BINDING OF THE $^{35}$S OF $^{35}$S-PROPYLTHIOURACIL
BY FOLLICULAR THYROGLOBULIN
IN VIVO AND IN VITRO

By
Petros D. Papapetrou, Stella Mothon and
W. Donald Alexander

ABSTRACT

Radioactivity was found bound to follicular thyroglobulin after administration of $^{35}$S-propylthiouracil (PTU) to rats. Denaturation of the thyroglobulin using various procedures could not separate the $^{35}$S from the protein; it was concluded that the $^{35}$S is bound to thyroglobulin covalently. Fractionation of saline-soluble thyroid proteins was performed by ultracentrifugation on sucrose gradients. The PTU-sulphur/thyroglobulin (S/Tg) molar ratio was calculated in all fractions. One hour after the injection of PTU the S/Tg molar ratio was the same for 19S thyroglobulin from rats on stock diet and 18S thyroglobulin from rats on low iodine diet. Injection of KI to the animals before administration of $^{35}$S-PTU significantly reduced the ratio. The highest S/Tg ratio 1.10 was noted at 19S thyroglobulin, 17 h after a single injection of PTU. Daily injection of PTU for six days increased the S/Tg ratio to 3.3. Inverse relationship between dose of PTU and S/Tg ratio was noted at one hour. In animals injected with large dose of $^{35}$S-PTU and sacrificed several hours later the S/Tg was higher at the 12S subunits than at the 19S protein. The amount of PTU bound to 3–8S subunits was minimal. Sulphite liberated 64% of the $^{35}$S bound to thyroglobulin which appeared as four.
compounds on thin layer chromatography plates. The main \(^{35}\)S compound liberated by sulphite was sulphate.

*In vitro* incubation of \(^{35}\)S-PTU or \(^{35}\)S-sulphate with bovine thyroglobulin showed that sulphate does not bind to thyroglobulin, while PTU or more probably an unknown metabolite of PTU (which contaminates even an oxygen free water solution of PTU) is attached to thyroglobulin by covalent bond.

It is concluded that the \(^{35}\)S of PTU or of an unknown metabolite of PTU different from sulphate binds to follicular thyroglobulin *in vivo*. The \(^{32}\)S of PTU is accepted by disulphide bonds of the thyroglobulin molecule.

Almost thirty years since the introduction of thiocarbamide type of antithyroid drugs (*Astwood et al. 1943*), the mechanism of action of these drugs remains unknown. One method to study this matter is to investigate the effect of the drug on the synthesis and secretion of thyroid hormone. This approach is limited by the incomplete knowledge of the mechanism of synthesis of thyroid hormones (*Astwood 1970*). A second approach to the problem is to study the intrathyroidal metabolism and fate of the thiocarbamides.

Following the initial observations of *Schulman* (1950) concerning the uptake and metabolism of thiourea by thyroid tissue, *Maloof & Soodak* (1963) presented a theory about the mechanism of the inhibition of the iodination of thyroglobulin by thiocarbamides based on their work. These investigators did not deal with the inhibitory effect of these drugs on the coupling of diiodotyrosine (*Richards & Ingbar 1959*).

Recently *Ferguson et al.* (1971) showed, using micro-autoradiography, that after administration of \(^{35}\)S-propylthiouracil to rats some radioactivity is concentrated in the lumen of the thyroid follicles.

We studied here the binding of \(^{35}\)S-6-N-propyl-2-thiouracil (PTU) by follicular thyroglobulin *in vivo* and *in vitro*.

**MATERIALS AND METHODS**

Male Sprague-Dawley rats, weighing 160–470 g (235 g), were used. The rats were fed stock diet (Purina Chow, iodine content 1.11 µg/g), and some were maintained on low iodine diet (LID) (special diet No. 30, Nutritional Biochemical Corporation, Cleveland, Ohio, iodine content 0.045 µg/g) for periods of time indicated in the text.

