THYROID HORMONE METABOLISM: IMPORTANCE OF DEIODINATION, CONJUGATION AND SIDE CHAIN MODIFICATION

Schildklierhormoon metabolisme: het belang van dejodering, conjugatie en zijketen modificatie

PROEFSCHRIFT

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Aan mijn ouders
In herinnering aan Tante

Uit 'Voetstappen in de tijd' (Jan H. de Groot)

Woorden dragen een verraderlijk gevoel
soms met driedubbele betekenissen
daarom hoor je telkens: 'ik bedoel'
uit vrees de juiste bedoeling te missen

Hier betuig ik mijn oprechte dank aan een ieder
die, direct of indirect, heeft bijgedragen
aan de totstandkoming van dit proefschrift.

DANK JULLIE WEL!

CONTENTS

LIST OF ABBREVIATIONS

Ac	N-acetyl
BHDB	butyl 4-hydroxy-3,5-diiodobenzoate
BrAc	N-bromoacetyl
DTT	dithiothreitol
EHC	enterohepatic circulation
G	glucuronide
GSH	reduced glutathione
HPLC	high performance liquid chromatography
IC_{50}	concentration giving half-maximum inhibition
ID	intestine-decontaminated
IRD	inner ring deiodination
IOP	iopanoic acid
iv	intravenous(ly)
K_i	inhibition constant
K_m	Michaelis constant
NS	sulfamate (i.e. N-sulfonate)
ORD	outer ring deiodination
PTU	6-propyl-2-thiouracil
RIA	radioimmunoassay
rT_3	reverse triiodothyronine (3,3',5'-triiodothyronine)
S	sulfate (i.e. O-sulfonate)
T_0	thyronine
T_1	monoiodothyronine
T_2	diiodothyronine
T_3	3,3',5-triiodothyronine
T_4	thyroxine (3,3',5,5'-tetraiodothyronine)
TA_0	thyroacetic acid
TA_1	monoiodothyroacetic acid
TA_2	diiodothyroacetic acid (diac)
TA_3	3,3',5-triiodothyroacetic acid (triac)
TA_4	3,3',5,5'-tetraiodothyroacetic acid (tetrac)
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin (transthyretin)
TSH	thyroid-stimulating hormone
UDP	uridine diphosphate
UDPGT	UDP-glucuronyltransferase
V_{max}	maximum velocity

Chapter I

INTRODUCTION

1 GENERAL INTRODUCTION

Thyroid hormones, chemically known as iodothyronines, are synthesized in the follicular cells of the thyroid gland (15,179). Reduced levels of circulating thyroid hormones induce the release of thyroid-stimulating hormone (TSH) from the thyrotrophic cells of the pituitary gland which in turn accelerates the many reactions leading to increased thyroid hormone production. The sequence of events resulting in secretion of iodothyronines into the circulation are 1) concentration of iodide by the thyroid gland, 2) iodination of tyrosyl residues of thyroglobulin, 3) coupling of mono- and diiodotyrosine residues with formation of iodothyronines, and 4) subsequent proteolytic digestion of the thyroglobulin with liberation of iodothyronines (15,179).

In healthy humans, the main secretory product of the thyroid gland is 3,3',5,5'-tetraiodothyronine (thyroxine, T_4), which has little intrinsic bioactivity and is generally regarded as a prohormone (57,84). The thyroid produces less than 20 % of circulating 3,3',5-triiodothyronine (T_3), which is the bioactive hormone, while the majority of T_3 is generated outside the thyroid by monodeiodination of the phenolic ring of T_4 (Fig. 1; Refs. 86,117,192,193). Likewise, other naturally occuring iodothyronines are produced by deiodination of T_4 and T_3 in peripheral tissues, besides negligible amounts secreted by the thyroid.

Thyroid hormones play key roles in the regulation of the differentiation processes in vertebrates, notable examples of which are the development of the brain and the metamorphosis of tadpoles. Its regulation of metabolic processes involved in temperature adaptation and calorigenesis has evolutionarily been a later phenomena (26,78). T_3 exerts its effect at the transcriptional level by binding to nuclear receptors (136-138,170, 176). These have been identified as the products of c-erbA proto-oncogenes, which control expression of thyroid hormone responsive-genes (58,135,203). The encoded proteins are involved in regulation of vital functions such as cellular differentiation and growth as well as energy consumption. In contrast to T_3, other natural iodothyronines, including T_4, show little activity at the receptor level. Therefore, the hormonal effects of T_4 *in vivo* are largely due to its conversion to T_3.

