conversion of 2-thiouracil to thio-UMP is catalyzed by enzymes in pathways normally utilizing uracil as a substrate, further metabolism of thio-UMP to a di- and triphosphate may also occur by uracil-utilizing enzyme systems. Maley *et al.* (5) described an enzyme preparation from rat liver which contains a mixture of nucleoside mono- and diphosphokinases. The enzyme mixture was able to utilize almost any of the naturally occurring nucleotides as a substrate and was recommended for the preparation of nucleoside di- and triphosphates. The utilization of 2-thio-UMP by this enzyme preparation has been investigated to determine if 2-thiouracil metabolism to the nucleoside di- and triphosphate can occur.

Materials and Methods. Thio-UMP, thio-UMP-^{14}C and thio-UMP-^{35}S were prepared by the action of UMP pyrophosphorylase on thiouracil as described by Lindsay *et al.* (6). Authentic thio-UMP and thio-UDP were kindly supplied by Dr. R. W. Chambers (New York University Medical Center) from a chemical synthesis according to Lengyel and Chambers (7). Phosphodiesterase (Russel's viper venom, lyophilized, B grade) was obtained from Calbiochem. Nucleoside mono- and diphosphokinases were prepared according to the method described by Maley *et al.* (5). Liver (22.5 gm) from a normal rat was homogenized with 75 ml of 1.15% KCl and centrifuged at 37,000*g* for 30 min. Five ml of 0.5 *M* potassium phosphate (pH 7.5) were added to the supernatant which was then heated to 85° in a water bath for 2 min. The suspension was cooled, then centrifuged and the supernatant fraction lyophilized. The residue was dissolved in a minimum volume of cold water and dialyzed against two 2-liter changes of 0.001 *M* potassium phosphate (pH 7.5). About 10 ml of active enzyme extract were obtained.

Complex ribonucleotides were separated by ascending two dimensional chromatography on polyethylenimine (PEI)- cellulose MN 300 precoated plastic sheets (Brinkmann Instruments, Inc.) with a solvent system modified from that described by Randerath and Randerath (8). Thin-layer chromatography in the first direction was carried out by sequential development with 0.1 *M* LiCl for 2 min, 0.5 *M* LiCl for 6 min, and 1.0 *M* LiCl until the solvent front reached a point 13 cm above the origin. The plate was then dried in a current of air and the LicCl re-

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moved by dipping in an anhydrous methanol bath for 15 min. After air drying again, chromatography in the second direction was carried out in 0.5 *M*, 2.0 *M* and 4.0 *M* sodium formate buffers, pH 3.2 with development being carried out, respectively, for 30 sec, 2 min and until the solvent front reached a point 15 cm above the origin. The plate was then dried and the nucleotides detected under UV light.

Radioactive spots on the thin-layer chromatograms were visualized by autoradiography. Thin-layer chromatograms were usually covered with Mylar sheets (15 μ in thickness) placed in contact with Dupont Cronex medical X-ray film and held in position with masking tape. The thin-layer plate was outlined on the film with pencil and the corner corresponding to the origin marked. Contact exposure was allowed to occur for various periods of time depending upon the type of radiation emitted and the quantity of radioactive material in the sample applied to the chromatogram.

Results. One of the problems previously encountered with thio-UMP was its instability and propensity to form the corresponding uracil derivative. Consequently, it was necessary to develop methods for the separation of uracil and thiouracil nucleotides before it could be established that thio-UMP conversion to thionucleoside di- or triphosphates could occur and could be clearly distinguished from the incorporation of UMP, a known degradation product, into uridine nucleotides. Separation of authentic uracil and thiouracil nucleotides was achieved by chromatography on AG-1 x8 anion exchange columns as indicated in Fig. 1. Both thio-UMP and thio-UDP were eluted from the column after the corresponding uracil nucleotide indicating that the thio compounds are more acidic than the corresponding oxygen analog, a situation generally encountered when –S or –SH is substituted for –O or –OH in organic compounds (9). An authentic thio-UTP standard was not available but, by analogy to the elution characteristics of thio-UMP and thio-UDP, would most likely be eluted after UTP.

