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3′-Monoiodothyronine Sulfate and Triac Sulfate Are Thyroid Hormone Metabolites in Developing Sheep

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ABSTRACT: We used novel 3′-monoiodothyronine sulfate (3′-T_1S) and 3,3′,5-triiodothyroacetic acid sulfate (TriacS) RIAs to characterize sulfation pathways in fetal thyroid hormone metabolism. 3′-T_1S and TriacS levels were measured in serum samples obtained from fetal (n = 21, 94–145 d gestational age), newborn (NB, n = 5), and adult sheep (AD, n = 5) as well as from fetuses after total thyroidectomy (Tx), or sham-operated twin fetuses controls, conducted at gestational age 110–113 d (n = 5). Peak levels (expressed as ng/dL) of both 3′-T_1S and TriacS occurred at 130 d gestation. These levels in fetuses were higher than those in NB and AD. In Tx fetuses, there was a significant decrease in the mean serum level of 3′-T_1S, but not TriacS. The decrease in 3′-T_1S in Tx is similar to that observed for thyroxine sulfate (T_4S) and 3,3′,5′-triiodothyronine sulfate (rT_3S), whereas TriacS levels were not altered in the hypothyroid state, similarly to 3,3′,5-triiodothyronine sulfate (T_3S). These data demonstrate that 3′-T_1S and TriacS are normal thyroid hormone metabolites in ovine serum and that TriacS is likely derived from T_3S or from the same precursor(s) as T_3S. (***Pediatr Res* 63: 149–153, 2008**)

We have identified the sulfated iodothyronines, thyroxine sulfate (T_4S), 3,3′,5-triiodothyronine (T_3S), 3,3′,5′-triiodothyronine sulfate (reverse T_3S) (rT_3S), and 3,3′-diiodothyronine sulfate (3,3′-T_2S), as important thyroid hormone metabolites in ovine and human fetal fluids (1–5). The relatively high concentrations of these sulfated iodothyronines in the developing fetus probably reflect the low type I deiodinase activities observed in fetal tissues (6,7). To further characterize metabolism of the sulfated iodothyronines in ovine fetuses, we developed sensitive and specific 3′-T_1S and 3,3′-5-triiodothyroacetic acid sulfate (TriacS) RIAs to quantify 3′-T_1S and TriacS levels in normal and hypothyroid fetal and maternal serum. In previous studies of hypothyroid fetuses, we found significant reductions of mean serum concentrations of T_4S and rT_3S, but not T_3S, suggesting that T_3, presumably the precursor of T_3S, was derived from T_3 in tissue. Hypothyroidism may result in a compensatory increase in activity of type II 5′-deiodinase, which tends to maintain tissue T_3 in a relatively normal range (8,9). The present study is to determine whether serum levels of TriacS and/or 3′-T_1S are reduced in fetal hypothyroidism.

MATERIALS AND METHODS

3′-T_1S and TriacS RIAs. 3′-T_1S and 3′-[^{125}I]T_1S as well as TriacS and [^{125}I]TriacS were prepared by the method of Eelkman-Rooda and co-workers (10,11). 3′-T_1S and TriacS in 0.025 N NaOH (4 mg/mL) were further purified and quantitatively recovered by reverse-phase HPLC with a preparative column (Biochrom 1010 ODS; Regis, Morton Grove, IL). The products were eluted isocratically with a mixture of acetonitrile and 20 mM ammonium acetate, pH 4.0 (22:78 vol/vol), at a solvent flow of 10 mL/min. 3′-T_1S and TriacS were recovered with purity >99%, as assessed by HPLC.

The RIA for 3′-T_1S used an anti-3′-T_1S antibody 3A-2, obtained from one of three New Zealand rabbits immunized with a 3′-T_1S-BSA conjugate (2). The RIA for TriacS used an antibody D1-3 against TriacS-BSA conjugants. Each rabbit was immunized with emulsions of 1 mL of a solution of the conjugate containing 2 mg BSA and an equal volume of complete Freund's adjuvant (Calbiochem, San Diego, CA) in multiple dorsal subcutaneous sites. Booster injections comprised of 1 mL conjugate and an equal volume of incomplete Freund's adjuvant were continued at 6-wk intervals. Moderate titer antibodies were detected in the sera of two of the three rabbits 3 wk after the fourth (3′-T_1S) and sixth (TriacS) immunizations. At a final dilution of 1:12,000 (in 1 mL of 0.075 M barbital buffer, pH 8.6, and 0.12% normal rabbit serum), anti-3′-T_1S antibody bound 35–45% of a tracer amount (= 8.3 fmol or 5 pg) of 3′-[^{125}I]T_1S (similarly for TriacS antibody, final dilution 1:5000). Ethanol did not appreciably inhibit the binding of 3′-[^{125}I]T_1S to 3A-2 antibody up to a final concentration of 22% (similarly for TriacS antibody D1-3). Therefore, we used ethanol (63%) extracts of sera to measure 3′-T_1S and TriacS concentrations. The final ethanol concentration in the assays was 19%.

