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SITE OF GLUCURONIDE CONJUGATION TO THE ANTITHYROID DRUG 6-*n*-PROPYL-2-THIOURACIL*

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Abstract—Chromatographyically pure, essentially salt-free radioactive 6-*n*-propyl-2-thiouracil (PTU) glucuronides were isolated from rat bile and urine and synthesized with guinea pig liver microsomes to determine if more than one PTU glucuronide was formed and to determine which group or groups in the PTU molecule was glucuronidated. Analyses on Bio-Gel P-2 and DEAE-Sephadex A-25 columns and on TLC sheets in five solvent systems demonstrated that the three glucuronide preparations were chromatographically identical. Furthermore, the reactivities of the three glucuronides with 1 N HCl, methyl iodide, sodium azide-iodine reagent, 2,6-dichloroquinone-chloroimide and H_2O_2 were also identical strongly indicating that a single PTU glucuronide was formed. The PTU glucuronide was partially hydrolyzed by 1 N HCl to 6-*n*-propyl-uracil (PU), a reaction typical of *S*-conjugated PTU; demonstrated greatly reduced reactivity with methyl iodide whereas the *S* of PTU was readily methylated; exhibited a negative reaction in the azide-iodine test which was a certain indication that the C—SH or C=S group was not present; failed to react with 2,6-dichloroquinone-chloroimide which reacts with the C=S of PTU and provides the basis for a colorimetric assay for PTU; and was not oxidized by H_2O_2 to form sulfate as are all PTU derivatives except *S* conjugates of PTU. Furthermore PU, which possesses identical potential conjugation sites with the exception of the *S*, was not glucuronidated under conditions in which PTU was readily conjugated. The results obtained strongly indicate that the glucuronide is conjugated to the *S* of PTU.

The antithyroid drug 6-*n*-propyl-2-thiouracil (PTU) is readily metabolized after administration to humans and rats and the major metabolite in urine, plasma and bile has been identified as PTU glucuronide [1–4]. Enzymatic conversion of PTU to the glucuronide has been demonstrated with guinea pig liver microsomes *in vitro* [5]. Glucuronide conjugation is also a major mechanism for biological inactivation since PTU glucuronide is only about 10 per cent as effective as PTU as an inhibitor of porcine thyroid peroxidase [6]. Marchant *et al.* [7, 8] concluded that man has a vastly greater ability to conjugate PTU than rats and suggested that this could contribute to the lesser effectiveness of PTU in man.

The glucuronide is apparently attached to an existing group in the PTU molecule since metabolism of PTU is not observed in microsomal preparations which readily conjugate PTU in the presence of UDPGA [5] and hydrolysis of PTU glucuronide with β-glucuronidase yields intact PTU [1, 5]. This is contrary to findings with methimazole (MMI), another important antithyroid drug. β-Glucuronidase hydrolysis of the major MMI glucuronide in rat bile yields a MMI metabolite rather than MMI [3] and guinea pig liver microsome UDP glucuronyl transferase, which readily conjugates PTU, does not utilize MMI as a substrate [5].

PTU possesses several potential sites at which glucuronide conjugation may occur. These are the sulfur at C-2, the enolic hydroxyl at C-4 and the two nitrogens in the heterocyclic ring. Sitar and Thornhill [4] reported that the PTU glucuronides in rat bile and urine have different chromatographic properties and suggested the formation of two conjugates with the glucuronide being conjugated to different sites in the PTU molecule. Early results in our laboratory with impure, salt-contaminated PTU glucuronides from the same two fluids also indicated a difference in their chromatographic properties.

One objective of the present study was to compare various chromatographic and chemical properties of purified, essentially salt-free PTU glucuronides isolated from rat bile and urine and synthesized with guinea pig liver microsomes to determine if more than one PTU glucuronide could be demonstrated. A second objective was to determine which group or groups in the PTU molecule were conjugated.

MATERIALS AND METHODS

Materials. [2-^{14}C]PTU was obtained from Mallinckrodt/Nuclear at a sp. act. of 3.78 μCi/μmole and [^{35}S]PTU from Amersham-Searle Corp. at a sp. act. of 45.2 μCi/μmole. [2-^{14}C] or [^{35}S]PTU glucuronide was synthesized with guinea pig liver microsomes as previously described [1, 5]. Isolation of PTU glucuronide from the incubation mixture and from rat bile and urine was carried out initially by chromatography on 2 × 115 cm Bio-Gel P-2 columns

