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Characterization of Iodothyronine Sulfatase Activities in Human and Rat Liver and Placenta

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In conditions associated with high serum iodothyronine sulfate concentrations, *e.g.* during fetal development, desulfation of these conjugates may be important in the regulation of thyroid hormone homeostasis. However, little is known about which sulfatases are involved in this process. Therefore, we investigated the hydrolysis of iodothyronine sulfates by homogenates of V79 cells expressing the human arylsulfatases A (ARSA), B (ARSB), or C (ARSC; steroid sulfatase), as well as tissue fractions of human and rat liver and placenta. We found that only the microsomal fraction from liver and placenta hydrolyzed iodothyronine sulfates. Among the recombinant enzymes only the endoplasmic reticulum-associated ARSC showed activity toward iodothyronine sulfates; the soluble lysosomal ARSA and ARSB were inactive. Recombinant ARSC as well as human placenta microsomes hydrolyzed iodothyronine sulfates with a substrate preference for 3,3′-diiodothyronine sulfate (3,3′-T_2S) ~ T_3 sulfate (T_3S) ≫ rT_3S ~ T_4S, whereas human and rat liver microsomes showed a preference for 3,3′-T_2S > T_3S ≫ rT_3S ~ T_4S. ARSC and the tissue microsomal sulfatases were all characterized by high apparent K_m values (>50 μM) for 3,3′-T_2S and T_3S. Iodothyronine sulfatase activity determined using 3,3′-T_2S as a substrate was much higher in human liver microsomes than in human placenta microsomes, although ARSC is expressed at higher levels in human placenta than in human liver. The ratio of estrone sulfate to T_2S hydrolysis in human liver microsomes (0.2) differed largely from that in ARSC homogenate (80) and human placenta microsomes (150). These results suggest that ARSC accounts for the relatively low iodothyronine sulfatase activity of human placenta, and that additional arylsulfatase(s) contributes to the high iodothyronine sulfatase activity in human liver. Further research is needed to identify these iodothyronine sulfatases, and to study the physiological importance of the reversible sulfation of iodothyronines in thyroid hormone metabolism. (*Endocrinology* 143: 814–819, 2002)

SULFATION IS AN important metabolic pathway that facilitates the inactivation and elimination of lipophilic exogenous and endogenous compounds, including thyroid hormones, by increasing their water solubility (1–3). A more important purpose for the sulfation of thyroid hormones is to facilitate their degradation by the type I iodothyronine deiodinase (D1) (4–7). D1 catalyzes the outer ring deiodination (activation) of T_4 to T_3 as well as the inner ring deiodination (IRD; inactivation) of T_4 to rT_3 and of T_3 to 3,3′-diiodothyronine (3,3′-T_2) (5). As IRD of sulfated T_4 and T_3 by D1 is accelerated 40- to 200-fold, whereas outer ring deiodination of T_4 sulfate (T_4S) is completely blocked (4–7), sulfation has an important role in the irreversible inactivation of thyroid hormone by D1. However, when D1 activity is low or clearance of iodothyronine sulfates is otherwise impaired, inactivation of thyroid hormone by sulfation may be reversible due to the expression of arylsulfatases in different tissues (8–10) or the presence of bacterial sulfatases in the intestine (11). Strongly elevated iodothyronine sulfate concentrations have been found in fetal and neonatal serum and in amniotic fluid in humans and sheep (12–16). Thyroid hormone is essential for the normal fetal development of several organs, in particular the brain (17–20). Therefore, it has been speculated that these iodothyronine sulfates, especially T_3S, function as a pool of inactive thyroid hormone from which the active hormone is released in a tissue-specific and development stage-dependent manner (7, 9, 12, 14, 21). Iodothyronine sulfatase activities are present in human fetal liver and lung, and become undetectable in lung after birth (22). In rats, which are born immature compared with humans, hepatic T_3S sulfatase activity progressively increases after birth until 2 months of age (23).