In humans, over 99 % of circulating T_4 and T_3 is bound to three plasma proteins, $\approx$75 % to thyroxine-binding globulin (TBG), $\approx$15 % to thyroxine-binding prealbumin (TBPA) and $\approx$10 % to albumin (26,30,85,160). The former is the least abundant but possesses the highest iodothyronine affinity. Among these circulating iodothyronine-binding proteins TBPA may play an essential role in tissue thyroid hormone supply (142). Species such as rat, dog, and sheep possess little TBG and their major plasma carrier for iodothyronines is TBPA.

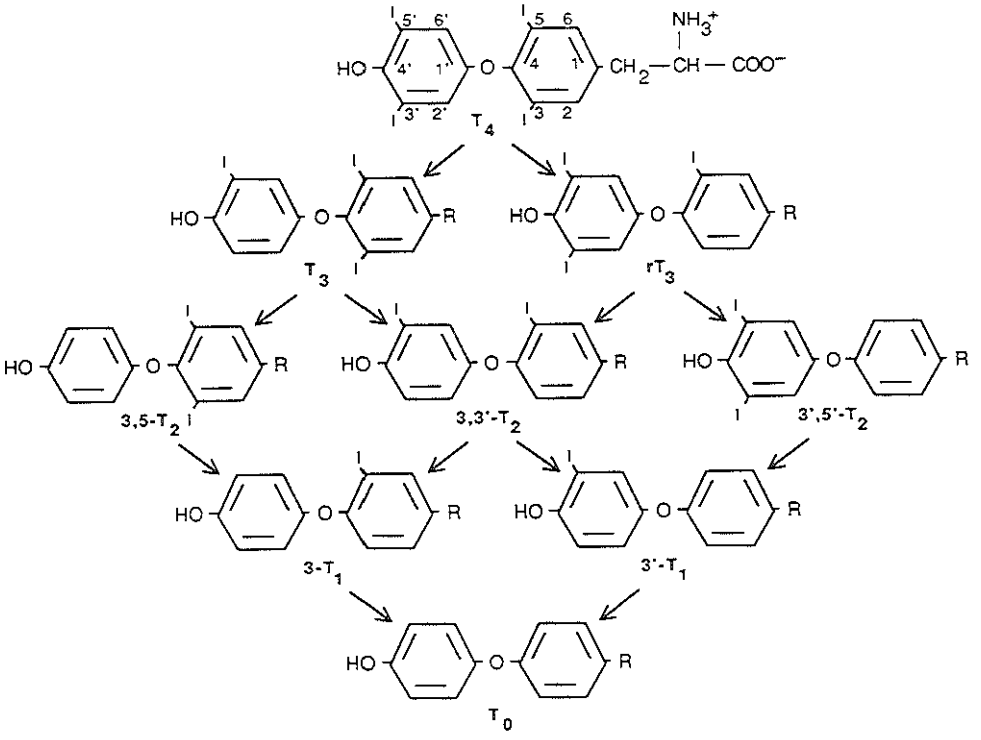

Figure 1. **Sequential deiodination of thyroxine (T_4)**

Although iodothyronines are hydrophobic molecules, they do not enter tissue cells by simple diffusion. Cellular uptake of iodothyronines is an active, saturable process mediated by specific, sodium- and energy-dependent carrier systems located in the cell membrane (49,108,146). Furthermore, it appears that T_4 and T_3 have separate transporters, although they can competitively inhibit each others uptake, at least in rat liver cells *in vitro*. As only nonprotein-bound thyroid hormone is taken up, the free iodothyronine concentration in serum ultimately determines hormone delivery to the tissue cells and is, therefore, a major determinant of T_4 and T_3 bioavailability (85,141). In addition, transfer of T_3 from the cytoplasm to the nucleus may also be a specific and energy-dependent process, determining the hormone levels at the receptor site (176).