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(200–400 mesh) eluting with water as previously described [1, 5]. The tubes corresponding to the radioactive glucuronide peaks were pooled and lyophilized. Bile and urine glucuronides were dissolved in H_2O and applied as 18 cm streaks on preparative cellulose TLC sheets which were developed in 1 M ammonium acetate:95% ethanol (15:75). The glucuronides were located by counting segments of a 1 cm strip of the TLC, eluted with water and lyophilized. All three glucuronides were further purified on 1 × 10 cm columns of DEAE-Sephadex A-25. The samples were dissolved in 1.0 ml freshly prepared 0.1 M ammonium carbonate and applied to columns previously equilibrated with the carbonate. The columns were eluted with 0.1 M ammonium carbonate and 3.0 ml fractions collected. The radioactive glucuronide peak for each preparations was located by counting 0.1 ml aliquots in a Packard Tri-Carb liquid scintillation spectrometer. The fractions containing the glucuronide from a single sample were pooled and lyophilized to vaporize the ammonium carbonate. The resulting glucuronides from each of the three sources gave a single peak after chromatography in four systems and were essentially salt-free. [2-^{14}C]-6-*n*-Propyluracil (PU) was synthesized as previously described [6] and purified by passage through a 2 × 115 cm Bio-Gel P-2 column eluting with H_2O [1].

PTU was purchased from Sigma Chemical Co. PU, *S*-methyl PTU, PTU disulfide, *N*-methyl PTU and *N*-acetyl PTU were synthesized as previously described [6]. Methyl iodide and 2,6-dichloroquinone-4-chloroimide were purchased from Aldrich Chemical Co. Bio-Gel P-2 (200–400 mesh) was obtained from Bio-Rad Labs and DEAE-Sephadex (A-25) from Pharmacia Fine Chemicals, Inc. TLC sheets of cellulose (No. 6064) and silica gel on plastic (No. 6061) were obtained from Eastman Kodak, sheets of Baker-flex cellulose DEAE from J. T. Baker Co. and silica gel impregnated fiber glass sheets from Gelman Instrument Co.

Chemical Modification of PTU-Glucuronide. Acid hydrolysis of PTU glucuronides was carried out with 1 N HCl in a boiling H_2O bath for 10 min. The mixture was lyophilized, the dry sample dissolved in 50 μl of H_2O and aliquots chromatographed on TLC plates. The *S* of PTU is completely stable under these conditions whereas *S*-conjugates are labile [9].

Oxidation of PTU glucuronides with 3% H_2O_2 was carried out at room temperature for 30 min. The samples were then lyophilized, dissolved in 50 μl of H_2O and aliquots spotted on TLC plates. H_2O_2 oxidation of PTU occurs primarily on the *S* which may be removed forming sulfate and primarily PU [1, 9]. Substitutions on the *S* (such as *S*-methyl PTU) protect the *S* from oxidation [9].

S-Methylation of [^{35}S]PTU and [^{35}S]PTU derivatives. PTU and PTU derivatives were *S*-methylated with methyl iodide by a modification of the method previously described [6, 9]. Samples containing approximately 100,000 dpm [^{35}S]PTU or [^{35}S]PTU glucuronide (3.78 μCi/μmole) were dissolved in 0.5 N NH_4OH in 95% ethanol and reacted with 570 μg of methyl iodide/5 μg PTU at 70° for 10 min. The reaction mixture was then lyophilized and dissolved and chromatographed in TLC systems previously described [9] to determine the extent of *S*-methylation. Aliquots of 0.8 ml of all samples were added to 25 ml Erlenmeyer flasks containing 1 mg of "cold" *S*-methyl PTU in 0.2 ml H_2O in the main compartment and 1.0 ml Hyamine in a removable center well. One ml 2 N HCl was added and the flasks were quickly capped with a rubber penicillin stopper covered with a thin plastic sheet. The vessels were then incubated at 50° for 20 hr and the methyl [^{35}S]mercaptan collected in Hyamine. [^{35}S]PTU remained stable under these conditions. After incubation, the Hyamine was quantitatively transferred to counting vials and the amount of methyl [^{35}S]mercaptan formation measured in a Packard Tri-Carb liquid scintillation spectrometer.

Iodine-azide spot test for C=S or C—SH groups. All PTU derivatives tested were dissolved in ethanol and 5-10 μl containing 0.5–10 nmoles of a single derivative pipetted onto a transparent spot plate and allowed to air dry. In each test, 0.5 nmoles of PTU was applied to a separate concavity and served as a positive control. Two drops of the iodine-azide solution were added and the formation of nitrogen bubbles observed with indirect light and a magnifying glass. The iodine-azide solution was composed of 3 gm sodium azide in 100 ml 0.1 N iodine in an aqueous solution. The reagent solution is slightly alkaline and hydrolytic splitting of disulfides and some other thio compounds may occur after they have been in contact with the reagent solution; however, immediate distinct production of nitrogen is considered certain proof of the C=S or C—SH groups and a lack of reaction a sure indication of the absence of such groups [10].

Colorimetric assay for C=S or C—SH groups. The reaction of PTU with 2,6-dichloroquinone-4-chloroimide is reported to require C=S as the reactive chemical group [11] and provides a convenient colorimetric assay for PTU. The method used was essentially that described by Ratliff *et al.* [11] with all PTU derivatives being dissolved in absolute ethanol.