Hydrolysis of sulfate conjugates is an enzymatic process, and multiple arylsulfatases have now been identified (24). Arylsulfatase A (ARSA) and arylsulfatase B (ARSB) are soluble enzymes, localized in lysosomes. To date, sulfated glycolipids have been identified as endogenous substrates for ARSA, whereas ARSB has a known substrate specificity for dermatan sulfate and chondroitin sulfate (25). These sulfatases are widely distributed, although in the pig ARSA activity is 20–60 times higher in the thyroid than in other tissues (26). Arylsulfatase C (ARSC), also termed steroid sulfatase, is located in the endoplasmic reticulum, and hydrolyzes steroid sulfates such as dehydroepiandrosterone sulfate (DHEAS), estrone sulfate (E1S), and cholesterol sulfate (25). We have recently demonstrated that iodothyronines are good substrates for estrogen sulfotransferase (27). Therefore, it seems logical to assume that iodothyronine sulfates are also good substrates for the steroid sulfatase ARSC. This

Abbreviations: ARSA, Arylsulfatase A; ARSB, arylsulfatase B; ARSC, arylsulfatase C; BTP, bis-Tris propane; DHEAS, dehydroepiandrosterone sulfate; E1S, estrone sulfate; IRD, inner ring deiodination; PTU, 6-*n*-propyl-2-thiouracil; 3,3′-T_2, 3,3′-diiodothyronine.

isoenzyme is expressed in many tissues, including placenta and liver (28–31). In the placenta, ARSC plays a major role in estrogen biosynthesis from DHEAS, which is mainly produced in the fetal adrenal gland and is converted to 16α-hydroxy-DHEAS by the fetal liver (32). Recently, a group of novel ARS genes was identified, clustered on Xp22.3 (33–35), near the ARSC gene. ARSDα and -β and ARSF have been localized in the endoplasmic reticulum, whereas ARSE is located in the Golgi apparatus (35, 36). The endogenous substrates for these arylsulfatases remain to be identified, although neither ARSE nor ARSF hydrolyzes steroid sulfates (33–36). ARSD does not appear to act as a conventional arylsulfatase, as no such activity has yet been determined for the recombinant protein (34, 35). They also differ from ARSC in that they are thermolabile.

Earlier studies demonstrated T_3S sulfatase activities in human and rat liver microsomes and in rat hepatocytes (9, 21). However, not much is known about which sulfatases are responsible for the hydrolysis of sulfated iodothyronines. Therefore, we studied the arylsulfatases ARSA, ARSB, and ARSC and the sulfatase activities in human and rat liver and placenta using iodothyronine sulfates as substrates to determine whether these arylsulfatases are involved in hydrolysis of thyroid hormone sulfates in tissues.

Materials and Methods

Materials

Rat livers were isolated from adult male Wistar rats, and normal human liver was obtained at surgery for liver tumors. Normal human placental tissue was obtained at spontaneous, full-term delivery, and rat placenta was obtained after cesarean section at 20 d gestational age. Approval was obtained from institutional committees. Cytosolic and microsomal fractions of the different tissues were prepared as previously described (4, 37). Human ARSA, ARSB, and ARSC cDNA clones were provided by Prof. K. von Figura (University of Göttingen, Göttingen, Germany) and expressed in V79 Chinese hamster lung fibroblast cells as previously described (25).

T_4, rT_3, 3,3′-T_2, and 3-iodothyronine were obtained from Henning Berlin GmbH & Co. (Berlin, Germany); T_3, E1S, 6-*n*-propyl-2-thiouracil (PTU) and bis-Tris propane (BTP) were purchased from Sigma (St. Louis, MO); [3′,5′-^{125}I]T_4, [3′-^{125}I]T_3, and [^{3}H]E1S were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK); [3′,5′-^{125}I]rT_3 and [3,3′-^{125}I]T_2 were prepared by radioiodination of 3,3′-T_2 and 3-iodothyronine, respectively (5). ^{125}I-Labeled and unlabeled T_4S, rT_3S, T_3S, and 3,3′-T_2S were prepared by reaction of labeled and unlabeled T_4, rT_3, T_3, and 3,3′-T_2 with chlorosulfonic acid in dimethylformamide. They were purified by LH-20 chromatography (38).