A mixture of nucleoside mono- and diphos-

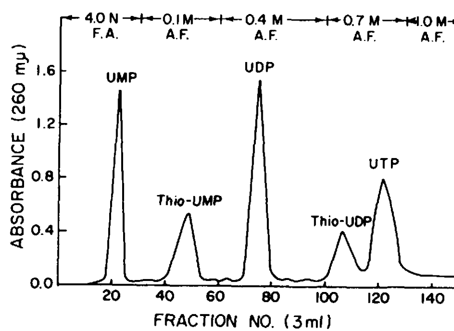

FIG. 1. Separation of authentic uracil and thiouracil nucleotides by column chromatography. Authentic nucleotides were applied to AG 1 $\times$ 8 (formate form, 200–400 mesh) and separated by gradient elution. The mixing vessel contained 22 ml H_2O initially with addition of formic acid (F.A.) or ammonium formate (A.F.) to the reservoir (a conical separatory funnel) as indicated at the top of the figure.

phokinases was prepared from rat liver by the method of Maley *et al.* (5) and incubated with UMP or thio-UMP. The results presented in Fig. 2A show that the enzyme preparation actively converted UMP to UDP and UTP, the only major metabolites formed. Thio-UMP was incubated with the enzyme extract under identical conditions with the results presented in Fig. 2B. Only one reaction product appeared to be formed and its absorbance was higher at 273 mμ than at 260 mμ, a trait characteristic of thiouracil compounds. The material in the peak was isolated, concentrated and subjected to UV absorption analysis. The absorption spectrum of this compound was identical to that of authentic thio-UDP in H_2O with both displaying maximum absorption at 275 mμ.

It was obvious from the relatively large amounts of uridine di- and triphosphates formed when compared to the very small amount of product from thio-UMP that thio-UMP utilization by these enzymes did not readily occur and was certainly not comparable to the rate of conversion of UMP. Since the primary objective of the experiment was to determine if this enzyme system could utilize thio-UMP for the formation of nucleoside di- and triphosphates, a more sensitive method of detecting product formation was required. This was accomplished with radi-

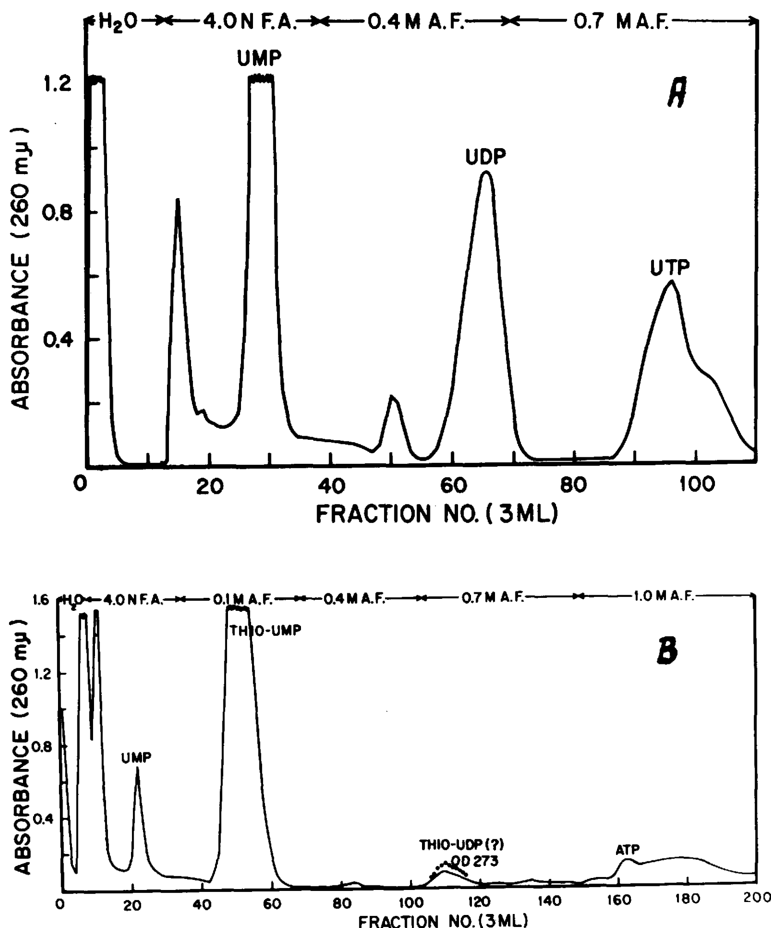

FIG. 2. Phosphorylation of UMP and thio-UMP by rat liver mono- and diphosphokinase. The reaction mixture contained 8 μmoles of UMP or 15.7 μmoles of thio-UMP; 50 μmoles of potassium phosphate, pH 7.5; 0.5 μmoles ATP; 3.0 μmoles of $MgCl_2$; 20 μmoles of creatine phosphate; 0.5 mg of creatine kinase; 10 μmoles of glutathione; 0.1 μmoles of Versene, 0.2 ml of enzyme preparation (6.6 mg enzyme protein) and 2 drops of toluene in a final volume of 1.1 ml. After 26 hr incubation at room temperature, the reaction mixture was applied to a 0.5 × 20 cm column of AG 1×8. Gradient elution was carried out as indicated at the top of each figure.

oactive thio-UMP and the results obtained with thio-UMP-^{35}S are presented in Fig. 3. Column chromatography after incubating the enzyme with thio-UMP-^{35}S revealed the formation of two major radioactive products.

