

Rapid Sulfation of 3,3',5'-Triiodothyronine in Native *Xenopus laevis* Oocytes

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ABSTRACT

Sulfation is an important metabolic pathway facilitating the degradation of thyroid hormone by the type I iodothyronine deiodinase. Different human and rat tissues contain cytoplasmic sulfotransferases that show a substrate preference for 3,3'-diiodothyronine (3,3'-T₂) > T₃ > rT₃ > T₄. During investigation of the expression of plasma membrane transporters for thyroid hormone by injection of rat liver RNA in *Xenopus laevis* oocytes, we found uptake and metabolism of iodothyronines by native oocytes. Groups of 10 oocytes were incubated for 20 h at 18°C in 0.1 ml medium containing 500,000 cpm (1–5 nM) [¹²⁵I]T₄, [¹²⁵I]T₃, [¹²⁵I]rT₃, or [¹²⁵I]3,3'-T₂. In addition, cytosol prepared from oocytes was tested for iodothyronine sulfotransferase activity by incubation of 1 mg cytosolic protein/ml for 30 min at 21°C with 1 μM [¹²⁵I]T₄, [¹²⁵I]T₃, [¹²⁵I]rT₃, or [¹²⁵I]3,3'-T₂ and 50 μM 3'-phosphoadenosine-5'-phosphosulfate. Incubation media, oocyte extracts, and assay mixtures were analyzed by Sephadex LH-20 chromatography for production of conjugates and iodide. After 20-h incubation, the percentage of added radioactivity present as conju-

gates in the media and oocytes amounted to 0.9 ± 0.2 and 1.0 ± 0.1 for T₄, less than 0.1 and less than 0.1 for T₃, 32.5 ± 0.4 and 29.3 ± 0.2 for rT₃, and 3.8 ± 0.3 and 2.3 ± 0.2 for 3,3'-T₂, respectively (mean ± SEM; n = 3). The conjugate produced from rT₃ was identified as rT₃ sulfate, as it was hydrolyzed by acid treatment. After injection of oocytes with copy RNA coding for rat type I iodothyronine deiodinase, we found an increase in iodide production from rT₃ from 2.3% (water-injected oocytes) to 46.2% accompanied by a reciprocal decrease in rT₃ sulfate accumulation from 53.7% to 7.1%. After 30-min incubation with cytosol and 3'-phosphoadenosine-5'-phosphosulfate, sulfate formation amounted to 1.8% for T₄, less than 0.1% for T₃, 77.9% for rT₃, and 2.9% for 3,3'-T₂. These results show that rT₃ is rapidly metabolized in native oocytes by sulfation. The substrate preference of the sulfotransferase activity in oocytes is rT₃ >>> 3,3'-T₂ > T₄ > T₃. The physiological significance of the high activity for rT₃ sulfation in *X. laevis* oocytes remains to be established. (*Endocrinology* 139: 596–600, 1998)

THE MAIN secretory product of the thyroid gland, T₄, is enzymatically converted in peripheral tissues to the biologically active hormone T₃ (1). This transformation concerns the elimination of an iodine from the phenolic ring of T₄, also termed outer ring deiodination (ORD). Deiodination of the tyrosyl ring [inner ring deiodination (IRD)] is an inactivation step by which T₄ and T₃ are converted to the inactive metabolites rT₃ and 3,3'-diiodothyronine (3,3'-T₂), respectively. The latter is also produced by ORD of rT₃ (2). These reactions are catalyzed by different iodothyronine deiodinases with distinct tissue distributions. The type I iodothyronine deiodinase (D1) is found predominantly in liver, kidney, and thyroid. It is a nonselective enzyme capable of ORD as well as IRD of different iodothyronines (1–3).

Besides deiodination, conjugation of the phenolic hydroxyl group with glucuronic acid or sulfate is another important step in thyroid hormone metabolism (2). In general, the purpose of these so-called phase II detoxification reactions is to increase the water solubility of lipophilic substances and, thus, to facilitate their excretion in bile and/or urine (4). The iodothyronine glucuronides are stable conju-

gates that are rapidly excreted in the bile (2). However, sulfate conjugation has been shown to facilitate the subsequent deiodination of iodothyronines by D1 in the liver (5, 6). Although sulfation blocks the ORD of T₄, it strongly facilitates the IRD of both T₄ and T₃, suggesting that sulfation is an important step in the irreversible inactivation of thyroid hormone (5, 6).