Sulfatase assay

Iodothyronine sulfatase activity was assayed by incubation of 0.1 μM unlabeled and 100,000 cpm ^{125}I-labeled T_4S, rT_3S, T_3S, or 3,3′-T_2S and 0.1 or 1 mM PTU (to block D1 activity) for 60 min at 37 C with the indicated amounts of tissue cytosol or microsomes or V79 cell homogenate in 0.2 ml buffer. Optimal assay conditions for the different sulfatases were determined by testing different buffers (0.1 M sodium acetate, sodium citrate, Tris-HCl, sodium phosphate, or BTP-HCl), pH values, and temperatures. The reactions were started by the addition of enzyme in ice-cold buffer and were stopped by the addition of 0.8 ml 0.1 M HCl. The mixtures were analyzed for T_4, rT_3, T_3, or 3,3′-T_2 formation by chromatography on Sephadex LH-20 minicolumns as previously described (39). Desulfation in complete reaction mixtures was corrected for background radioactivity detected in the corresponding Sephadex LH-20 fractions of control incubations without enzyme.

Estrogen sulfatase activity was analyzed by incubation of 0.1 μM [^{3}H]E1S for 0 (blank) or 30 min at 37 C with the indicated amounts of tissue microsomes or V79 cell homogenate in 0.1 ml 0.1 M Tris-HCl (pH 7.2). The reactions were stopped by the addition of 0.4 ml 0.1 M Tris-HCl (pH 8.8), and the mixtures were extracted with 2.5 ml chloroform. Sulfate hydrolysis was quantified by counting 0.25 ml of the aqueous phase. The amount of E1S still present in complete reaction mixtures after 30 min at 37 C was compared with the amount of E1S present in the corresponding nonincubated reaction mixtures.

Results

Figure 1 shows the pH profiles of the desulfation of 0.1 μM 3,3′-T_2S by rat liver and human liver microsomes and recombinant human ARSC obtained using acetate and BTP-HCl buffers. Rat liver microsomal sulfatase showed an optimum at pH 6.0–6.5 (Fig. 1A), human liver microsomal sulfatase at pH 6.0–7.5 (Fig. 1B), and ARSC at approximately pH 7.0 (Fig. 1C). At neutral pH, the different enzymes showed similar sulfatase activities in BTP-HCl and Tris-HCl buffers, but much lower activities in phosphate buffer (Fig. 2A). At acidic pH values, incubations of the different enzymes, in particular human liver, in citrate buffer strongly inhibited their 3,3′-T_2S sulfatase activities compared with incubations in acetate buffer (Fig. 2B). Similar results were obtained in buffers with or without 2 mM EDTA (not shown).

Figure 3 demonstrates the effects of temperature on the desulfation of 0.1 μM 3,3′-T_2S by human and rat liver microsomes. The optimal temperature for human liver microsomes is 50 C, and that for rat liver microsomes is 70 C or higher.

Figure 4 presents the desulfation of 0.1 μM T_4S, T_3S, rT_3S, and 3,3′-T_2S by recombinant human ARSC and human and rat placenta and liver microsomes at pH 7.2. ARSC showed similar activities toward 3,3′-T_2S and T_3S, whereas both rT_3S and T_4S were poor substrates for this enzyme. The substrate specificity of human placenta microsomes was similar to that of ARSC. Very high desulfation rates were observed in human liver microsomes, with a strong substrate preference for 3,3′-T_2S, which was hydrolyzed about 4 times faster than T_3S (*i.e.* desulfation rates of ~7.2 and 1.8 pmol/min·mg) and more than 10 times faster than the relatively poor substrates rT_3S and T_4S. Rat liver microsomes also showed a substrate preference for 3,3′-T_2S, which was desulfated twice as fast as T_3S; in rat placenta microsomes low desulfation rates (*i.e.* <0.1 pmol/min·mg) were observed with all iodothyronine sulfates. We also tested steroid sulfatase activities of ARSC and of human placenta and liver microsomes. Table 1 compares E1S and T_2S sulfatase activities of ARSC and the different human tissue microsomes. The low ratio of E1S to T_2S hydrolysis in liver microsomes differs largely from the high preference for E1S *vs.* T_2S hydrolysis by ARSC and human placenta microsomes. Tested at the optimum pH 5.5, soluble ARSA and ARSB as well as rat and human liver cytosols showed very low activity toward all iodothyronine sulfates (results not shown).

Figure 5 shows the desulfation of 3,3′-T_2S by ARSC, human liver, and human placenta microsomes as a function of the substrate concentration. As no saturation was reached even at the highest concentration of 50 μM, K_m and maximum velocity values could not be calculated. Similar results were obtained when T_3S was used as substrate. Apparently all of these sulfatases have low affinity for iodothyronine sulfates, with K_m values greater than 50 μM.