Knowing that this enzyme system readily converts UMP to UDP and UTP, these products appeared to be thio-UDP and thio-UTP. The chromatographic characteristics on AG 1 were consistent with this tentative identification since the peaks were eluted after the corresponding uridine nucleotides UDP and UTP.

The two radioactive peaks were isolated, desalted, lyophilized and subjected to two dimensional, thin-layer chromatography. In the system used, UMP, thio-UMP, UDP, thio-UDP and UTP are well separated (Fig. 4A) Thio-UMP travels more slowly than UMP in both solvents and thio-UDP more slowly than UDP. Authentic thio-UTP was not available, but based on the positions of the other two thio-nucleotides, it could be predicted that thio-UTP would travel more slowly than UTP in both solvent systems.

The peak from Fig. 3 tentatively labeled

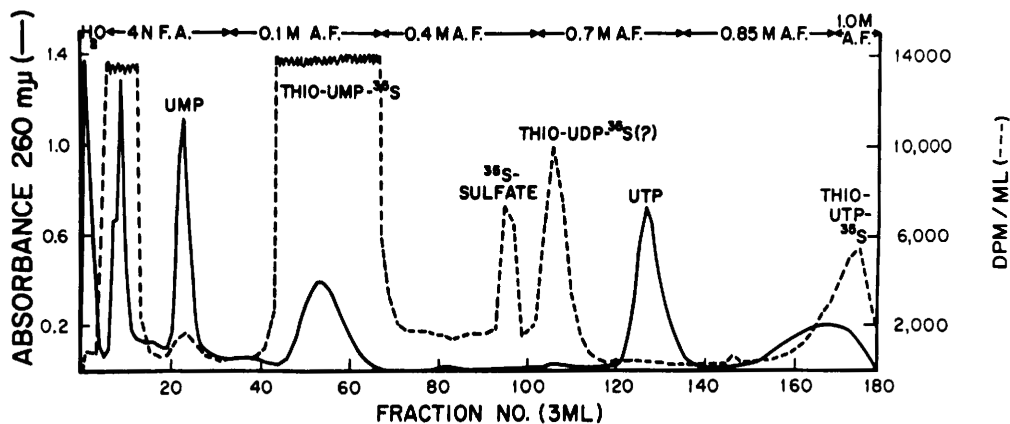

FIG. 3. Phosphorylation of thio-UMP-^{35}S. The reaction mixture was essentially the same as in Fig. 1 except for the addition of 5.0 μCi of thio-UMP-^{35}S (2.5 μmoles) as substrate. UMP and UTP were added prior to chromatography as references. Gradient elution on AG 1 × 8 columns was carried out as indicated at the top of the figure.

thio-UTP was chromatographed on the thin-layer plates (Fig. 4A). The greatest portion of the radioactivity was concentrated in a region nearer the origin than UTP and corresponded to the position predicted for thio-UTP. A second major radioactive spot was in a position corresponding to inorganic sulfate and a small amount of radioactivity was coincidental with the spot occupied by non-radioactive authentic thio-UDP. Since separation of thio-UDP and the "thio-UTP" compound was readily achieved by the column chromatographic system used, the thio-UDP is most probably a degradation product. The inorganic sulfate must also be formed during manipulation and indicates the instability of the compound.

When incubated in a weak acid medium (Fig. 4B), the "thio-UTP-^{35}S" was partially hydrolyzed to form thio-UDP-^{35}S. Acid also appeared to remove the phosphate from thio-UDP-^{35}S since a small amount of radioactivity was also found in the thio-UMP region. Treatment with snake venom phosphodiesterase, which catalyzes the removal of phosphate or pyrophosphate from nucleoside di- or triphosphates, converted "thio-UTP-^{35}S" to thio-UMP-^{35}S as shown in Fig. 4C. Inorganic sulfate was also present in these chromatograms.