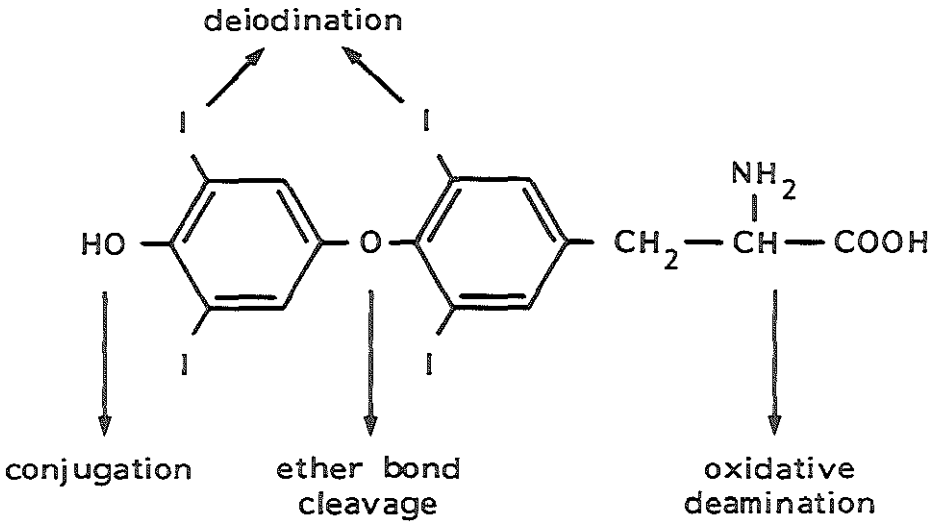

Figure 2. Metabolism of thyroxine

All physiological processes that influence intracellular T_3 levels have a role in control of hormonal activity (37,76,83,191). Apart from thyroidal secretion regulated by TSH, and the exchange of T_4 and T_3 between tissues and plasma, the bioavailability of T_3 depends on the intracellular iodothyronine metabolism (Fig. 2). The next section deals with the major pathways, that is 1) removal of iodine substituents by deiodination, 2) conjugation of the phenolic hydroxyl group of the iodothyronines with glucuronic acid or sulfate and, 3) oxidative deamination and decarboxylation of the alanine side chain (16,57,86,106,197). Cleavage of the diphenyl ether bond (Fig. 2) is negligible under normal conditions (57) and is therefore not further discussed. Apart from the conversion of T_4 to T_3, all other pathways lead to inactivation of thyroid hormone. The contribution of the various metabolic routes to the daily iodothyronine turnover highly depends on the pathophysiological conditions (36,57,83,197) and the use of certain drugs (23,24,32). Normally, the metabolic pathways lead to irreversible clearance of the generated metabolites from the body with feces and urine. However, glucuronidation is a reversible process which represents a first step in the enterohepatic circulation of T_4 and T_3 (197)

2 METABOLIC PATHWAYS OF IODOTHYRONINES

2.1 Oxidative deamination and decarboxylation

Transformation of the alanine side chain of an iodothyronine to the corresponding iodothyroacetic acid derivative is a distinct, but relatively minor pathway in the normal iodothyronine metabolism. *In vitro*, oxidative deamination and decarboxylation of thyroid hormones have been demonstrated in homogenates of mammalian liver, kidney and brain (57,70,182,183). The reaction sequence was elucidated in preparations of rat kidney mitochondria and the first step consists of oxidative deamination of the alanine side chain to the pyruvic acid analog; the latter is then converted by decarboxylation to the acetealdehyde, which is subsequently oxidized to the acetic acid derivative ($RCH_2CH(NH_2)COOH \longrightarrow RCH_2COCOOH \longrightarrow RCH_2CHO \longrightarrow RCH_2COOH$; Ref. 183).

Iodothyroacetic acids as well as their conjugates have been observed in tissues and body fluids of experimental animals (16,18,63,68,70,153,154,156,157,177) as well as in humans after equilibration with radioiodide or injection with radiolabeled T_4, T_3 or 3,3'-T_2 (19,22,129,145). Interestingly, 3,3',5-triiodothyroacetic acid (TA_3) binds more firmly to the nuclear receptor than T_3 but expresses less thyromimetic activity (31,96,138). 3,3',5,5'-Tetraiodothyroacetic acid (TA_4) and TA_3 have higher affinities for TBPA than T_4 and T_3, respectively, but lower affinities for TBG (2,26).