The 3′-T_1S and TriacS RIA procedures, developed using antisera 3A-2 and D1-3, respectively, were modifications of the RIA procedure described previously for the measurement of T_4S and T_2S in sera (2,4). The 3′-T_1S and TriacS RIAs were carried out in duplicate or triplicate in 10 × 75-mm disposable glass tubes containing 0.053 M barbital buffer (pH 8.6), 0.07% sodium azide, 0.088% normal rabbit serum, and 19% ethanol (containing either the unknown or standard amounts of unlabeled 3′-T_1S or TriacS). Standard curves were constructed with 1 pg to 1000 pg of 3′-T_1S (2.1 fmol to 2.09 pmol) or TriacS (1.4 fmol to 1.43 pmol), optimal amount of 3A-2 or D1-3 antibody (final dilution 1:12,000 for 3A-2 and 1:5000 for D1-3), and 18,000–22,000 cpm 3′-[^{125}I]T_1S or [^{125}I]TriacS, in a final volume of 1 mL. The tubes were thoroughly mixed and incubated at 4°C overnight. A sufficient amount of a previously titered goat anti-rabbit gamma globulin (second

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Abbreviations: AD, adult; **NB,** newborn; **PR,** production rate; **rT_3S,** 3,3′,5′-triiodothyronine sulfate (reverse T_3S); **SULT,** sulfotransferase; **Tx,** thyroidectomy; **Triac,** 3,3′,5-triiodothyroacetic acid; **TriacS,** 3,3′,5-triiodothyroacetic acid sulfate; **T_3,** 3,3′,5-triiodothyronine; **T_3S,** 3,3′,5-triiodothyronine sulfate; **T_4S,** thyroxine sulfate; **3′-T_1,** 3′-monoiodothyronine; **3′-T_1S,** 3′-monoiodothyronine sulfate; **3,3′-T_2S,** 3,3′-diiodothyronine sulfate

antibody) was then added. The tubes were mixed, incubated at 4°C overnight, and centrifuged at 2000 *g* for 20 min. Supernatants were carefully aspirated, and radioactivities in the precipitates were quantified using an Isodata 20/20 gamma counter (Isodata, Palatine, IL). Nonspecific binding (determined in tubes without added 3A-2 or D1-3 antibody) was <3% and was subtracted from counts of bound 3′-[^{125}I]T_1S or [^{125}I]TriacS. Standard curve plots and other calculations were undertaken as described previously (5,8).

Animal preparation and samples. Western mixed-breed time-dated pregnant ewes with twin pregnancies were obtained from the Nebeker Ranch (Lancaster, CA) and acclimated to our laboratory conditions and food. Animals were studied in the following periods of gestation: 94 d ($n = 5$), 110–111 d ($n = 6$), 130–131 d ($n = 6$), and 145 d ($n = 6$). These gestational ages were chosen because of differences in thyroid hormone secretion and metabolism during this period of development (12,13). In addition, sera from newborn (NB) ($n = 5$) and adult (AD) ($n = 5$) animals, including pregnant and nonpregnant ewes, were studied.

To assess the effect of fetal hypothyroidism on 3′-T_1S and TriacS levels, a group of five ewes (110–113 d gestation) with twin fetuses were selected. The ewes were sedated (1.2 mg atropine and 700 mg ketamine i.m.), and a continuous infusion of ketamine (100 mg/h) was started *via* a jugular venous catheter. After local anesthesia of the abdominal wall (2% lidocaine), a midline incision was followed by palpation of the uterus and fetal parts and identification of the fetal head. A hysterotomy was performed over the fetal neck, which was exteriorized with attention to avoiding loss of amniotic fluid. The fetal neck was infiltrated with 1% lidocaine, followed by dissection and complete removal of the thyroid gland (Tx). The neck incision was closed, and the second fetus was handled in a similar manner, except that a sham operation was conducted, and the thyroid gland was left intact (control). The ewes were treated for 3 d postoperatively with oxacillin (2 g) and gentamicin (80 mg) given intramuscularly in divided doses. The fetuses were studied 13 d after the initial operation. The 13-d interval from Tx to death was chosen based on the serum half-life of T_4 in the ovine fetus (24 h) and to avoid long-term effects of Tx on cell number and body weight (12–14).