Sulfate conjugation of various compounds is catalyzed by sulfotransferases, which represent a group of homologous enzymes with overlapping substrate specificities that occur in the cytosolic fraction of different tissues, in particular liver (7). For all these enzymes 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acts as the sulfate donor. Iodothyronine sulfotransferase activities in rat and human tissues show a substrate preference for 3,3'-T₂ >>> T₃ > rT₃ > T₄ (8–12).

In experiments to express plasma membrane transport proteins for thyroid hormone by injection of RNA from rat tissues in *Xenopus laevis* oocytes (13), we found that native oocytes transport and sulfate iodothyronines, in particular rT₃. We have, therefore, investigated the uptake and metabolism of T₄, T₃, rT₃, and 3,3'-T₂ by *X. laevis* oocytes as well as the sulfation of these compounds by isolated oocyte cytosol.

Materials and Methods

Materials

T₄, T₃, PAPS, and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). rT₃, 3,3'-T₂, and 3-iodothyronine (3-T₁) were obtained from Henning Berlin (Berlin, Germany). [3',5'-¹²⁵I]T₄ [43 megabecquerels (MBq)/nmol], [3'-¹²⁵I]T₃ (>66 MBq/nmol), and carrier-free

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Na¹²⁵I (80 MBq/nmol) were purchased from Amersham (Aylesbury, UK). [3',5'-¹²⁵I]rT₃ and 3,[3'-¹²⁵I]T₂ were prepared from Na¹²⁵I and 3,3'-T₂ or 3-T₁, respectively, using the chloramine-T method, followed by purification on Sephadex LH-20 (12). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

Capped rat D1 copy RNA (cRNA) was prepared from the complementary DNA in pBluescript (14) (provided by Dr. P. Reed Larsen), linearized with *Xho*I (Boehringer Mannheim, Mannheim, Germany), using the AmpliScribe T₃ transcription kit (Epicentre Technologies, Madison, WI) according to the protocol of the supplier. For capping, the m⁷G[5']ppp[5']G cap analog was used. cRNA pellets were dissolved in water (0.04 μg/μl) and stored at -80 C.

Animals

Two- to 3-yr-old adult *X. laevis* females were obtained from the Hubrecht Laboratory (Utrecht, The Netherlands). Frogs were maintained in a water-filled tank with three dark sides at a temperature of 18–22 C. A 12-h light, 12-h dark cycle was maintained to reduce seasonal variations in oocyte quality. Frogs were fed twice a week, and water was changed immediately after feeding.

Oocyte isolation and RNA injection

Oocytes were prepared as described previously (13). After isolation, the oocytes were sorted manually on morphological criteria, such as size, polarization, pigmentation, and absence of follicular layer debris. Healthy-looking stage V–VI oocytes (15) were transferred to six-well tissue culture plates and incubated in the dark at 18 C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.4 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 2.4 mM NaHCO₃, and 10 mM HEPES (pH 7.4), containing 10 IU/ml penicillin and 10 μg/ml streptomycin]. The next day, oocytes were injected with 23 nl water containing 0.92 ng D1 cRNA using the Nanoject system (Drummond Scientific, Broomall, PA). Injected and uninjected oocytes were maintained for 3–4 days at 18 C in modified Barth's solution, with daily change of medium.

Uptake and metabolism assays

Groups of 10 oocytes were transferred to a 96-well tissue culture plate and subsequently incubated in the dark at 18 C with 4 nM [¹²⁵I]T₄, 5 nM [¹²⁵I]T₃, 2 nM [¹²⁵I]rT₃, or 2 nM [¹²⁵I]T₂ in 0.1 ml sodium-containing incubation medium (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM Tris, pH 7.5). After 20 h, incubation medium was collected, and the oocytes were transferred to tubes and washed four times with 2.5 ml ice-cold sodium buffer containing 0.1% BSA. Two groups of 5 oocytes from each group of 10 were transferred to new tubes, counted, and lysed with 0.1 ml 0.1 M NaOH. Lysates were cleared by centrifugation. Lysates (in duplicate) and incubation media were acidified with 0.1 M HCl and analyzed by Sephadex LH-20 chromatography (16). The products were separated by successive elutions with 1 ml 0.1 M HCl (iodide) twice, 1 ml water (conjugates) 6 times, and 1 ml 1% NH₄OH in ethanol (iodothyronines) three times.