In healthy humans, a daily production rate of 1.6 nmol TA_4 accounts for only 2 % of total T_4 turnover, and negligible amounts of TA_4 (<3 %) are converted to TA_3 (22,145). Administration of [^{14}C]-T_4 to humans has demonstrated a daily urinary excretion of 30 nmol thyroacetic acid (TA_0) (144), which clearly indicates the physiological significance of the acetic acid pathway in the metabolism of especially the lower iodinated thyronines (22). Moreover, at least 14 % of T_3 metabolism can be ascribed to side chain modification in athyreotic, T_3-substituted humans, in which a daily production rate of approximately 17 nmol TA_3 was determined (72). Levels of TA_3 in human plasma vary from 0.03 to 0.24 nM (72,133), which are in the same range as the most reliable measurements of TA_4 (35,147).

Iodothyroacetic acids are metabolized via the same deiodination and conjugation pathways as their parent iodothyronines, giving rise to a variety of iodothyroacetic acid derivatives (16,57). Their metabolic degradation by deiodination is discussed in Chapter IX.1, while the interrelation of deiodination and sulfation of these compounds is further outlined in Chapter IX, sections 2.2 and 3.2.

2.2 Deiodination

Cascade of successive deiodinations (Fig. 1)

The crucial reaction in the regulation of thyroid hormone expression is the transformation of the prohormone T_4 to bioactive T_3. This concerns the elimination of an iodide from the phenolic ring, called outer ring deiodination (ORD), which is regarded as an activating step. In contrast, deiodination of the tyrosyl ring of T_4 is regarded as an inactivating step, because it destroys the potential thyromimetic activity of the prohormone. This inner ring deiodination (IRD) of T_4 yields 3,3',5'-triiodothyronine (reverse T_3, rT_3), a compound without hormonal activity. For reviews, see references 86, 106, 117, 193.

Approximately 115 nmol T_4 and 45 nmol of each T_3 and rT_3 are produced daily in healthy adults. The unique source of T_4 production is the thyroid, which secretes only about 9 nmol T_3 and 2 nmol rT_3 each day. This implicates that most T_3 and virtually all rT_3 are produced by extrathyroidal conversion of T_4. The sum of these deiodinations account for the majority of the T_4 disposal in humans (57,84). Like T_4, T_3 is inactivated by IRD; this yields 3,3'-diiodothyronine (3,3'-T_2), the same metabolite as produced by ORD of rT_3. The other diiodothyronines 3,5-T_2 and 3',5'-T_2 are minor metabolites. Further stepwise deiodination of these diiodothyronines results, via the T_1 intermediates, ultimately in the formation of T_0. It has been reported that in humans only 44 % of the disposal of the triiodothyronines is accounted for by monodeiodination and, therefore, alternative pathways equally contribute to their metabolic clearance (22,57,197).

Rats, equilibrated with radioiodinated T_4 excrete 50-60 % of the administered radioactivity as iodide in urine (126,184). In humans as much as 85 % of administered radioiodinated T_4 is ultimately recovered as urinary radioiodide (57,114), indicating that deiodination of iodothyronines is a more important pathway in humans than in rats (114,193).

Iodothyronine deiodinases

In vitro studies of thyroid hormone metabolism, predominantly in rat tissues, have resulted in the discovery of at least three different iodothyronine-deiodinating enzymes. Extensive reviews of the characteristics of the iodothyronine deiodinases have been recently published (86,106,117,118,192,196). These deiodinases have in common that they are membrane-associated proteins, located in the tissue microsomal fractions. Furthermore, in the absence of cytosol they all require sulfhydryl compounds as cofactor for their catalytic activity, such as low-molecular weight thiols. The synthetic dithiol, dithiothreitol (DTT), is one of the most potent cofactors *in vitro*. The enzymes are discriminated on the basis of their different 1) iodothyronine substrate specificity, 2) tissue distribution, 3) catalytic mechanism and extent of cofactor requirement, 4) susceptibility to enzyme inhibitors, and 5) regulation by the thyroid status.

The so-called type I iodothyronine deiodinase is most abundant in the endoplasmic reticulum of liver, the plasma membrane of kidney, and membrane fraction of the thyroid. It is a nonselective enzyme, because it is capable of deiodinating the inner ring as well as the outer ring of iodothyronines. It is generally believed that in healthy individuals the type I enzyme of liver - and kidney - is largely responsible for both the production of plasma T_3 as well as the clearance of plasma rT_3 (117,197).