Glucuronidation of [^{14}C]PTU and [^{14}C]PU by UDP glucuronyl transferase. [^{14}C]PTU or [^{14}C]PU (0.1 μmoles) were incubated with guinea pig liver microsomes under the conditions previously described [5]. The reaction was terminated with 2 vol. of 95% ethanol and the mixture centrifuged. The supernatant was lyophilized, reconstituted with 0.3 ml H_2O, and applied to 2 × 115 cm Bio-Gel P-2 columns (200–400 mesh) previously equilibrated with H_2O. Chromatography was carried out as previously described [5].

[^{14}C]PTU and [^{14}C]PU glucuronides in rat bile and urine. The appearance of glucuronides of PTU and PU in rat bile and urine was examined as previously described [1, 5] after the administration of [^{14}C]PTU or [^{14}C]PU. All radioactive peaks separated by chromatography on 2 × 115 cm columns of Bio-Gel P-2 were reacted with β-glucuronidase as previously described [1, 5] and the formation and identification of any products determined by TLC [5].

RESULTS

The results of Sitar and Thornhill [4] and our early results with impure PTU glucuronides suggested that

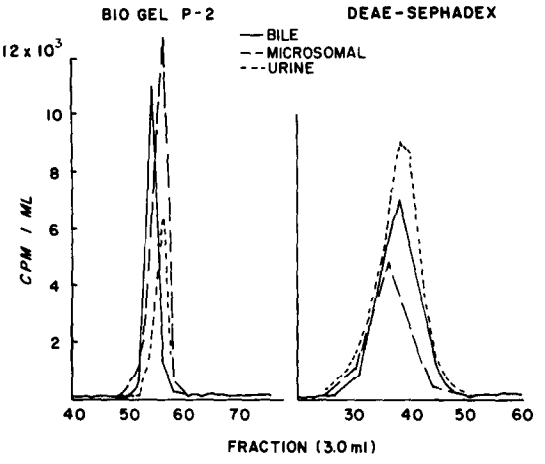

Fig. 1. Chromatographic properties of purified, essentially salt-free ^{14}C-labeled PTU glucuronides isolated from rat bile and urine and prepared enzymatically with guinea pig liver microsomes. Aliquots of each radioactive glucuronide were chromatographed on 2 × 115 cm Bio-Gel P-2 columns eluting with water and on 1 × 10 cm DEAE-Sephadex A-25 columns eluting with freshly prepared 0.1 M ammonium carbonate. See Materials and Methods for details of purification and chromatography.

the glucuronides from urine and bile had different chromatographic properties. Subsequently radioactive PTU glucuronides from three sources, rat bile and urine and synthesized with guinea pig liver microsomes, were purified to obtain samples which were essentially salt free and which formed a single radioactive peak in the column systems in Fig. 1 and the TLC systems in Table 1.

The chromatographic properties of the three purified glucuronide samples on separate 2 × 115 cm columns of Bio-Gel P-2 and on separate 1 × 10 cm columns of DEAE-Sephadex were compared with the results shown in Fig. 1. All three glucuronide samples had essentially the same elution properties from each column with the slight variation seen being well within the column reproducibility for a single compound. Furthermore, simultaneous chromatography of all three PTU glucuronide samples on each of five different TLC plates as shown in Table 1 demonstrated that migration of the three glucuronides was identical in each TLC system. Thus the chromatographic properties of the three purified PTU glucuronide samples in seven different chromatographic systems are identical and do not provide any evidence for more than one glucuronide of PTU.

The purified ^{14}C-labeled glucuronides were partially hydrolyzed with 1 N HCl and further analyzed by TLC. Qualitative and quantitative conversion to hydrolysis products was examined with each purified glucuronide in the appropriate solvent system with DEAE cellulose, cellulose, silica gel on plastic sheets and silica gel impregnated glass fiber sheets. The results obtained were essentially the same with the three glucuronides and are illustrated in Fig. 2 with the bile preparation chromatographed on silica gel plastic TLC sheets. [^{14}C]PTU is very stable to HCl hydrolysis as illustrated in Fig. 2C and 2D while PTU glucuronide is partially hydrolyzed (Fig. 2A and 2B) to PTU, PU and an unidentified derivative. PU is the major product of hydrolysis and is the usual product formed by HCl hydrolysis of *S* substituted PTU's [1, 9]. The unknown was not observed when [^{35}S]PTU glucuronides were hydrolyzed demonstrating that it is a desulfurated product like PU.

N-Methyl PTU is unaltered by HCl treatment under the conditions employed above [9] suggesting that a glucuronide conjugated to the *N* of PTU would not labilize the *S* to HCl and that a glucuronide *C* to *N* bond would be stable to HCl hydrolysis. Confirmation of the stability of the *C—N* bond was obtained with 1,2-dimethyl-PTU in which methyl groups are attached to both the *N* and the *S*. HCl had no effect on the *N*-methyl group but quantitatively hydrolyzed the *S*-methyl group to yield *N*-methyl PU (data not shown). Consequently, HCl hydrolysis of PTU glucuronide to PU suggests that the *S* is conjugated and is the primary site of glucuronidation in the PTU molecule.