The \(^{35}\)S-6-N-propyl-2-thiouracil was obtained from the Radiochemical Centre, Amersham, England, with a specific radioactivity of 14–10 mCi/mmol at the time it was used for these experiments; its purity was tested by thin-layer chromatography (*Papapetrou et al. 1972*) and it was found to be 99% PTU. Each rat received, ip, 10–20 µCi of \(^{35}\)S-PTU. The \(^{35}\)S-PTU was dissolved in 0.03 N NaOH; the solution was made up shortly before its administration. The dosage is expressed in the text as mg per 800 g rat. The animals were sacrificed by removal of blood from the ab-
dominal aorta after anaesthesia with ether. The thyroid gland in the majority of cases was immediately perfused in situ with chilled normal saline as described by Inoue & Taurog (1967). The gland was then homogenized in 0.5 ml phosphate buffer (0.01 M, pH 6.8, containing 0.15 M NaCl) in an all glass hand homogenizer. This buffer will be mentioned as isotonic phosphate buffer (IsoPB) in the rest of the text. All the procedures were done at 4°C.

The thyroid glands from two rats injected and sacrificed in identical fashion were always pooled during homogenization and all the individual values of this study are derived therefore from at least two pooled glands. After homogenization the volume of the specimen was increased to 5.0 ml with IsoPB and the debris and subcellular particles were removed by centrifugation at 105,000 g for one hour. The clear supernatant was made up to 50% with ammonium sulphate, and left for one hour in ice (Thomson & Goldberg 1968). The precipitated thyroid proteins were then sedimented by centrifugation at 15,000 r.p.m. for 10 min. The supernatant containing the unbound intrathyroidal radioactivity (which is about 92 to 75% of the total thyroid radioactivity (Marchant et al. 1971) was discarded. Many specimens were dialyzed twice against 1 litre of IsoPB for 22 h at 4°C before precipitation with ammonium sulphate in order to remove totally the unbound radioactivity trapped in the protein precipitate. The majority of specimens was fractionated in the sucrose gradient without previous dialysis. The unbound radioactivity did not migrate below fraction No. 24; it did not interfere therefore with the 12S protein peaks located at fraction No. 19 or 20. Dialysis was necessary in order to evaluate the protein-bound radioactivity to 3–8S peaks.

The thyroid proteins obtained after precipitation with ammonium sulphate were re-dissolved in 0.8 ml IsoPB and applied to the top of the sucrose gradient. Storage of thyroglobulin below 0°C was avoided.

For the fractionation of the soluble thyroid proteins the methods of Inoue & Taurog (1968a) were employed. Ten to 40% linear sucrose gradients were prepared in 5 ml centrifuge tubes and centrifuged for 16 h at 37,000 r.p.m. in a Beckman L2-65B centrifuge using the rotor SW 65K. Thirty-three to 34 fractions (10 drops) were collected in scintillation vials. Twenty µl from each fraction was transferred to a test tube for protein estimation using the method of Lowry et al. (1951) and hag thyroglobulin as standard. The remaining (0.13 ml) was mixed with 0.5 ml hyamine hydroxide (Packard), and after the addition of 10 ml of Bray's scintillator it was counted in a liquid scintillation counter (Nuclear Chicago). Corrections for quenching were made when necessary. The number of counts obtained from the fractions corresponding to protein peaks was at least 4000 per 10 min. Along with each series of counted fractions, an aliquot of the solution of 35S-PTU administered to the animals was counted and its specific radioactivity was calculated (counts per µg of PTU). The number of counts from each fraction was thus expressed as µg of PTU or moles of sulphur (S) (1 mole of S derived from 1 mole of PTU). The sulphur thyroglobulin (S/Tg) ratio was calculated from the apex of each protein peak. For the 19S or 18S peaks molar ratios were derived considering the molecular weight of thyroglobulin as 660,000. The method of Inoue & Taurog (1968a) was used for assignment of the S value to the protein peaks.

Thyroglobulin from rats injected with 35S-PTU 12 h before sacrifice was freed from unbound 35S by dialysis and re-dissolved in a small volume of phosphate buffer pH 6.8, tris buffer pH 7.4 or tris buffer pH 10.8. An aliquot of this solution of thyroglobulin was counted for 35S and known portions of the rest were treated with 9 M
Three groups of rats injected with 0.2 mg $^{35}$S-PTU per 300 g rat and sacrificed one hour later. The third group was injected with 0.5 mg KI per rat one hour before the injection of PTU. The horizontal bars represent the arithmetic mean. Each point represents two pooled thyroid glands.