The type II iodothyronine deiodinase acts only on the outer ring, as demonstrated by its conversion of T_4 to T_3 and of rT_3 to 3,3'-T_2. It is present in pituitary, brown adipose tissue and the central nervous system, especially in the neurons of cerebral cortex and cerebellum. Within these tissues, the type II enzyme is important for the local supply of T_3 (98). Furthermore, in case of hypothyroidism, T_3 production via type II conversion of T_4 may become an important source of circulating T_3 (175).

The type III enzyme is a true inner ring deiodinase, and catalyzes conversion of T_4 to rT_3 and of T_3 to 3,3'-T_2. In the central nervous system it is mainly localized in the glial cells of the cerebral cortex, and it is also found in placenta, skin, fetal rat intestine and chick embryo liver. According to recent data, some type III-like enzyme activity also apparently exists in adult rat liver (56,197). Brain and skin may be the major tissues for the extrathyroidal rT_3 production from T_4 and for clearance of plasma T_3 in adults.

The activities of the three deiodinases are differently affected by the thyroid status, providing an optimal regulation of intracellular T_3 within critical tissues, such as the central nervous system (99,106,117,193). This thesis deals primarily with the iodothyronine type I deiodinase, the key enzyme in plasma T_3 production. The type I enzyme of rat liver and kidney has been best characterized (86,106,118,196). In addition, type I deiodinase activity has been identified in human thyroid (91), liver (79,194) and kidney (17,205) and found to resemble the rat enzyme with regard to catalytic mechanism and substrate specificity. The structure of the type I deiodinase of rat liver has only recently been elucidated, showing that it contains a selenocysteine residue (see below; Ref. 13).

Reaction mechanism

The type I deiodination of the inner as well as the outer ring of iodothyroinines follows 'ping-pong' reaction kinetics (117). This means that substrate and cofactor react with different interconvertable forms of the enzyme. The identification of an essential selenocysteine residue in the catalytic centre of the type I deiodinase explains earlier observations of impaired enzyme activity in Se-deficient rats (3,10). Therefore, the previously proposed mechanism of the enzymatic reaction involving an essential sulfhydryl group, is accordingly adjusted (Fig. 3). The selenol (SeH) group of the native enzyme reacts with the iodothyronine substrate by accepting an iodonium ion (I^+). The generated enzyme-selenenyl iodide intermediate (E-SeI) is reduced with cofactor to E-SeH (199).

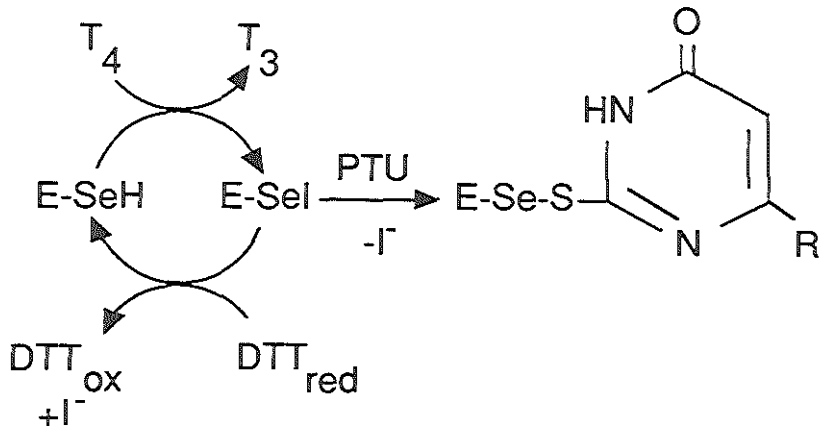

Figure 3. **Mechanism of type I iodothyronine deiodinase**

The physiological cofactor(s) of the various deiodinases is still unknown (196). The potent reductant dihydrolipoamide seems insignificant as a natural cofactor because its intracellular concentration is negligible. Although reduced glutathione (GSH) is the most abundant thiol present in liver and other tissues, it hardly stimulates microsomal deiodinase activity, even in the presence of NADPH and glutathione reductase. However, the stimulation of type I deiodination is greatly enhanced by the combination of GSH with glutaredoxin, a natural polypeptide dithiol. In comparison, the analogous thioredoxin system shows little potency in stimulating deiodination rates *in vitro*. Therefore, glutaredoxin in combination with GSH appears to be the major endogenous cofactor of type I deiodinase (196).