Involvement of the *S* in conjugation was further investigated by determining the availability for methylation of the *S* group in PTU glucuronides. If the *S* were conjugated by glucuronide, it would not react with methyl iodide to form an *S*-methyl compound while *N* or *O* glucuronide conjugation is unlikely to affect the reactivity of the *S*.

^{35}S-Labeled PTU and the three purified glucuronides were reacted with methyl iodide in an attempt to form the *S*-methyl analog with the results shown in Table 2. *S*-Methylation of PTU, measured after separation of PTU and *S*-methyl PTU by TLC [9],

Table 1. Chromatographic properties of purified, essentially salt-free PTU glucuronides isolated from rat urine and bile and synthesized with guinea pig liver microsomes

Source of PTU Glucuronide	Rf's of Radioactive PTU Glucuronides in TLC Systems*				
			Silica Gel-P		
	Cellulose	DEAE-Cell.	(A)	(B)	Silica Gel-FG
Rat Urine	0.48	0.21	0	0.68	0
Rat Bile	0.46	0.23	0	0.69	0
Synthesized	0.46	0.21	0	0.67	0

* The results shown are averages obtained from 7 TLC's with each TLC system. TLC cellulose sheets were developed in 1 M ammonium acetate-95% ethanol (15:75), DEAE-cellulose in 0.05 M ammonium carbonate, silica gel on plastic sheets in (A) benzene-isopropanol (60:10) or (B) 95% ethanol-glacial acetic acid (9:1), and silica gel impregnated glass fiber sheets in hexane–acetone (50:10).

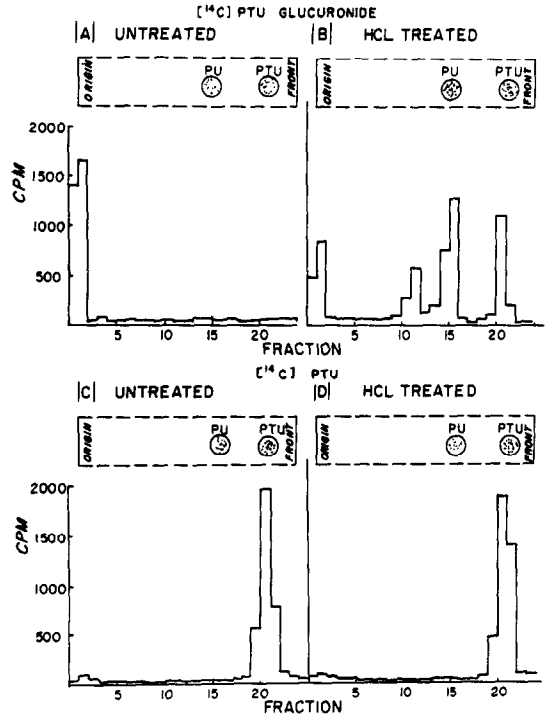

Fig. 2. HCl treatment of purified, essentially salf-free [^{14}C]PTU glucuronide isolated from rat bile. The PTU glucuronide or PTU was hydrolyzed with 1 N HCl for 10 min in a boiling water bath, the mixture lyophilized and the dry sample dissolved in 50 μl of H_2O. Aliquots were chromatographed with "cold" authentic PTU and PU standards on silica gel plastic TLC strips developed in benzene-isopropanol (60:10). The TLC strip, with the position of the uv absorbing cochromatographed standards circled, is outlined with broken lines; the distribution of radioactivity on the TLC strip is shown in solid lines.

readily occurred with an 83.1 per cent yield being obtained. Chromatography of the reaction mixtures containing the three glucuronides did not demonstrate any evidence of *S*-methylation. The reaction mixtures were further treated with HCl to convert any *S*-methyl moieties to volatile methyl [^{35}S]mercaptan which was collected in Hyamine. The results obtained with PTU demonstrate that 92.3 per cent of the radioactive *S*-methyl PTU (77 per cent of the original PTU) was recovered as methyl mercaptan. Under identical conditions, less than 14 per cent of the radioactivity originally present as the [^{35}S]PTU glucuronides was recovered in the Hyamine. Similar results were obtained with the three glucuronide preparations suggesting that they were probably identical. Consequently, the *S* group in the three PTU glucuronide preparations does not appear to react with methyl iodide suggesting that the reaction is blocked by a group conjugated to the *S*.

A very sensitive and specific spot test for C—SH or C=S has been described by Feigl [10] in which organic compounds with these groups catalyze a reaction between sodium azide and iodine producing nitrogen. Feigl has pointed out that if adequate sample is tested, an immediate distinct production of nitrogen can be taken as certain proof of the presence of C—SH or C=S groups and that a complete lack of reaction is certain proof of the absence of such groups. Slow hydrolytic cleavage of some compounds may occur with formation of C—SH or C=S groups resulting in a positive test that develops slowly after contact of the test substance with the iodine-azide reagent solution.