To obtain serum from fetuses, the ewes were sedated as previously outlined and given spinal and epidural anesthesia (5 mL of 0.5% Marcaine and 5 mL of 2% lidocaine). After a paramedian abdominal incision and hysterotomy, each twin was delivered and immediately killed with an overdose of i.v. pentobarbital sodium. After obtaining samples from each fetus, the ewe was similarly killed. All experiments were approved by the Harbor-University of California at Los Angeles Medical Center Animal Use Committee.

Preparation of test serum. Samples were extracted with two volumes of 95% ethanol before assay. Preliminary experiments showed that the extraction efficiencies of 3′-T_1S and TriacS in serum were 90–98% and 88–95%, respectively (mean ± SE, 93 ± 4 and 91 ± 5, respectively, in 6–8 experiments with different, known amounts of unlabeled 3′-T_1S or TriacS added). Final values of 3′-T_1S and TriacS concentration were not corrected for recovery efficiency. The preliminary experiments demonstrated that the immunoreactive 3′-T_1S and TriacS in ethanol extracts of fetal and maternal serum co-chromatographed with the corresponding synthetic compounds on HPLC.

Source of materials. 3,3′-T_2, 3′,5′-T_2, D-T_3, T_3, rT_3, 3-monoiodothyronine (3-T_1), 3′-T_1, 3,5-diiodothyroacetic acid (Diac), 3,3′,5-triiodothyroacetic acid (Triac), 3,3′,5,5′-tetraiodothyroacetic acid (Tetrac), and thyroxine (T_4) were purchased from Henning-Berlin (Berlin, Germany). Thyronine (To), 3,5-T_2, 3,3′,5-triiodothyropropionic acid (Triprop), monoiodotyrosine (MIT), diiodotyrosine (DIT), BSA, and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO). 3′-[^{125}I]T_1 and [^{125}I]TriacS were prepared by radioiodination of To and Diac, respectively, using the method described previously (15). T_4S, T_3S, and rT_3S were synthesized by the method described previously (10,11). Chlorosulfonic acid, 99%, was purchased from Aldrich Chemical (Milwaukee, WI).

Statistical analysis. ANOVA was used for multi-group comparisons. If significant differences were detected, Dunnett's multicomparison test was used to compare the control or baseline mean and the mean values of other groups (16). Significance was defined as $p < 0.05$. Results are reported as mean ± SE.

RESULTS

3′-T_1S and TriacS RIAs: Specificity and sensitivity. Of the various thyroid hormone analogs studied, only T_4S and T_3S cross-reacted significantly (0.3% and 0.01%, respectively) in the 3′-T_1S RIA; T_4, T_3, rT_3, and 3,3′-T_2 showed <0.0002% cross-reaction with the antiserum. The antiserum to TriacS cross-reacted significantly with T_4S (5.9%), 3,3′-T_2S (3.0%), rT_3S (1.1%), T_3S (0.09%), and 3′,5′-T_2S (0.001%); all other analogs cross-reacted <0.0001% (Table 1). The lower limit of detection was 2 ng/dL (or 41.8 pmol/L and 28.5 pmol/L, respectively, for 3′-T_1S and TriacS) in a 300-μL ethanol extract.

Table 1. *Cross-reactivities*

RIAs/analogs	3′-T_1S	TriacS
3′-T_1	<0.0001%	<0.0001%
3,3′-T_2	<0.0002%	<0.0001%
T_3	<0.0002%	<0.0001%
rT_3	<0.0002%	<0.0001%
T_4	<0.0002%	<0.0001%
3′-T_1S	—	<0.0001%
3,3′-T_2S	<0.0001%	3.0%
3′,5′-T_2S	<0.0001%	0.001%
T_3S	0.01%	0.09%
rT_3S	<0.0001%	1.1%
T_4S	0.3%	5.9%

Parallelism, recovery, and reproducibility. The dose-response curves for inhibition of binding of 3′-[^{125}I]T_1S to antibody 3A-2 produced by the ethanol extracts of serum were compared with the standard curve. The mean deviation from predicted values in various dilutions was 6.2% and 4.1% in two mean concentrations studied, 86 ng/dL and 175 ng/dL, respectively. The mean recovery of nonradioactive 3′-T_1S added to serum extracts in concentrations ranging from 20 to 200 ng/dL in six experiments averaged (±SE) 93 ± 4%. The mean coefficients of variation for different 3′-T_1S concentrations in sera tested ($n = 5$) in the same assay were 9.7% for 25 ng/dL, 6.0% for 100 ng/dL, and 7.4% for 150 ng/dL. The mean interassay coefficients of variation of 3′-T_1S concentrations were 16.5% at 25 ng/dL, 8.5% at 100 ng/dL, and 15.4% at 150 ng/dL.