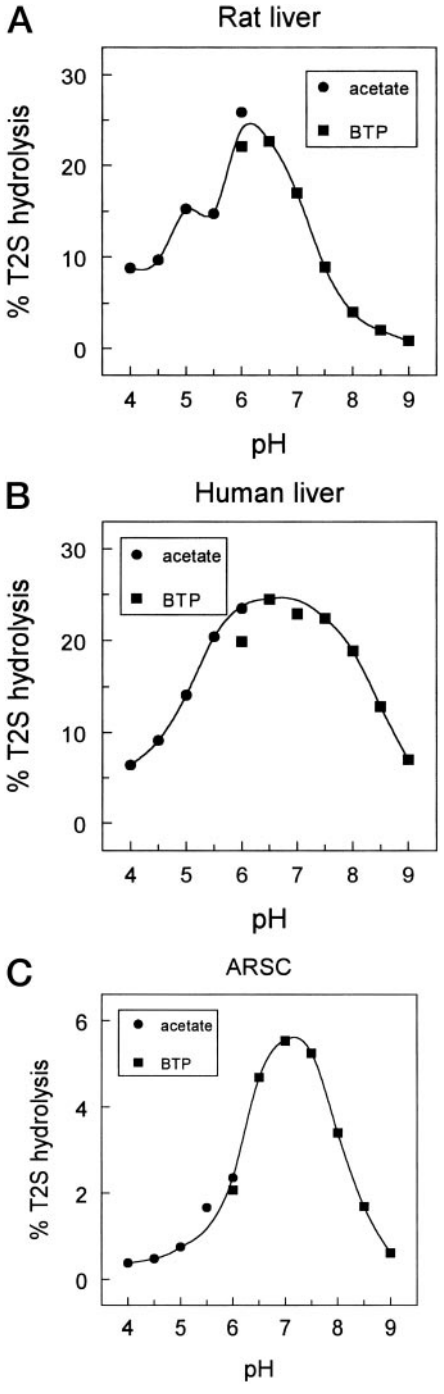

FIG. 1. Effects of pH on 3,3′-T_2S desulfation by rat liver or human liver microsomes, or ARSC. Reaction conditions were 0.1 μM ^{125}I-labeled 3,3′-T_2S, 0.25 (A and C) or 0.05 (B) mg protein/ml, 0.1 mM PTU, and 60-min incubation in 0.1 M sodium acetate or BTP-HCl. Results are the means of triplicate determinations from a representative experiment.

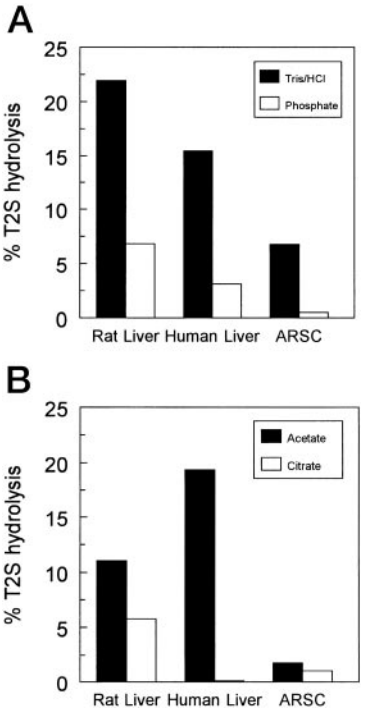

FIG. 2. Effects of buffer on 3,3′-T_2S desulfation by rat liver, and human liver microsomes, and ARSC at pH 7.2 (A) or 5.5 (B). Reaction conditions were 0.1 μM ^{125}I-labeled 3,3′-T_2S, 0.25 (rat liver and ARSC) or 0.05 (human liver) mg protein/ml, 0.1 mM PTU, and 60-min incubation in 0.1 M sodium phosphate (pH 7.2), 0.1 M Tris-HCl (pH 7.2), 0.1 M sodium acetate (pH 5.5), or 0.1 M sodium citrate (pH 5.5). Results are the means of triplicate determinations from a representative experiment.