In vitro experiments

Bovine thyroglobulin (Sigma) was incubated in phosphate buffer pH 7.4 at a concentration 5–10 mg/ml with $^{35}$S-PTU (10$^{-2}$–10$^{-4}$ M), at 37°C for 30 min. At the end of the incubation 90–95% of the radioactivity in the incubation medium was found by thin layer chromatography (Papapetrou et al. 1972) to be PTU, up to 1% SO$_4$ and up to 10% unknown compound X (Papapetrou et al. 1972; Marchant et al. 1971). This compound was present even in O$_2$ free incubation system. After the end of the incubation the thyroglobulin was freed of the unbound $^{35}$S by precipitation with ammonium sulphate and dialysis and the S/Tg ratio was measured as described above. S/Tg molar ratios between 0.4 and 4.0 were obtained in these in vitro experiments. The Tg-$^{35}$S complex was then washed with ethylacetate containing 25% dioxan, treated with 5% trichloroacetic acid (TCA) for 2 h at room temperature, 6 M guanidine, 0.1 M sulphite or 0.1 dithiothreitol for 30 min at 37°C at pH 7.4. After treatment with the last three reagents the unbound $^{35}$S was removed by dialysis and the new S/Tg was measured.
RESULTS

Three groups of rats were injected with 0.2 mg $^{35}$S-PTU and sacrificed one hour later (Fig. 1). In the group on stock diet the S/Tg (19S) molar (molS/molTg) ratio at the 19S peak was $0.12 \pm 0.05$ (mean ± sd). In the rats on LID for 33 days the S/Tg (18S) molar ratio was $0.12 \pm 0.02$. The rats of the third group were fed stock diet and injected ip with 0.5 mg KI per rat one hour before the injection of $^{35}$S-PTU (i.e., 2 h before sacrifice). The S/Tg (19S) molar ratio was $0.08 \pm 0.01$, significantly lower than in both previous two groups by the Rank test ($P < 0.05$).

In a group of rats on stock diet all the animals were sacrificed one hour after the administration of various doses of $^{35}$S-PTU between 0.2 mg and 2.0 mg. The thyroglobulin was 19S in all cases. The S/Tg (19S) molar ratio showed the highest values in rats injected with 0.3 mg and decreased gradually in rats injected with larger doses, reaching a plateau above the dose of 1.0 mg (Fig. 2).

Some rats on stock diet were injected with either 0.2 mg or 1.0 mg of $^{35}$S-PTU and sacrificed at various time intervals between 1 and 24 h. The thyroglobulin was 19S in all cases. In rats given the small dose, the S/Tg (19S) molar ratio rose gradually and attained a peak value of 0.75 11 h after a single injection of $^{35}$S-PTU and then gradually fell. In rats injected with the large dose the S/Tg (19S) molar ratio increased gradually, until it reached a plateau value of 1.0 at about 16 h after the injection.

![Fig. 2](https://example.com/fig2.png)

Rats on stock diet sacrificed one hour after injection with various doses of $^{35}$S-PTU. Each point represents two pooled thyroid glands. The line was drawn through the arithmetic mean of each group.

252
Thyroglobulin from rats on low iodine diet for 73 days, injected with 0.2 mg $^{35}$S-PTU and sacrificed two hours later. Same specimen dialyzed in phosphate buffer (0.01 M, pH 6.8, with 0.15 M NaCl) and in tris buffer pH 10.8. The Tg bound $^{35}$S is expressed as “PTU”.

Very little radioactivity was bound to 3–8S peak of proteins whether the rats were on stock or LID, sacrificed at one hour or longer time intervals after the injection (Fig. 3).

The binding of PTU by 12S subunits of thyroglobulin was studied by causing dissociation of the 19S or 18S dimer to 12S subunits either by dialysis for 22 h at 4°C in low ionic strength buffer (0.01 M phosphate, pH 6.8, without NaCl) (LISPB) (Edelhoch 1960), or by dialysis for 22 h in tris buffer (0.01 M, pH 10.8 with 0.15 M KCl) at 4°C (Cavalieri et al. 1970). The results are summarized in Table 1. In specimens from rats injected with 1.0 mg $^{35}$S-PTU and sacrificed at 16–18 h, dialysis in LISPB resulted in the formation of small significant protein peaks at 12S. The S/Tg (μg/μg) ratio was lower at 19S than at 12S (sample No. 2, Table 1). In rats killed 1 or 2 h after administration of PTU the S/Tg ratio was the same at 19S or 18S and 12S protein (Table 1).