Reaction of PTU and PTU derivatives in the iodine-azide test is shown in Table 3. As little as 0.5 nmoles of PTU catalyzed an immediate and prominent production of nitrogen bubbles whereas more than 10 times as much of each of the PTU glucuronide preparations produced a negative reaction. At 7.9 nmoles, the bile glucuronide formed nitrogen bubbles which appeared slowly and were less pronounced than with 0.5 nmoles of PTU suggesting hydrolytic cleavage. As expected, neither propyluracil nor *S*-methyl PTU produced a reaction due to the absence of *S* in propyluracil and the presence of a thioether (*R*—*S*—*R*) group in *S*-methyl PTU rather than C—SH or C=S. The unstable PTU disulfide produced a slight reaction, which was slow to appear, at an amount 10 times that producing a strong reaction with PTU suggesting hydrolytic splitting. The two *N*-substituted PTU derivatives readily catalyzed nitrogen formation in the test demonstrating that conjugation to the heterocyclic *N* of PTU does not affect the reactivity of the C=S or C—SH group in this test. The results presented in Table 3 provide additional strong support for the conclusion that the

Table 2. Availability of *S* group of PTU and PTU glucuronide for *S*-methylation*

Compound	Initial DPM's	% Radioactivity in Reaction Product: *S*-methyl derivative	methyl mercaptan	residual
[^{35}S]PTU	85,600	83.1 ± 2.3	76.7 ± 3.1	11.1 ± 0.7
[^{35}S]PTU glucuronide				
synthesized	61,400	None	7.5 ± 0.8	92.1 ± 4.0
bile	75,100	None	13.5 ± 1.0	79.9 ± 3.7
urine	68,950	None	12.5 ± 1.2	84.5 ± 3.9

* [^{35}S]PTU and [^{35}S]PTU glucuronides were reacted with methyl iodide as described in Materials and Methods. An aliquot of the reaction mixture was applied to TLC sheets to quantitate conversion to the ^{35}S-labeled methyl derivative and another aliquot treated with 1 N HCl to convert any ^{35}S-labeled methyl derivative to methyl [^{35}S]mercaptan which was collected in Hyamine. The residual fraction contained the non-volatile radioactivity remaining after HCl treatment.

Table 3. Reaction of PTU and PTU derivatives in the iodine-azide test for C=S or C—SH groups

PTU Derivative	Amount (nmoles)	Nitrogen Formation
PTU	0.5	positive
PTU-glucuronide		
synthesized	5.4	negative
urine	5.4	negative
bile	5.4	negative
bile	7.9	trace*
Propyluracil	10.0	negative
S-methyl PTU	10.0	negative
PTU-disulfide	5.0	trace*
	10.0	positive
N-methyl PTU	1.0	positive
N-acetyl PTU	1.0	positive

* Nitrogen bubbles less pronounced than with 0.5 nmoles PTU and develop more slowly indicating possible hydrolysis to PTU.

S of PTU is altered due to glucuronide conjugation at this site and that it does not exist as a C=S or C—SH group as it does in the parent PTU molecule.

The absence of a reactive *S* group was confirmed with the colorimetric assay for PTU described by Ratliff *et al.* [11] which depends upon the reaction of 2,6-dichloroquinone-4-chloroimide with the C=S group of PTU. Neither of the three PTU glucuronides, PU nor *S*-methyl PTU produced a colored reaction product whereas *N*-methyl and *N*-acetyl PTU formed more color than PTU (results not shown) to confirm that conjugation to the heterocyclic *N* of PTU does not suppress the reactions of the PTU sulfur.

The results presented thus far are consistent with glucuronide conjugation to the *S* of PTU forming a thioether (glucuronide-*S*-PTU) or to conjugation on the heterocyclic NH or enolic OH of a PTU in which oxidation of the C—SH group has occurred to form a PTU-SO_2H or PTU-SO_3H derivative. If the *S* is oxidized, HCl hydrolysis would form PU (if the conjugated glucuronide was removed) and the *S* would not react with methyl iodide or 2,6-dichloroquinone-4-chloroimide and would not catalyze the iodine-azide reaction.