The dose-response curves for inhibition of binding of 3′-[^{125}I]TriacS to antibody D1-3 produced by the ethanol extracts of serum were compared with the standard curve. The mean deviations from predicted values in various dilutions were 3.9% and 15.4% in two mean concentrations studied, 8.7 ng/dL and 34.1 ng/dL, respectively. The mean recovery of nonradioactive TriacS added to serum extracts in concentrations ranging from 20 to 200 ng/dL in seven experiments averaged (±SE) 91 ± 5%. The mean within assay coefficient of variation for TriacS (concentration 25 ng/dL $n = 5$) was 9.9%. The mean interassay coefficient of variation of TriacS (concentrations 30 ng/dL, $n = 6$) was 15.6%.

3′-T_1S and TriacS concentrations. Serum 3′-T_1S and TriacS concentrations measured in each age group of fetuses, newborns, and adults are shown in Figure 1. Fetal serum 3′-T_1S and TriacS levels increased progressively from 94 to 130 d, reaching a peak of 229 ± 24 ng/dL and 114 ± 13 ng/dL, respectively. These 130-d values were significantly higher than values in near-term animals (145 d gestation; 68 ± 3.8 and 44 ± 8, respectively; $p < 0.05$) and in newborns (105 ± 5 and 12.5 ± 2.8, respectively; $p < 0.05$).

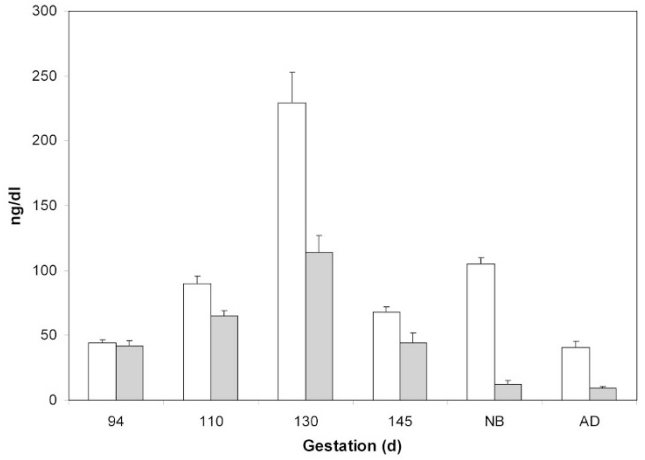

Figure 1. Serum concentration of 3′-T_1S (*open*) and TriacS (*shaded*) in ng/dL of fetuses of different gestational ages (in days), of NB and AD sheep. Vertical bars and lines represent means ± SE. Each bar represents mean of 5–6 animals. For conversion to nmol/L 3′-T_1S, multiply by 0.0209; nmol/L TriacS, multiply by 0.0140.

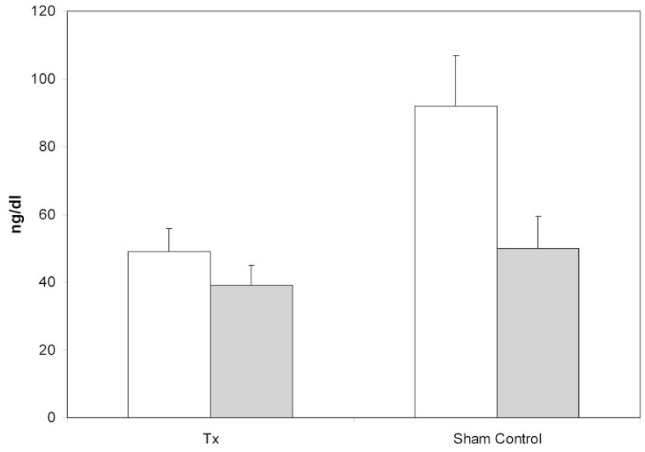

Figure 2. Effect of Tx on 3′-T_1S (*open*) and TriacS (*shaded*) concentrations in serum from ovine fetuses 13 d after Tx at gestational age 110–113 d. Controls were sham-operated twin fetuses.