rT₃ conjugate hydrolysis

Acid hydrolysis of rT₃ conjugate was tested by incubation for 1 h at 80 C in 1 M HCl (17). The reaction was stopped by placing the mixtures on ice, and the products were analyzed by Sephadex LH-20 chromatography as described above.

Sulfotransferase assay

Oocytes were homogenized on ice in 2–3 vol 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA, and 1 mM dithiothreitol, and cytosol was isolated after centrifugation for 1 h at 100,000 × g. The protein concentration was determined by the method of Bradford (18), using BSA as the standard.

Iodothyronine sulfotransferase activities were measured by incubation of 1 μM T₄, T₃, rT₃, or 3,3'-T₂ and 100,000 cpm of the ¹²⁵I-labeled compound for 30 min at 37 C (optimal temperature for warm-blooded animals) or 21 C (optimal temperature for cold-blooded animals) with 0.1 or 1 mg cytosolic protein/ml in the presence (in triplicate) or absence

(blank) of 50 μM PAPS in 0.2 ml 0.1 M phosphate buffer (pH 7.2) and 2 mM EDTA, as described previously (11, 12). The reaction was started by the addition of diluted cytosol and was stopped by the addition of 0.8 ml 0.1 M HCl. Iodothyronine sulfate formation was analyzed by Sephadex LH-20 chromatography as described above.

Statistics

Uptake and metabolism studies were performed with groups of 10 oocytes. Data are expressed as the percent uptake of total radioactivity per 10 oocytes and are presented as the mean ± SEM. Statistical significance was evaluated by Student's *t* test for unpaired observations.

Results

Uptake and metabolism of iodothyronines in oocytes

The percentage of added radioactivity present in the media and, after washing, in the oocytes after 20-h incubation amounted to 19.4 ± 3.7 and 67.1 ± 7.0 for T₄, 3.3 ± 0.3 and 51.5 ± 0.7 for T₃, 50.9 ± 1.4 and 35.0 ± 1.4 for rT₃, and 24.2 ± 0.5 and 68.1 ± 0.7 for 3,3'-T₂ (Fig. 1). The incomplete recovery of radioactivity, in particular with [¹²⁵I]T₃, is primarily due to adsorption of label to the tube as the incubation medium did not contain protein. For T₄, T₃, and 3,3'-T₂, almost all radioactivity present in the oocytes was in the form of the added iodothyronine. However, only 15.4 ± 0.5% and 4.9 ± 0.1% of the added [¹²⁵I]rT₃ was recovered as intact iodothyronine from the medium and oocytes, respectively. After incubation with [¹²⁵I]rT₃, most of the added radioactivity was