It is highly unlikely that the *S* of the PTU glucuronide is oxidized since β-glucuronidase hydrolysis yields unaltered PTU [1, 5] which is readily separated from PTU-SO_2H and PTU-SO_3H in the TLC systems used to identify the PTU product (R. H. Lindsay, unpublished observations) and the conditions of incubation with β-glucuronidase favor oxidation of the *S* rather than reduction. However, further evidence was needed to clarify the state of the *S* group in PTU glucuronide and was provided by H_2O_2 oxidation. If the *S* were partially oxidized or unconjugated, H_2O_2 oxidation would yield sulfate. The results obtained when H_2O_2 treated and untreated samples were chromatographed in TLC systems are illustrated in Fig. 3 with the synthesized preparation chromatographed on cellulose TLC strips and demonstrate that PTU (Fig. 3C and D) is almost completely oxidized by H_2O_2 with sulfate being the major product. Under identical conditions, none of the [^{35}S]PTU glucuronides was oxidized to sulfate (Fig. 3A and B). Previous studies with *S*-methyl PTU [9] demonstrated that substitution on the heterocyclic nitrogen of PTU did not affect the susceptibility of the *S* to H_2O_2 oxidation whereas substitution

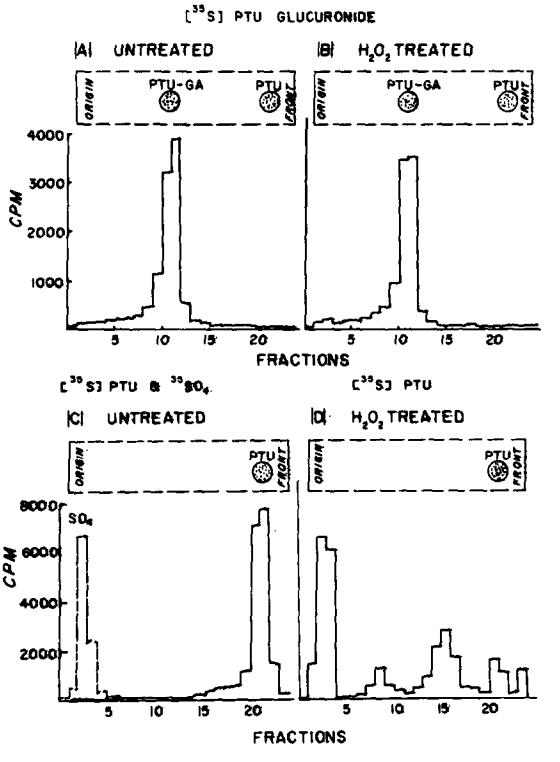

Fig. 3. H_2O_2 treatment of purified, salt-free, microsome synthesized [^{35}S]PTU glucuronide. [^{35}S]PTU or [^{35}S]PTU glucuronide was incubated with 3% H_2O_2 for 30 min at room temperature. The sample was lyophilized and dissolved in 50 μl of H_2O. Aliquots were cochromatographed with "cold" PTU and/or PTU glucuronide on cellulose TLC strips developed with 1 M ammonium acetate-95% ethanol (15:75). The TLC strip, with the position of the uv absorbing cochromatographed standards circled, is outlined with broken lines; the distribution of radioactivity on the TLC strips from the H_2O_2 treated and untreated samples are shown in solid lines. The position of a $^{35}SO_4$ standard is shown with broken lines in 3C.

on the *S* stabilized this group and prevented oxidation by H_2O_2. Consequently the results obtained with the PTU glucuronides confirm that the *S* in each of the three preparations was not partially oxidized but was blocked, as could logically occur in the PTU glucuronide only by glucuronide conjugation to the *S*, preventing H_2O_2 oxidation of the *S*.

Further evidence that the *S* of PTU was the site of glucuronide conjugation was obtained by comparing the utilization of PTU and PU in glucuronidation experiments. The structures of these two compounds are shown in Fig. 4. PU is identical to PTU except that an *O* in PU replaces the *S* in PTU. Since PU is actually an oxidation product of PTU, all other potential glucuronide conjugating sites in the two compounds are identical. [^{14}C]PU and [^{14}C]PTU were compared as substrates for guinea pig liver microsomal UDP glucuronyl transferase with the results shown in Fig. 5. Chromatography on Bio Gel columns of reaction mixtures incubated with and without UDPGA demonstrated that PTU was an excellent substrate for the enzyme with approximately 10 per cent of the available PTU being converted to PTU glucuronide. Under identical conditions, no measurable glucuronidation of PU was observed. The absence of PU glucuronidation was confirmed by TLC chromatography in the 5 systems used in Table 1.

Similar results were obtained after radioactive PU and PTU were administered to rats and the bile and urine examined. Chromatography on Bio Gel columns and in various TLC systems demonstrated the formation of PTU glucuronide but uniformly failed to provide any evidence for the glucuronidation of PU.