Four rats on stock diet received 2.0 mg $^{35}$S-PTU ip once daily for 6 days. They were sacrificed 18 h after the last injection. Pooled thyroglobulin from these rats showed a peak at 19S with a “shoulder” at 18S. The S/Tg molar ratio was 3.3.

In the in vivo experiments the thyroglobulin-bound $^{35}$S resisted completely treatment with 9 M urea, 6 M guanidine, washing with several volumes of ethyl-
Table 1.
Binding of $^3$S of $^{35}$S-PTU by 12 subunits of thyroglobulin.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Dose of $^{35}$S-PTU (mg/300 g)</th>
<th>Time of sacrifice (hours)</th>
<th>Average from thyroids No.</th>
<th>&quot;PTU&quot;/Tg ($\mu$g/$\mu$g) at protein peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>1</td>
<td>5</td>
<td>$34.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>16-18</td>
<td>4</td>
<td>$255.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>1-2</td>
<td>6</td>
<td>$76.5 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

The data of this table are derived from rats on stock diet; similar data were obtained from rats on LID (not shown).
The procedure used for dissociation of thyroglobulin to 12S protein was dialysis in low ionic strength buffer for sample Nos. 1 and 2 and dialysis at pH 10.8 for sample 3.

**ETHANOL / AMM. ACETATE 1M**
7.5/3.0

![Figure 4](image)

Fig. 4.
Thyroglobulin-$^3$S complex obtained *in vitro* was applied at the origin of a cellulose plate and developed in a solvent system composed of ethanol-ammonium acetate (1 M, v. 5/3). Heavy proteins remain at the origin in this thin layer chromatography system. This figure shows schematically (as peaks) radioactive spots located by autoradiography and counted in scintillation vials. In the control specimen the protein bound $^{35}$S remained at the origin. The other two compounds are unknown. Treatment with sulphite released at least two new compounds. No. 1 is sulphate. The other compounds are unknown. Similar results were obtained in *in vivo* experiments.
acetate, dialysis for 22 h in phosphate buffer, pH 6.8, or tris buffer, pH 10.8; it was not displaced by 50 % ammonium sulphate. MSH removed 40 % of the thyroglobulin bound radioactivity. Sulphite removed 27 %.

In the in vitro obtained Tg-S35 complex ethylacetate-dioxan removed 15 % of the protein bound 35S, TCA removed 12 % and 6 m guanidine did not change the S/Tg ratio. Sulphite and dithiothreitol displaced 64 % and 52 % of the protein bound 35S respectively. Thin layer chromatography in an ethanol-ammonium acetate 1 m (7.5/3.0) system (Papapetrou et al. 1972) showed that sulphite released two new 35S compounds (Fig. 4). The compound near the origin, which was the most important quantitatively, had the Rf 0.16 which is the Rf of sulphate and thiosulphate in this system. This compound was completely precipitated by 0.3 m BaCl2. This concentration of BaCl2 precipitated very little thiosulphate, while it precipitated completely sulphate in some pilot experiments. It was concluded therefore that the main 35S compound released by sulphite from Tg-35S complex was sulphate. Incubation of 35S sulphate (10⁻² m) with thyroglobulin in vitro resulted in no binding of 35S. The 35S compounds present in the thyroid of rats injected with 35S-PTU are PTU, an unknown compound X and sulphate (Marchant et al. 1971). The compound X was eluted in pure form from the TLC plates and it was incubated with thyroglobulin. The 35S of “X” was bound to Tg covalently. It seems that the sulphate released by sulphite is formed during interaction of this agent with thyroglobulin.