Discussion

Normally, serum T_4S and T_3S levels are low (12, 40, 41). This is explained by the very rapid deiodination of these conjugates, as sulfation strongly induces the D1-catalyzed IRD of both T_4 and T_3 (7). However, under certain (patho)-physiological conditions, *e.g.* during fetal development and nonthyroidal illness, possibly due to diminished D1 activity, plasma concentrations of iodothyronine sulfates are increased (12, 13, 16, 41). T_3S is considered to be biologically inert, as it has lost its affinity for the T_3 receptors (42). It could, however, serve as a reservoir from which active thyroid hormone is regenerated by tissue sulfatases or bacterial sulfatases in the intestine (8–11).

Recently, much research has been performed to develop inhibitors of steroid sulfatase (ARSC) because of their potential for the treatment of estrogen-dependent breast cancers (43–54). These studies have revealed some important structure-activity relations for compounds binding to the active site of ARSC. Furthermore, the crystal structures of arylsulfatases A and B have recently been elucidated (55, 56). Although the overall amino acid sequence homology is only about 20–30% between different arylsulfatases, the protein structures of all sulfatases share some important features. The active site of eukaryotic sulfatases contains a metal ion, probably Mg^{2+} (56, 57), and a formylglycine, generated by posttranslational modification of a cysteine residue (58–61). Residues interacting with Mg^{2+} and formylglycine are conserved among the members of the sulfatase family. Uhlhorn-Dierks *et al.* (57) proposed a catalytic mechanism for the hydrolysis of sulfates by sulfatases based on their structure and mutational analyses. An intermediate enzyme-sulfate complex is formed by the covalent binding of sulfate to the hydrated formylglycine (*i.e.* dihydroxyalanine). When the active site formylglycine is replaced by a serine (*i.e.* hydroxy-

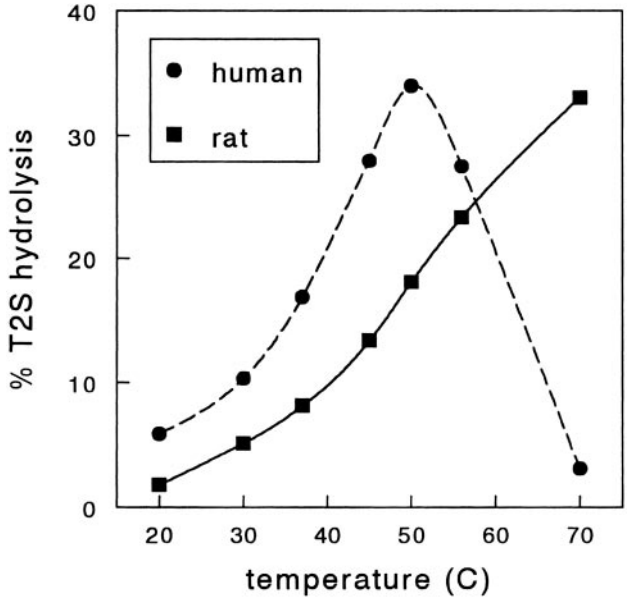

FIG. 3. Effects of temperature on desulfation of 3,3′-T_2S by human or rat liver microsomes. Reaction conditions were 0.1 μM [3,3′-^{125}I]T_2S, 0.25 (rat liver) or 0.025 (human liver) mg protein/ml, 1 mM PTU, and 60-min incubation in 0.1 M Tris-HCl (pH 7.2) at 20–70 C. Results are the means of two closely agreeing experiments.