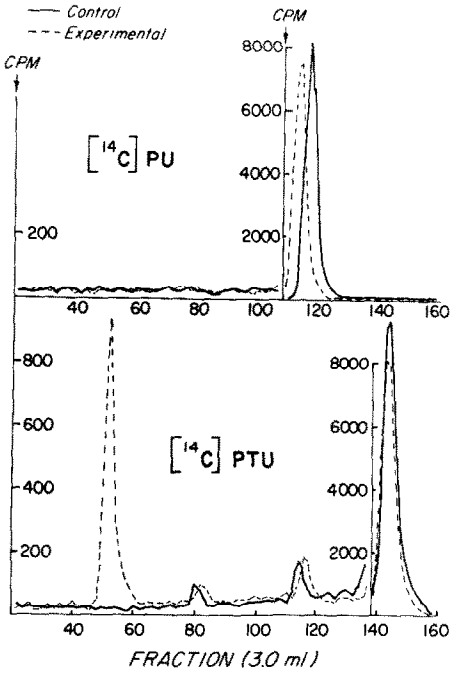

Fig. 5. Glucuronidation [^{14}C]PTU and [^{14}C]PU by guinea pig microsomes. The compounds were incubated with guinea pig liver microsomes for 1 hr in the presence (broken line) and absence (solid lines) of UDPGA, as described in Materials and Methods, and aliquots of the incubation mixture chromatographed on 2 × 115 cm Bio-Gel P-2 columns eluting with water.

DISCUSSION

In studies of PTU metabolism in rats, Sitar and Thornhill [4] observed that the PTU glucuronide in urine was eluted much earlier from DEAE-Sephadex columns than the glucuronide from bile and that their chromatographic properties in one TLC system were different. These apparent differences were interpreted as indicating that the PTU glucuronides in urine and bile were different and probably represented metabolites in which the glucuronide was conjugated to different sites in the PTU molecule. The implication was that the metabolite in urine consisted primarily of glucuronide attached to PTU at one site while the metabolite in bile consisted primarily of glucuronide attached to PTU at a different site. This concept requires a high degree of selectivity by the excretory systems in which one PTU glucuronide is excreted only in urine while the other is excreted only in bile. This would be a highly unusual occurrence and it would be contrary to the pertinent observations of Bastomsky and Marchant [12] in which an i.v. injection of biosynthetic radioactive PTU glucuronide (presumably isolated from either bile or urine) into rats resulted in the appearance of 61 per cent of the unchanged PTU glucuronide in urine and 27 per cent in bile after 3 hr. Thus selective excretion of a single PTU glucuronide into urine or bile did not occur.

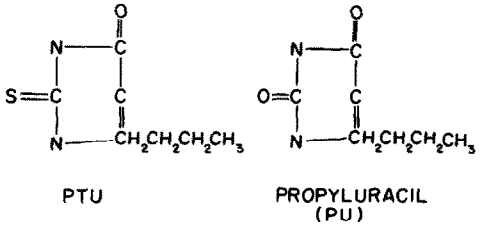

Fig. 4. Structures of PTU and PU.

Nevertheless, preliminary experiments in our laboratory with impure PTU glucuronide preparations isolated after the passage of rat bile and urine through Bio-Gel columns also demonstrated different chromatographic properties for the two samples on DEAE cellulose TLC strips. The urine preparation had a high salt content while the bile preparation was almost salt-free. It had been observed that the chromatographic properties of the PTU metabolites in several chromatographic systems were salt sensitive and the differences that we observed between urine and bile PTU glucuronides were thought to be salt effects. The results presented in the present study with highly purified PTU glucuronides from rat bile and urine and a preparation synthesized with liver microsomal UDP glucuronyl transferase demonstrate that the chromatographic differences we had noted previously were not observed with the pure preparations which were chromatographically identical in seven chromatographic systems. Furthermore, the PTU glucuronides from the three different sources yielded

similar products in similar yields after HCl hydrolysis and the reactivity of the *S* in the PTU glucuronide from each source was drastically altered preventing reaction with methyl iodide, 2,6-dichloroquinone-4-chloroimide and the iodine-azide reagent and preventing oxidation by H_2O_2.

The absence of chromatographic differentiation and the identical reactivity of the PTU glucuronides in the reactions summarized above provide strong evidence that the PTU glucuronides are identical and that conjugation probably occurs to a single site in the molecule.

A consideration of the observed properties of PTU glucuronide and PTU derivatives provides essential information regarding the precise moiety in the PTU molecule conjugated. Conjugation to an enolic hydroxyl group, such as the one at C-4 of the PTU molecule, is very common and PTU was thought to have a high potential for conjugation at this site. MMI, which is not glucuronidated by UDP glucuronyl transferase, does not possess a comparable group and conjugation at the C-4 hydroxyl of PTU would explain the difference in the utilization of these two drugs. However, three important observations indicate that this is not the site of conjugation. First, glucuronidation of PTU markedly reduces biological activity with PTU glucuronide being approximately 10 per cent as active as PTU as an inhibitor of thyroid peroxidase [6]. The enolic hydroxyl at C-4 is not essential for the antithyroidal activity of PTU and conjugation at this site would be very unlikely to produce the alteration in biological activity observed. Second, glucuronidation of PTU drastically altered the reactivity of the *S* in the PTU molecule as demonstrated in the present study. Conjugation at the C-4 hydroxyl would have only minor effects on the reactivity of the *S* and would not abolish the reactions typical or specific for the C=S or C—SH group. Third, PTU was readily conjugated after administration to rats and by UDP glucuronyl transferase whereas PU, which differs from PTU only in the replacement of the *S* with *O*, was not conjugated. Thus in spite of the ready availability of the C-4 hydroxyl group in a molecule almost identical to that of PTU, conjugation did not occur.