The only compounds that could be converted to thio-UDP and thio-UMP by these two procedures are thio-UTP, thio-UDP and thio-UDP conjugates. Thio-UDP was ruled out since it is separated from the "thio-UTP" product by both anion exchange and thin-layer chromatography. Thio-UDP conjugates can also be ruled out since no UDP conjugates were observed when UMP was used as a substrate confirming that enzymes forming conjugates were not present in the enzyme preparation used. Consequently, the parent compound must have been thio-UTP.

When thio-UMP-^{14}C was substituted for thio-UMP-^{35}S in the incubation with mono- and diphosphokinases, two labeled products were formed which were identical to the two ^{35}S-labeled products observed in Fig. 3. Chromatography of the "thio-UTP-^{14}C" product on thin-layer sheets demonstrated that the major portion of the radioactivity was localized at the same site as "thio-UTP-^{35}S" (Fig. 4A) with some activity coincidental with thio-UDP and thio-UMP. Hydrogen peroxide treatment of the "thio-UTP-^{14}C" product, which replaces the S of the thiouracil moiety with 0 to form uracil, caused most of the radioactivity to appear coincidentally with the UTP standard (Fig. 4D) with some appearing in the UMP spot. This conversion to UTP could have occurred only if the parent compound was thio-UTP. Thus the chromatographic properties and the products formed after partial degradation

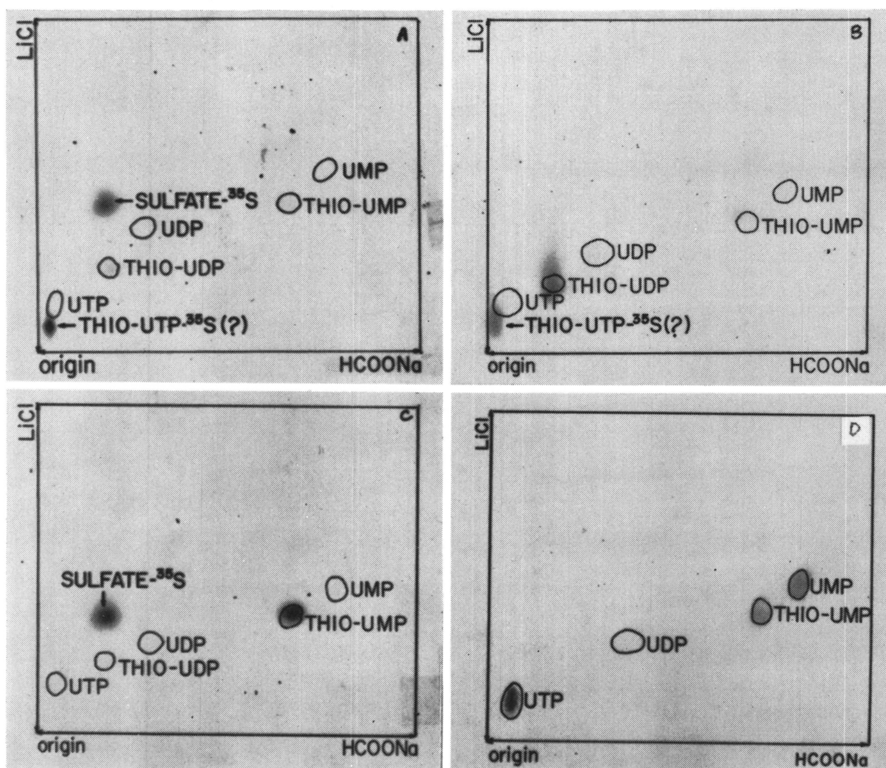

FIG. 4. Thin-layer chromatograms of the "thio-UTP-^{35}S" or thio-UTP-^{14}C product observed in Fig. 3. UMP, UDP, UTP, thio-UMP and thio-UDP were authentic standards cochromatographed with the sample, visualized under UV light and indicated as circles. The radioactivity was detected by autoradiography and is shown as dark spots The details of chromatography are described in *Materials and Methods*. Figs. A, B and C represent chromatography of 10 μl of the "thio-UTP-^{35}S" fraction from Fig. 3 incubated 30 min at 37° with 25 μl H_2O (Fig. A) 10 μl 1 *N* HCl (Fig. B) and 25 μl snake venom phosphodiesterase (18 μg of enzyme protein), Fig. C. Fig. D represents a chromatogram of 10 μl of "thio-UTP-^{14}C" incubated 30 min at 37° with 10 μl of 6% hydrogen peroxide.

provide strong evidence that the "thio-UMP" product is indeed thio-UTP.