The mean serum level of 3′-T_1S in NB sheep was 105 ± 5 ng/dL, compared with 41 ± 4.4 ng/dL in AD ($p < 0.0005$); in contrast, TriacS levels were similar in NB (12.5 ± 2.8 ng/dL) and AD (9.3 ± 1.2 ng/dL; $p > 0.05$).

Effect of Tx on 3′-T_1S and TriacS concentrations. Figure 2 summarizes the effect of Tx on 3′-T_1S and TriacS concentrations in serum from ovine fetuses (125 d of gestation). Serum 3′-T_1S was found to be significantly decreased in Tx fetuses (49 ± 6.8 ng/dL) compared with sham-operated control fetuses (92 ± 15 ng/dL; $p < 0.02$). No such decrease was observed in TriacS levels between Tx fetuses (39 ± 6.0 ng/dL) and sham-operated control fetuses (50 ± 9.5 ng/dL, $p > 0.05$).

DISCUSSION

There is general agreement that sequential monodeiodination is the major mechanism regulating the bioavailability

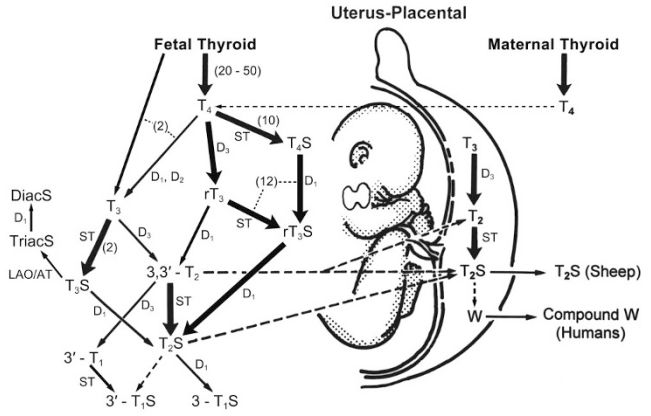

Figure 3. Postulated metabolic pathways for ovine fetal thyroid hormones. *D1, D2,* and *D3*: type I, type II, and type III iodothyronine deiodinases; *ST*: iodothyronine sulfotransferases; *LAO*: L-amino acid oxidase; *AT*: thyroid hormone aminotransferase. LAO and AT are postulated to convert T_3S to TriacS. Heavy solid lines indicate pathways that are more active in fetuses than in adults; thin solid lines, pathways that are less active in fetuses. The upper horizontal light dotted line depicts T_4 of maternal origin moving to the fetal compartment in the first trimester, before the fetal thyroid begins functioning. Other broken lines represent unconfirmed pathways. Numbers in parentheses indicate published production rates (μg/kg/d).

of thyroid hormones in tissues. However, the alternate pathways may also play a role in some circumstances. Sulfoconjugation of iodothyronines, for example, is an important pathway in developing animals (7,17) (Fig. 3), and sulfated iodothyronines can also be deiodinated, at an even faster rate (7). Likewise, iodothyroacetates can be sulfated and further deiodinated.

Previously, we demonstrated high levels of T_4S, T_3S, rT_3S, and 3,3′-T_2S in ovine fetal serum, bile, and amniotic fluid (1–5). Our present results indicate that 3′-T_1S and TriacS are normal metabolites in thyroid hormone metabolism in sheep and that the sulfation pathway is more prominent in ovine fetuses than in adults. In earlier studies of the effect of Tx on thyroid metabolism in fetal sheep, serum T_4 and T_3 levels fell to <0.7 μg/dL and <10 ng/dL, respectively (18). Labeled hormone kinetic studies showed minimal maternal to fetal transfer of T_4 or T_3 and estimated T_4 and T_3 turnover rates approximating 0.6 and 0.7 μg/24 h. These amounts represent <0.2% and <50% of the mean daily T_4 and T_3 turnover rates of 46 and <1.5 μg/kg in euthyroid third trimester fetal sheep (18). Reductions in T_4S, rT_3S, and 3,3′-T_2S were also observed in later studies but there was no decrease in serum T_3S levels (5,8). The relatively constant levels of T_3S in these animals led to the postulation that T_3S may be derived from T_3 in tissue. Hypothyroidism results in a compensatory increase in activity of type II 5′-deiodinase, which would tend to maintain tissue T_3 in a relative normal range, important for brain development. There is also a limited maternal to fetal placental transfer of T_3 (13). In addition, Tx may reduce the clearance of T_3S by a decrease in type I deiodinase activity (8). The Tx fetus would presumably have a severe reduction of type I deiodinase activity in a state analogous to targeted disruption of the type 1 selenodeiodinase gene (Dio1) in mice,

in which the serum T_3 levels remained unchanged compared with wild-type animals (9).