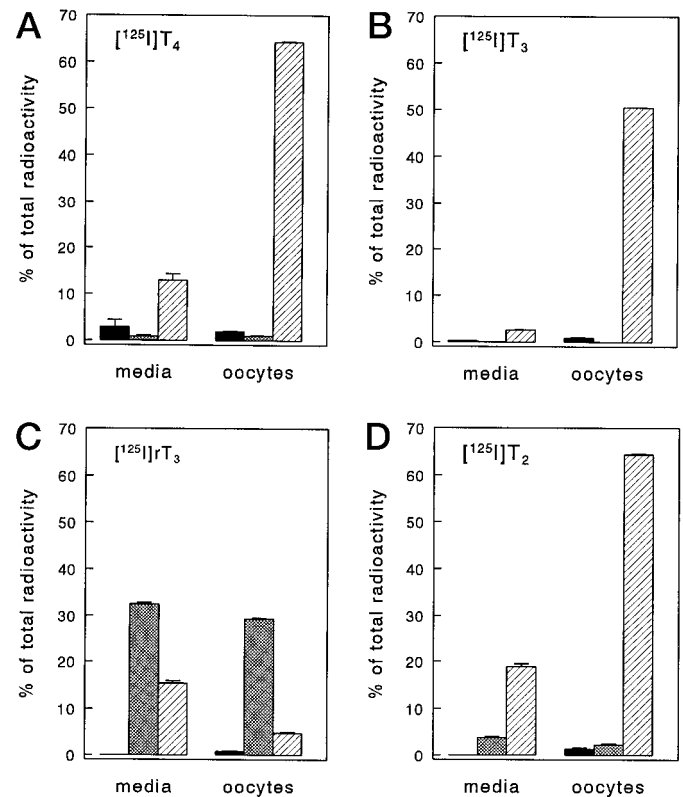


FIG. 1. Uptake and metabolism of [¹²⁵I]T₄ (A), [¹²⁵I]T₃ (B), [¹²⁵I]rT₃ (C), and [¹²⁵I]3,3'-T₂ (D) during incubation for 20 h at 18 C with 10 oocytes. Percentages of iodide (■), conjugates (▨), and iodothyronines (□) in the incubation buffer and in the homogenized oocytes, analyzed by Sephadex LH-20 chromatography. Bars represent the mean ± SEM (n = 3).

recovered as conjugate, *i.e.* $32.3 \pm 0.4\%$ in the medium and $29.3 \pm 0.2\%$ in the oocytes, indicating active conjugation of rT₃ in the oocytes. With none of the iodothyronines was significant iodide production observed in the oocytes.

Native iodothyronines are regenerated from their sulfate conjugates by acid-catalyzed hydrolysis (17). The conjugate formed after incubation of rT₃ with oocytes was completely hydrolyzed after treatment for 1 h at 80 C with 1 M HCl (Fig. 2), indicating that rT₃ was indeed sulfated by the oocytes.

Injection of cRNA coding for rat D1 resulted, after incubation of the oocytes for 20 h with [¹²⁵I]rT₃, in a large increase in iodide formation from 2.3% to 46.2% of the added radioactivity, which was accompanied by a reciprocal decrease in rT₃ sulfate (rT₃S) accumulation from 53.7% to 7.1% compared with that in water-injected oocytes (Fig. 3). Water-injected oocytes showed the same rate of rT₃ sulfation as native (uninjected) oocytes (Figs. 1C and 3). Figure 3 also shows that the amount of iodide in the D1 cRNA-injected oocytes incubated with [¹²⁵I]rT₃ was 3 times higher than that in the medium.

Sulfotransferase assay

Iodothyronine sulfotransferase activities were measured in cytosol from native oocytes at incubation temperatures of 37 and 21 C, producing the same results (not shown). Figure 4 presents the results of the experiments performed at 21 C. Although sulfation rates were higher at 1 than at 0.1 mg cytosolic protein/ml, the pattern of sulfation of the different iodothyronines was independent of the protein concentration. The sulfotransferase activity in oocyte cytosol showed a preference for rT₃ \gg 3,3'-T₂ > T₄ > T₃.

Discussion

In experiments using *X. laevis* oocytes to express rat liver cell membrane transporters for thyroid hormones (13), we found that native *X. laevis* oocytes transport iodothyronines and sulfate rT₃. We also found that transport and sulfation of rT₃ in oocytes injected with rat liver messenger RNA (mRNA) were similar to those in uninjected oocytes (data not shown). Thus, the possible induction of an exogenous trans-

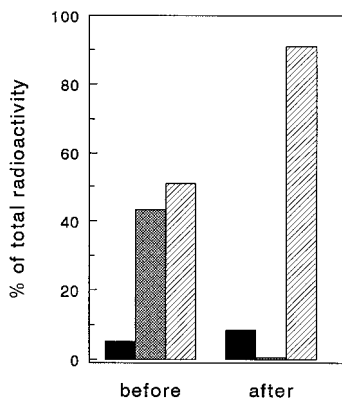


FIG. 2. Acid-catalyzed hydrolysis of rT₃ conjugate produced after incubation of [¹²⁵I]rT₃ for 20 h at 18 C with 10 oocytes. The percentages of iodide (■), conjugate (▣), and rT₃ (▨) in the incubation medium and oocytes before and after the acid-catalyzed hydrolysis were determined by Sephadex LH-20 chromatography. Results are derived from a duplicate incubation in a representative experiment.