Substrate specificity

Reverse T_3 is the preferred substrate for the type I iodothyronine deiodinase known sofar, as its conversion to 3,3'-T_2 is roughly three orders of magnitude more efficient than the deiodinations of T_4 and T_3. This is especially due to its high affinity for the type I enzyme, since the apparent K_m value of rT_3 (0.06 μM) is markedly lower than the K_m values (>2 μM) of the other iodothyronines (117,193). In homogenates or microsomes of rat liver as well as in isolated rat hepatocytes, T_4 undergoes both IRD and ORD but no significant rT_3 accumulation is observed, because it is instantaneously deiodinated in the outer ring to 3,3'-T_2. In contrast, T_3 is relatively stable in these systems (86,193,194,197). Thus, little rT_3, produced by type I deiodination of T_4 in tissues such

as liver and kidney *in vivo*, will escape into the circulation due to its rapid deiodinative breakdown.

Many naturally occuring and synthetic iodothyronine derivatives are deiodinated by the type I enzyme. Some of these are even better substrates than the corresponding iodothyronines (22,104,198). As discussed in detail in Chapter IX, type I deiodination is markedly stimulated by sulfate conjugation of the phenolic hydroxyl group and, although to a smaller extent, by modifications that produce a net negative charge in the alanine side chain.

Type I deiodinase inhibitors

For reviews, the reader is referred to references 86, 106, 117, 192, 196.

Substrate competitors. A wide variety of competitive inhibitors of the type I deiodination of iodothyronines are known, usually all aromatic substances with halogen substituents in the ortho position to hydroxyl or amino groups.

1) The inhibition by iodothyronines derivatives, which are alternative substrates, is independent of whether or not they undergo the same deiodination (IRD or ORD) as the substrate under study. This inhibition is characterized by identical K_m and K_i values.
2) Competitive inhibitors, other than alternative substrates, comprise:
   a) substances isolated from plants, such as coumarin compounds and polyaromatic flavenoids with K_i values in the μM range;
   b) many of the widely used dyes and acid-base indicators, such as the weak inhibitor phenol red. Bromophenol blue, the food-coloring agent erythrosine, and rose-bengal are potent inhibitors ($K_i \approx 0.05$ μM);
   c) the X-ray contrast agents iopanoic acid (IOP) and ipodate, which belong to the group of simple iodinated phenols and aniline derivatives. IOP inhibits the type I deiodinase as well as type II and III, and induces profound changes in peripheral thyroid hormone metabolism.

Protein reagents. Covalent modification of the type I deiodinase with certain amino-acid selective reagents results in a loss of enzyme activity.

1) Iodoacetate is one of the most potent reagents for nucleophiles such as SH or SeH groups. In addition, the CO_2^- group of iodoacetate may interact with basic residues of the enzyme active centre, which explains why it is a ten-fold more potent inhibitor than iodoacetamide. This kind of enzyme inactivation is competively prevented by substrates or competitive inhibitors which protect the active centre. The most potent inactivators of the type I enzyme are N-bromoacetyl-iodothyronines. For instance, N-bromoacetyl-T_3 ($BrAcT_3$) has an apparent K_i of 0.1 nM, i.e roughly 10^5 times lower than the apparent K_m of T_3. $BrAcT_3$ irreversibly inactivates the enzyme by covalent attachment to a functional group in the active centre (see below).

2) 6-Propyl-2-thiouracil (PTU) and methimazole are thiourea derivatives which are widely applied to treat hyperthyroidism, because they block thyroid hormone biosynthesis by inhibition of the thyroid peroxidase. PTU - but not methimazole - additionally blocks the type I deiodinase, presumably due to reaction with the enzyme intermediate E-SeI. Formation of a stable enzyme-PTU selenosulfide prevents the regeneration of native enzyme (Fig. 3). Enzyme inactivation by PTU is competitively prevented by cofactor. Further, deiodinase activity is restored by reaction of the enzyme-PTU complex with excess DTT (117).

Properties of the type I iodothyronine deiodinase

The type I iodothyronine deiodinase is a cytoplasm-oriented transmembrane protein with an apparent total molecular weight of 50-60 kDa (101,118,169,192,196). A 27 kDa protein of rat liver and kidney microsomes is identified as the functional deiodinase or a subunit by affinity-labeling with BrAc[^{125}I]T_3, -T_4 or -rT_3 (see above; Refs. 12,107,172) as well as by ^{75}Se-protein labeling (3).