In the present study, fetal Tx decreased 3′-T_1S while TriacS levels were unchanged. The similar trends for T_3S and TriacS suggest that TriacS is likely derived from T_3S or from the same precursors as T_3S (8). There is evidence to indicate that T_3S can be converted to TriacS by oxidative degradation, possibly involving two enzymes, L-amino acid oxidase (LAO) and thyroid hormone aminotransferase (AT) (Fig. 3) (7,17). Alternatively, the relatively stable level of TriacS could be due to a reduction in the removal of TriacS by outer-ring deiodination, mediated by type I deiodinase, to 3,3′-DiacS (19–21). The role of glucuronidation of Triac is less well characterized in sheep (21,22); in addition, the enzyme activity is significantly less in ovine fetal liver (23). The observed rapid reduction of serum TriacS levels in newborns compared with fetuses (Fig. 1) may reflect a maturation of glucuronosyltransferase in the clearance of Triac.

The formation of TriacS may have clinical relevance because Triac has a higher affinity than T_3 to thyroid hormone receptors TRβ_1 and TRβ_2 in tissue (7). In humans, Triac is found to derive mainly from T_3 (21). TriacS has been found in mammalian circulation following the intravenous infusion of Triac, which is known to be a preferred substrate for sulfotransferase (SULT) 1A1 (21). In humans, SULT1A1 shows the highest affinity for both iodothyronines and 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the universal sulfonate donor. The role of SULT1A1 in ovine tissue is not known. Sulfation of Triac would presumably reduce its binding to thyroid receptors, similar to sulfated T_3 (21). As there is some evidence that TriacS can be desulfated to yield Triac (21), TriacS may serve as a reservoir for Triac even though its physiologic role in the fetus is not known.

The decrease in 3′-T_1S in the Tx fetuses in the present study resembles the earlier observed trends in T_4S, rT_3S, and 3,3′-T_2S (5,8). The precursor for 3′-T_1S likely is 3,3′-T_2S. Although outer-ring deiodination to 3-T_1S is known (21), the conversion of 3,3′-T_2S to 3′-T_1S has not been demonstrated. Alternatively, 3′-T_1S could be a sulfated product of 3′-T_1, which could be derived from inner-ring deiodination of 3,3′-T_2; the type III deiodinase is known to be active in fetal tissue. From the high potency of 3′-T_1 in inhibiting sulfoconjugation of 3,3′-T_2 in human SULT1A1 and SULT1A3, 3′-T_1 may be an excellent substrate to be sulfated (24). After the infusion of outer-ring-labeled T_3 in the euthyroid fetus, 3,3′-T_2S was found to be the predominant metabolite, followed by lesser amounts of 3,3′-T_2 and T_3S; but no 3′-T_1 or its sulfoconjugate was seen (25). The kinetics of labeled T_3 in Tx ovine fetuses have not been studied with HPLC or other available RIAs specific to sulfated iodothyronines developed recently (7).

A proposed scheme for thyroid hormone metabolism in developing sheep is outlined in Figure 3. The high production rate (PR) for sulfated iodothyronines reflects the dominant pathway, sulfoconjugation, in ovine fetuses. The PR for T_4 and T_3 are 46 and <1.5 μg/kg/d, respectively, in the euthyroid third trimester fetus while the PR for rT_3S, T_4S, and T_3S are 12, 10, and 2 μg/kg/d, respectively (18,26). The kinetic studies predicted that 3,3′-T_2S also is a major thyroid hormone metabolite and this was later confirmed qualitatively (25). 3,3′-T_2S was also found to be the major metabolite in maternal circulation following the fetal infusion of radioactive T_3 (26); a similar immunoreactive product, Compound W, was found peaked at term in pregnant women disappearing 7–10 d after delivery (4,27). Kinetic studies on the PR of 3,3′-T_2S, 3′-T_1S, and TriacS would be important to quantitatively evaluate the fetal thyroid hormone metabolism and fetal-maternal transfer of thyroid hormone metabolites that may be an invaluable tool in the noninvasive detection of fetal thyroid function. The elucidation of fetal and maternal exchange of thyroid hormone and/or its metabolites may have significant clinical implications.

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