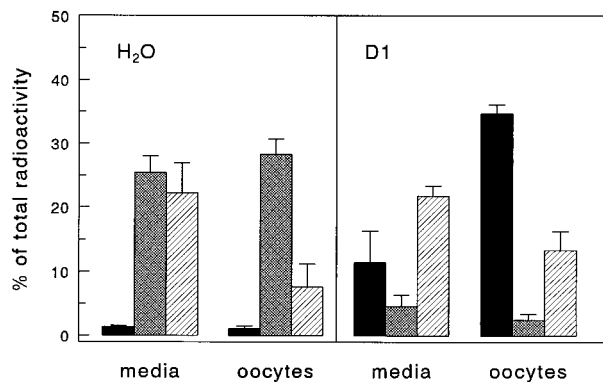


FIG. 3. Metabolism of rT₃ in D1 cRNA-injected and water-injected oocytes during incubation of [¹²⁵I]rT₃ for 20 h at 18 C with 10 oocytes. Percentages of iodide (■), conjugates (▣), and rT₃ (▨) in the incubation medium and oocytes were determined by Sephadex LH-20 chromatography. Bars represent the mean \pm SEM (n = 3).

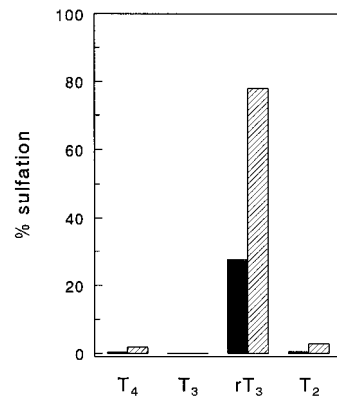


FIG. 4. Sulfation of 1 μ M iodothyronines during incubation for 30 min at 21 C with oocyte cytosol in the presence of 50 μ M PAPS. The cytosolic protein concentration amounted to 0.1 mg/ml (■) and 1.0 mg/ml (▨). Results are the means of two closely agreeing experiments.

port protein for rT₃ by the injection of rat liver mRNA was completely masked by the endogenous transport system for rT₃ in the oocytes. This is in contrast to previous findings concerning the expression of hepatic cell membrane transporters for T₃ and T₄ (13), showing that injection of rat liver mRNA or partially purified fractions thereof induces a significant 2- to 3-fold increase in T₃ and T₄ transport into oocytes.

After 20-h incubation with [¹²⁵I]-labeled T₄, T₃, or 3,3'-T₂, most radioactivity was associated with the oocytes as unmetabolized iodothyronines. As after incubation the oocytes were extensively washed in BSA-containing medium, cell-associated radioactivity most likely represents internalized iodothyronine. This conclusion is supported by the observation that uptake of iodothyronines by *X. laevis* oocytes is a Na⁺-dependent process (13). On the contrary, after 20-h incubation with [¹²⁵I]rT₃, most radioactivity was recovered from the medium, largely as rT₃S. Taking medium and oocytes together, more than 50% of added rT₃ was sulfated. Sulfation of iodothyronines is catalyzed by cytoplasmic sulfotransferases (7–12), which enzymes are apparently also present in oocytes. These results, therefore, support the conclusion that rT₃ was internalized by the oocytes and sulfated

intracellularly. Subsequently, (part of) the rT₃S formed is transported back to the medium. The finding that after 20-h incubation of oocytes with rT₃ and other iodothyronines almost no iodide is formed indicates that *X. laevis* oocytes possess little or no endogenous D1 activity, in agreement with the findings of St. Germain *et al.* (19). In fact, D1 activity is not expressed at any stage of development in amphibians (20). On the other hand, injection of cRNA coding for rat D1 leads to a large increase in iodide production from rT₃ at the expense of rT₃S accumulation. As, like native D1 in mammalian cells, exogenous D1 in oocytes is probably located intracellularly (1–3), these results again strongly support the presence of an endogenous transporter(s) for iodothyronines in *X. laevis* oocytes.