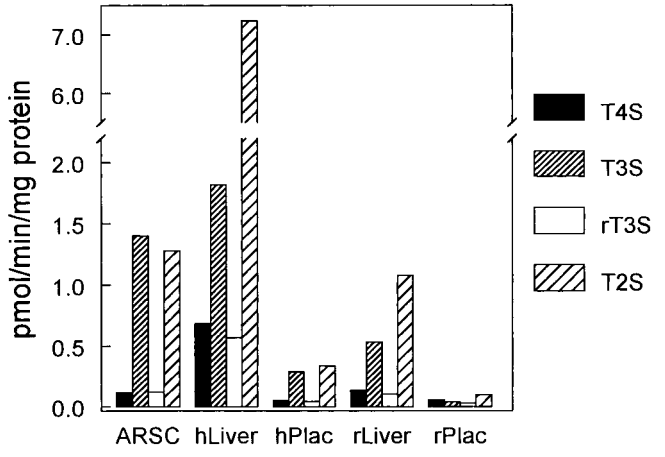

FIG. 4. Desulfation of iodothyronine sulfates by ARSC, human or rat liver microsomes, or human or rat placenta microsomes. Reaction conditions were 0.1 μM ^{125}I-labeled T_4S, T_3S, rT_3S, or 3,3′-T_2S; 0.25 (ARSC and rat liver), 0.05 (human liver), or 0.5 (human and rat placenta) mg protein/ml; 0.1 mM PTU; and 60-min incubation in 0.1 M Tris-HCl (pH 7.2). Results are the means of triplicate determinations from a representative experiment.

alanine), the intermediate enzyme-sulfate complex is trapped (62), which indicates that the second hydroxyl group of hydrated formylglycine is needed for sulfate release (57, 62).

Crystallographic analyses (55, 56, 63) also revealed structural homology between alkaline phosphatases and arylsulfatases. A functional relationship between the enzymes was shown by O'Brien *et al.* (64), who demonstrated that alkaline phosphatase exhibits a low level of sulfatase activity. They also showed inhibition of the phosphatase as well as the sulfatase activities of alkaline phosphatase by inorganic phosphate (64). Anderson *et al.* reported on steroidal and nonsteroidal phosphates that inhibited steroid sulfatase activity (43). We demonstrated inhibition of the iodothyronine sulfatase activities by inorganic phosphate and citrate. Perhaps these anions block the active site of iodothyronine sulfatases. An alternative explanation is that citrate and phosphate inhibit iodothyronine sulfatase activity by complexing the enzyme-bound Mg^{2+}. However, we did not observe inhibition of iodothyronine sulfatase activity in the presence of EDTA. Our results strongly suggest that iodothyronine sulfatase activities determined in phosphate buffers, as reported by others (9, 23), represent a marked underestimation of true enzyme levels.

TABLE 1. Hydrolysis of E1S and 3,3′-T2S by human ARSC, liver and placenta

Enzyme source	Hydrolysis		
	E1S	3,3′-T_2S	E1S/3,3′-T_2S
	(pmol/min·mg)		
ARSC-V79 homogenate	100	1.3	81
Human liver microsomes	1.6	7.2	0.21
Human placenta microsomes	52	0.34	150

Reaction conditions were: 0.1 μM 3,[3′-^{125}I]T_2S, 0.1 mM PTU and 0.25 (ARSC), 0.05 (liver) or 0.5 (placenta) mg protein/ml, or 0.1 μM [^{3}H]E1S and 0.005 (ARSC), 0.5 (liver) or 0.01 (placenta) mg protein/ml, and 60-min incubation in 0.1 M Tris-HCl (pH 7.2). Results are the means of triplicate determinations from a representative experiment.

Whereas significant sulfatase activity toward 3,3′-T_2S and T_3S was found in human placenta, in rat placenta activity toward these iodothyronine sulfates was much lower. This difference in activity may be due to the species difference. It should be noted, however, that whereas the rat placental tissue used in this study consisted of both the fetal as well as the maternal side of the placenta, the human placental tissue mainly consisted of the fetal side of the placenta, as most of the maternal side (decidua) is not expelled at spontaneous delivery. Furthermore, the rat placenta was isolated at embryonic d 20, *i.e.* 1 d before birth, whereas human placenta was obtained at full-term delivery. Therefore, besides the species difference, these differences in tissue composition and developmental stage may also contribute to the different sulfatase activities found in human and rat placenta.

We showed a pH optimum for rat liver microsomes at pH 6.0–6.5, for human liver microsomes at pH 6.0–7.5, and for arylsulfatase C at pH 7.0. The broader peak for the human and rat liver microsomes might indicate that different sulfatases, with different pH optima, are involved in the desulfation of 3,3′-T_2. Kung *et al.* (9) observed T_3S sulfatase activities in human and rat liver microsomes. E1S and DHEAS, both substrates for ARSC, inhibited T_3S hydrolysis with IC_{50} values of approximately 10 μM. The fact that high levels of E1S only partially inhibited T_3S desulfation, whereas high DHEAS concentrations produced complete inhibition, supports the involvement of multiple sulfatases, possibly including ARSC (9). However, these analyses were performed in phosphate buffer (9), which may strongly affect the contributions of different sulfatases.