**DISCUSSION**

The findings reported here indicate that 35S is bound to follicular thyroglobulin in vivo after administration of 35S-PTU to rats. The amount of sulphur originated from PTU bound to 19S thyroglobulin one hour after administration of the drug in rats on stock diet was small. Obviously only a small fraction of 19S thyroglobulin is available for binding PTU one hour after the administration of the drug. The degree of iodination of thyroglobulin does not influence the magnitude of 35S binding by this protein (Fig. 1). It has been shown (Simon et al. 1966; Lissitzky et al. 1965; Sellin & Lippoldt 1964) that the newly iodinated, immature thyroglobulin dimer of 12S formed a short time before the sacrifice of the rats is labile to freezing or alkali and easily dissociated to 12S subunits. In thyroglobulin from rats on stock or low-iodine diet sacrificed one or two hours after administration of 35S-PTU exposed to low ionic strength buffer or dialyzed at pH 10.8, the “PTU”/Tg (μg/μg) ratio was practically the same at 19S or 18S and the 12S protein peaks (Table 1). However, in the samples obtained from rats sacrificed 16–18 h after injection of 1.0 mg 35S-PTU (sample 2, Table 1, Fig. 5) the “PTU”/Tg (μg/μg) ratio
Thyroglobulin from two rats on stock diet injected with 1.0 mg $^{35}$S-PTU and sacrificed 16 h later. Thyroids not perfused and not dialyzed. Homogenization in phosphate buffer (0.01 M, pH 6.8 without NaCl). Note the higher "PTU"/Tg ratio at 11.5S peak compared to 19S. See also Table 1, sample 2.

was higher at 12S than at 19S protein peak. This indicates that if sufficient time elapses after injection of large amount of $^{35}$S-PTU, the $^{35}$S is found at higher concentrations in the newly formed immature thyroglobulin represented here by the 12S subunits. Whether this accumulation of PTU-sulphur at the 12S protein is responsible for delayed maturation of the protein it is not possible to say at present.

Fig. 2 shows that excess PTU inhibits its own binding by 19S thyroglobulin in vivo, one hour after its administration. Marchant et al. (1971) have found that excess PTU inhibits its own uptake by the thyroid gland of the rat.

Potassium iodide decreased the binding of PTU by thyroglobulin at one hour (Fig. 1). Iodides decrease the concentration of $^{35}$S-PTU by the thyroid of the rat (Marchant et al. 1971). Propylthiouracil or metabolite X interact with thyroglobulin in vitro; enzymatic action is not necessary for this reaction.

Concerning the nature of the linkage between thyroglobulin and $^{35}$S, it is reasonable to conclude that the bond is mainly covalent, since it resisted denaturation of the thyroglobulin with 9 M urea, 6 M guanidine, ethylacetate, dioxan, dialysis at pH 6.8 and pH 10.8 and treatment with 50% ammonium sulphate. The radioactivity did not separate from the protein under various
conditions of thin layer chromatography in systems containing alcohol or chloroform (unpublished observations).

Sulphite, which cleaves disulphide bonds, displaced 64% of the thyroglobulin-bound radioactivity. MSH was used in our experiments in a concentration known to reduce all the disulphide bonds of thyroglobulin (Pitt-Rivers & de Crombrugghe 1965); this removed 40% of the thyroglobulin-bound $^{35}$S. These results are suggestive that a proportion of the $^{35}$S is bound to thyroglobulin by disulphide bonds.

The amount of $^{35}$S bound to 3–8S subunits of thyroglobulin was found to be negligible (Fig. 3). The 3–8S protein can be obtained from 19S thyroglobulin by reduction of disulphide bonds with mercaptoethanol (de Crombrugghe 1965). It is possible that 3–8S subunits contain a smaller number of disulphide bonds than the 19S molecules and this may account for the marked difference in the affinity of these two proteins for the $^{35}$S of PTU.

The significance of the binding of $^{35}$S of PTU by thyroglobulin is not clear at present.

Several investigators (Simon et al. 1966; Inoue & Taurog 1968b; Cramarossa et al. 1971) have studied the dissociation of thyroglobulin caused by PTU or mercaptoimidazole in vivo and in vitro. From the data presented here we can only speculate that the $^{35}$S of PTU bound to thyroglobulin could be of some importance in the phenomena studied by the above authors. There is experimental evidence that inhibition of the coupling of diiodotyrosine to form thyroxine is the main action of thiocarbamides (Richards & Ingbar 1959). The mechanism of coupling is unknown and largely hypothetical. PTU or one of its metabolites may interfere with coupling by destroying some crucial disulphide bonds in the molecule of thyroglobulin.

ACKNOWLEDGMENTS

We are grateful to Dr. E. B. Astwood for his help and encouragement during this work and to Dr. S. Reichlin for helpful comments. We should like to thank Dr. Arnold Marglin for helpful discussions and Dr. Andrew McAuley for technical assistance.

REFERENCES


Inoue K. & Taurog A.: Endocrinology 83 (1968a) 816.


Received on July 2nd, 1974.