D1 is a nonselective enzyme capable of both ORD and IRD of iodothyronines (1–3). The enzyme is most effective in the ORD of rT₃, but the deiodination of different iodothyronines is dramatically affected by the sulfation of these compounds (5, 6). Thus, IRD of both T₄ and T₃ by rat D1 is accelerated 40–200 times after their sulfation, suggesting that sulfation is an important step, leading to the irreversible inactivation of thyroid hormone (5, 6). In contrast, ORD of T₄ is completely blocked by sulfation. This is not a general phenomenon, as ORD of rT₃ by rat D1 is not affected by sulfation of this substrate, whereas ORD of 3,3'-T₂ is facilitated 50-fold by sulfation (5, 6). In contrast to the facilitated deiodination of iodothyronine sulfates by D1, deiodination of iodothyronines by the type II (D2) and type III (D3) iodothyronine deiodinases is inhibited by sulfation (21) (Visser, T. J., unpublished observations). As rT₃ and rT₃S are deiodinated equally well by rat D1, it is uncertain to what extent iodide production from rT₃ in rat D1 cRNA-injected oocytes proceeds via rT₃S or represents direct ORD of rT₃. The iodide formed remains trapped inside the oocytes, indicating the absence of transporters for both influx and efflux of iodide in native oocytes, in accordance with the studies of Dai *et al.* (22).

Our experiments have not directly addressed the possible IRD of the different iodothyronines in the oocytes. However, St. Germain *et al.* (23) have shown that native *X. laevis* oocytes kept in Barth's medium do not express D3. Moreover, we did not find conjugate formation after incubation of T₄ with oocytes, which would have been expected if T₄ underwent IRD to rT₃. Therefore, it is also unlikely that T₃ was metabolized by IRD in the oocytes.

Sulfate conjugation of iodothyronines is catalyzed by phenol sulfotransferases located in the cytosolic fraction of different tissues (7). In both rats and humans, iodothyronine sulfotransferase activities show a substrate preference for 3,3'-T₂ >> T₃ > rT₃ > T₄, with rT₃ being a relatively poor substrate (8–12). Our results show that sulfotransferase activity in *X. laevis* oocyte cytosol has a clear substrate preference for rT₃, whereas 3,3'-T₂, T₃, and T₄ are hardly sulfated at all. The physiological importance of thyroid hormone sulfation is still unknown. As discussed above, sulfation is an irreversible pathway of thyroid hormone metabolism when D1 activity is high, as sulfated iodothyronines are rapidly degraded by this enzyme (5, 6). However, sulfation is a reversible pathway of thyroid hormone inactivation when D1 activity is low because of the regeneration of free iodothyronines by sulfatases produced in different tissues and by

intestinal bacteria (5, 6). It has been speculated that due to the low D1 activity during fetal development in mammals, sulfation/desulfation of T₃ is an important mechanism for the tissue-specific and time-dependent regulation of thyroid hormone bioactivity (6, 21). Indeed, high concentrations not only of T₃S, but also of T₄S, rT₃S, and 3,3'-T₂S have been detected in fetal sheep serum and human cord serum (24, 25).

It is remarkable that during embryonic and fetal development in different organisms, two active pathways exist for the inactivation of thyroid hormone: IRD of T₄ to rT₃ and of T₃ to 3,3'-T₂ by D3, and sulfation (1–3, 24, 25). That these are true inactivation pathways is suggested by the findings that rT₃, 3,3'-T₂, and T₃S have little or no affinity for the T₃ receptor (26, 27). Thyroid hormone plays an important role not only in the embryonic development of tadpoles, but also in the metamorphosis of the tadpole to the froglet (20). This requires the tissue-specific and development stage-dependent regulation of the balance between thyroid hormone activation by D2 and inactivation by D3 (20). We hypothesize that sulfation/desulfation contributes to the regulation of thyroid hormone bioactivity during embryonic development and metamorphosis in frogs. Although direct evidence is lacking, this hypothesis is supported by the absence of D1 in frogs (20). Why, then, the sulfotransferase(s) involved shows a profound substrate preference for rT₃ remains unknown. The possibility that rT₃S exerts a biological function by binding to a transcription factor other than the T₃ receptor deserves investigation.

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