Attempts to confirm the identification of the other thio-nucleotide product seen in Fig. 3, which had been tentatively labeled thio-UDP, were inconclusive. Cochromatography of the "thio-UDP-^{35}S" compound with authentic thio-UDP on thin-layer sheets produced the results shown in Fig. 5. The migration of the "thio-UDP" was not comparable to the authentic standard but moved considerably further in the first dimension and more slowly in the second. Cochromatography of the "thio-UDP-^{35}S" fraction with authentic thio-UDP on an Ag 1 anion exchange column also demonstrated that these compounds were chromatographically different. The presence of inorganic sulfate-^{35}S in both these chromatograms demonstrated the labile nature of the thionucleotide which was a serious problem throughout the study.

Discussion. The conclusion that thio-UMP can be converted to thio-UTP by enzymes from mammalian tissues is very strongly supported by the evidence presented. Although UV absorption analysis and the nature of the enzyme reaction suggested that thio-UDP was also formed, the chromatographic results failed to support its identification. On the other hand, the results of the chromatograms should not rule out the formation of thio-UDP since Lindsay *et al.* (unpublished ob-

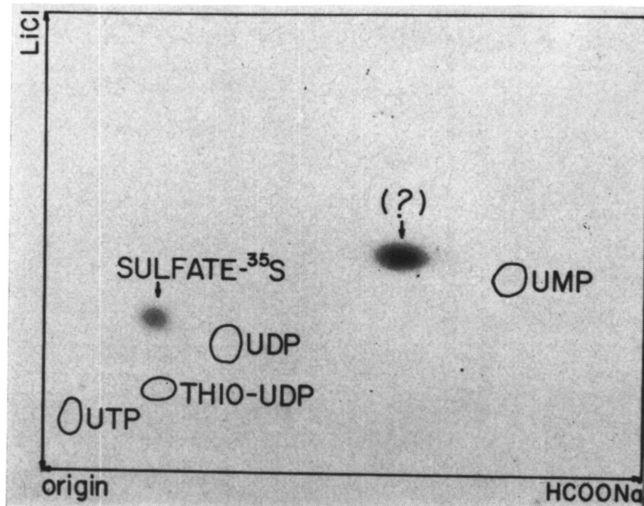

FIG. 5. Thin-layer chromatogram of the "thio-UDP-^{35}S" fraction from Fig. 3. Authentic standards are circled.

servations) demonstrated complex formation between thiouracil and thiouridine with the complex behaving differently chromatographically from either parent compound. The material in the "thio-UDP-^{35}S" fraction may actually be a similar complex. Formation of thio-UDP by the nucleoside mono- and diphosphokinase system must have occurred even though it was not identified since the nucleoside diphosphate is a mandatory intermediate in the formation of the nucleoside triphosphate.

It is interesting to note that the mixture of nucleoside mono- and diphosphokinases employed in this study utilized both UMP and thio-UMP as substrates. There is no definite proof that the same enzymes are involved in the conversions of UMP and thio-UMP but results with other systems suggest that this is probably the case. All enzyme or enzyme systems thus far reported to metabolize thiouracil or a thiouracil derivative also utilize the corresponding uracil compound. Another characteristic of the systems utilizing thiouracil, a characteristic shared by the nucleoside mono- and diphosphokinase system, is that the uracil derivative is more actively metabolized than the corresponding thiouracil substance.

The pathways for thiouracil conversion to thio-UMP utilize the same enzymes involved in uracil conversion to UMP. Further conversion of UMP and thio-UMP to nucleoside triphosphates also involves the same, or very similar, enzymes. Thus the pathways for uracil and thiouracil conversion to the nucleoside triphosphate are either identical or very similar.

The demonstration of pathways in mammalian tissues for thiouracil metabolism to thio-UTP suggest that thiouracil incorporation into mammalian RNA might occur. Furthermore, the pyrimidine nucleoside triphosphates are utilized to form pyrimidine nucleoside diphosphate conjugates which play many important roles in physiological processes. Thio-UTP may be similarly utilized.

Summary. The results presented demonstrated that a mixture of rat liver mono- and diphosphokinases metabolized 2-thio-UMP to two substances. One was identified as thio-UTP. The other had some properties similar to thio-UDP but was chromatographically different. The rate of UMP conversion to UDP and UTP by this enzyme system greatly exceeded that of thio-UMP to nucleoside triphosphate. Thio-UMP conversion to thio-UTP demonstrates that pathways are present in animal tissues for the metabolism of 2-thiouracil to 2-thio-UTP. The enzymes involved appear to be identical or very similar to those converting uracil to UTP.

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