Glucuronidation of a heterocyclic nitrogen is especially rare and the authors are aware of only one report of conjugation to this site and that involves the heterocyclic nitrogen of sulfisoxazole [13]. The heterocyclic nitrogens of PTU are constituents of the thionamide moiety which is essential for the antithyroidal activity of the drug [14] and conjugation to the heterocyclic nitrogen could abolish the antiperoxidase activity as observed with *N*-methyl PTU [6]. However, conjugation to a heterocyclic nitrogen in the thiourylene moiety of PTU is not compatible with the observed properties of the PTU glucuronide. PTU glucuronide is readily and rapidly hydrolyzed by β-glucuronidase [1, 5] whereas *N*-glucuronides are either not affected by the enzyme or are hydrolyzed only after prolonged treatment by large amounts of enzyme [15, 16]. Hydrolysis of PTU glucuronide with 1 N HCl yielded PU as a major product but *N* substituted PTU (*N*-methyl PTU) is stable to HCl which neither removes the *N*-methyl group nor alters the *S* [9]. It was demonstrated in this presentation that glucuronidation of PTU essentially abolishes the reaction of the *S* in the molecule whereas *N*-methyl and *N*-acetyl substituted PTU reacted equally as well as PTU in the iodine-azide test and with 2,6-dichloroquinone-4-chloroimide. Earlier reports also demonstrated that the *S* of *N*-methyl substituted PTU was readily oxidized by H_2O_2 [6, 9] and readily *S*-methylated by methyl iodide [6]. Consequently, a substitution or conjugation to a heterocyclic nitrogen of PTU is highly unlikely to affect the reactivity of the *S*. Finally, PU, with heterocyclic nitrogens identical to those in PTU was not glucuronidated.

In contrast to the above, all of the data obtained with PTU glucuronide are consistent with and strongly support the conclusion that conjugation of the glucuronide is to the *S* of PTU. It was observed that PU was formed by hydrolysis of the glucuronide with 1 N HCl. PU is known to be a hydrolytic product of PTU only when the *S* is substituted or oxidized. Oxidation was ruled out by the formation of PTU upon β-glucuronidase hydrolysis and by the lack of desulfuration with H_2O_2. Thus the *S* must be conjugated. It was observed that the *S* in PTU glucuronides did not participate in reactions which are typical for the *S* of PTU. The only molecular alterations which abolish *S* reactivity are *S* substitution (*S*-methyl PTU), oxidation or removal. Oxidation has been ruled out above. Since radiolabeled *S* remains in the molecule after glucuronidation and β-glucuronidase hydrolysis yields PTU, the *S* remains in the molecule and must be conjugated. It was observed that PU was not glucuronidated under conditions readily conjugating PTU. Since PU is identical to PTU, except for the replacement of the *S* of PTU by *O*, all potential conjugating sites are identical with the exception of the *S*. Thus all potential sites for glucuronidation other than the *S* appear to be ruled out.

On the basis of the evidence presented above it was concluded that the glucuronide is conjugated to the *S* of PTU to form a *S*-glucuronide.

S-Glucuronide conjugation is not common but it does occur and several *S*-glucuronides have been reported [17, 18]. In further studies of the *S*-glucuronide of diethyldithiocarbamate, Dutton and Illing [18] observed that the *S*-glucuronide was readily formed by a mouse UDP-glucuronyl transferase and was readily hydrolyzed by β-glucuronidase. Similar properties have been observed for the PTU glucuronide [1, 5].

The *S* is the most metabolically active moiety in the PTU molecule since all metabolites of PTU identified thus far involve an alteration of the *S*. PU, *S*-methyl PTU, PTU-*S*-glucuronide and sulfate are all metabolites of PTU and appear in various body fluids [1–4]. The formation of PTU disulfide has also been suggested [19], but not confirmed, and the oxidative metabolites PTU-SO_3H and PTU-SO_2H have been identified in rat thyroid extracts (Lindsay *et al.*, unpublished observations).

The *S* of PTU is also a component of the thionamide moiety of PTU and as such is required for the biological activity of PTU. It has been repeatedly demonstrated that conjugation or substitution on the *S* abolishes the anti-thyroidal activity of the thionamide. Consequently, it is no surprise that the *S*-glu-

curonide of PTU has little effect on thyroid peroxidase activity [6].

MMI is a thionamide antithyroid drug like PTU but the biological reactivity of the *S* group is apparently qualitatively different. It was previously demonstrated that PTU, but not MMI, was *S*-methylated by a thiol transmethylase [9] and that PTU, but not MMI, was glucuronidated by UDP glucuronyl transferase [5]. The present report identifies this as an *S*-glucuronide of PTU indicating that the *S* of PTU participates in enzymatic transformations not utilizing MMI.

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