The optimal temperature of iodothyronine sulfatase activities is 50 C in human liver microsomes and at least 70 C in rat liver microsomes. The high thermostability of these sulfatases is in agreement with the temperature optimum of 60 C for ARSC (65). ARSC and the sulfatase activities in

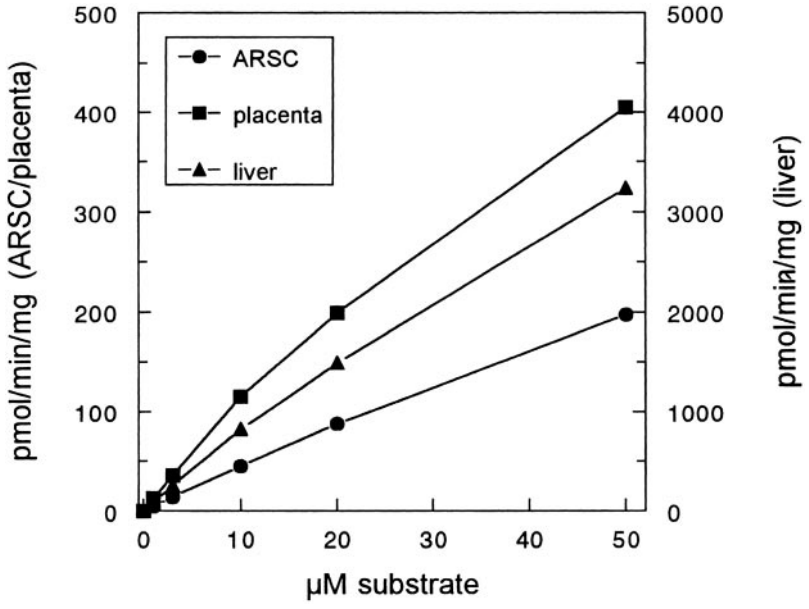

FIG. 5. Effects of substrate concentration on the desulfation of 3,3′-T_2S by ARSC, human placenta, or human liver microsomes. Reaction conditions were 1–50 μM [3,3′-^{125}I]T_2S; 0.05 (liver), 0.25 (ARSC), or 0.5 (placenta) mg protein/ml; 1 mM PTU; and 60-min incubation in 0.1 M Tris-HCl (pH 7.2). Results are the means of triplicate determinations from a representative experiment.

human liver and placenta microsomes have high K_m values for iodothyronine sulfates. The different substrate specificities of the iodothyronine sulfatase activity in human liver *vs.* ARSC and placenta as well as the finding that the ratio of E1S to T_2S hydrolysis in human liver differs greatly from that in ARSC and placenta suggest that in human liver additional sulfatases to ARSC contribute to the hydrolysis of iodothyronine sulfates, in particular T_2S. However, ARSD, ARSE, and ARSF are not likely candidates, as 1) both ARSE and ARSF are thermolabile, whereas iodothyronine sulfatase activity appears thermostable (33, 34); and 2) ARSD does not possess arylsulfatase activity (34, 35).

It is remarkable that although 3,3′-T_2 is the preferred substrate for sulfotransferases, 3,3′-T_2S is the preferred substrate for (human liver) sulfatase. Thus, reversible sulfation/desulfation seems a more important metabolic pathway for 3,3′-T_2 than for T_4, T_3, and rT_3. This may reflect restrictions in the active sites of the sulfotransferase and sulfatase to accommodate bulky substrates with more than two iodine substituents. However, a physiological role for 3,3′-T_2 is not excluded. Although its affinity for the nuclear thyroid hormone receptors is low (17), 3,3′-T_2 has been shown to stimulate mitochondrial respiration in different tissues (66).

In conclusion, we have identified arylsulfatase C as a high K_m iodothyronine sulfatase that is most likely the main enzyme responsible for the hydrolysis of iodothyronine sulfates in human placenta and to some extent in human liver. Further investigations are needed to determine the possible importance of other, still unidentified, microsomal sulfatases in hydrolysis of iodothyronine sulfates in the liver and perhaps other tissues. This information may contribute to the understanding of the role of sulfation-desulfation in the regulation of thyroid hormone bioactivity, in particular during fetal development.

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