The type I enzyme is a basic protein, with a pI value of 9.3 in the delipidated state (124). Although purification, using conventional biochemical techniques, have resulted in a 2400-fold increase in specific enzyme activity (125), the deiodinase has still not been completely purified (12,169). Very recently, Berry *et al.* (13) have identified the complete nucleotide and deduced amino acid sequence of the type I deiodinase, by expressing rat liver cDNA transcripts in Xenopus oocytes and screening for rT_3 deiodinase activity. They discovered the presence of a selenocysteine in the active site, a rare amino-acid in mammalian enzymes. The reported sequence corresponds to a basic protein of 29.5 kDa.

It is speculated that, like iodoacetate (see above), BrAcT_3 also reacts with this essential selenocysteine. Possible other targets for BrAcT_3 at the active enzyme centre are the amino group of lysine or the imidazole of histidine, the latter essential for deiodinase activity (104,118,122,192,196). Incorporation studies using radiolabeled BrAcT_3 (122) or iodoacetate (116) have yielded values for the type I deiodinase content of rat liver and kidney microsomes of approximately 2.5 pmol per mg protein. The enzyme, therefore, represents about 0.01 % of total microsomal protein in these tissues (125). Hopefully, the recent cloning of the type I deiodinase will result in the large scale production of pure (and cristalline) enzyme in order to deduce its three-dimensional structure and its exact catalytic mechanism.

2.3 Conjugation

The second important pathway in the metabolism of thyroid hormones is conjugation of the phenolic hydroxyl group (4'-OH) with glucuronic acid or sulfate (Figs. 2 and 4; reviews in Refs. 22,197,198). This so-called phase II reaction couples functional groups

such as OH, COOH, NH_2 and SH with glucuronic acid or sulfate, which increases the water-solubility of lipophilic substances and thus facilitates their excretion in bile and urine (51,77,102,127). For this, specific enzymes have evolved in response to the need to eliminate potentially toxic endobiotics and environmentally produced xenobiotics (181).

Glucuronidation

Glucuronidation is catalyzed by UDP-glucuronyltransferases (UDPGTs), a family of closely related enzymes, located in the endoplasmic reticulum of especially the liver but also in kidney, intestine and other tissues (21,51,180,181). The cofactor UDP-glucuronic acid is the donor of the sugar moiety and is generated by oxidation of UDP-glucose, which is produced by reaction of UTP and ubiquitous glucose-1-phosphate. Neither in man nor in the rat, the identity and substrate specificity of the UDPGTs for iodothyronine derivatives have been established (197), although separate isozymes appear to be involved for T_4 and T_3. The latter is strongly suggested by very recent findings in rat liver (11), showing that glucuronidation of T_3, but not T_4, is closely correlated with androsterone UDPGT, while glucuronidation of T_4 seems to be catalyzed by both phenol and bilirubin UDPGTs (see below).

Sulfation

Sulfate conjugation of various phenolic substances is performed by phenol sulfotransferases, a group of homologous enzymes present in the cytosolic fraction of predominantly the liver, kidney, small intestine, brain as well as platelets (127). The universal sulfate donor is 3'-phosphoadenosine-5'-phosphosulfate (PAPS), which requires 2 ATP molecules and inorganic sulfate for its synthesis (127,128). The type I and II phenol sulfotransferases identified in rats (92) resemble the more recently characterized 'thermostable' phenol sulfotransferase in human (206), having in common that they are sensitive to inhibition by 2,6-dichloro-4-nitrophenol (DCNP). This thermostable form is very effective in sulfation of 'simple' phenols, but displays little activity towards catecholamines. These latter are, amongst others, substrates for the rat type IV and human 'thermolabile', DCNP-resistant phenol sulfotransferases.

With respect to iodothyronines, Sekura *et al.* (173) demonstrated that the efficiency of sulfation of 3'-T_1 and 3,3'-T_2 was roughly 28 times higher than that of T_3 using partially purified rat liver phenol sulfotransferases, but was nearly negligible for rT_3 and T_4. These investigators also reported that sulfation of the naturally occuring deaminated analogs of T_3, such as TA_3, was 7-9 times more rapid than for T_3. Recent studies of Young *et al.* (206) provided strong evidence, that the 'thermostable' phenol sulfotransferase in the cytosol of human liver cells, is most important for the sulfation of T_3, and probably for other iodothyronines as well. These data are in agreement with those reported by De Herder *et al.* (40), showing the involvement of a DCNP-sensitive, T_3

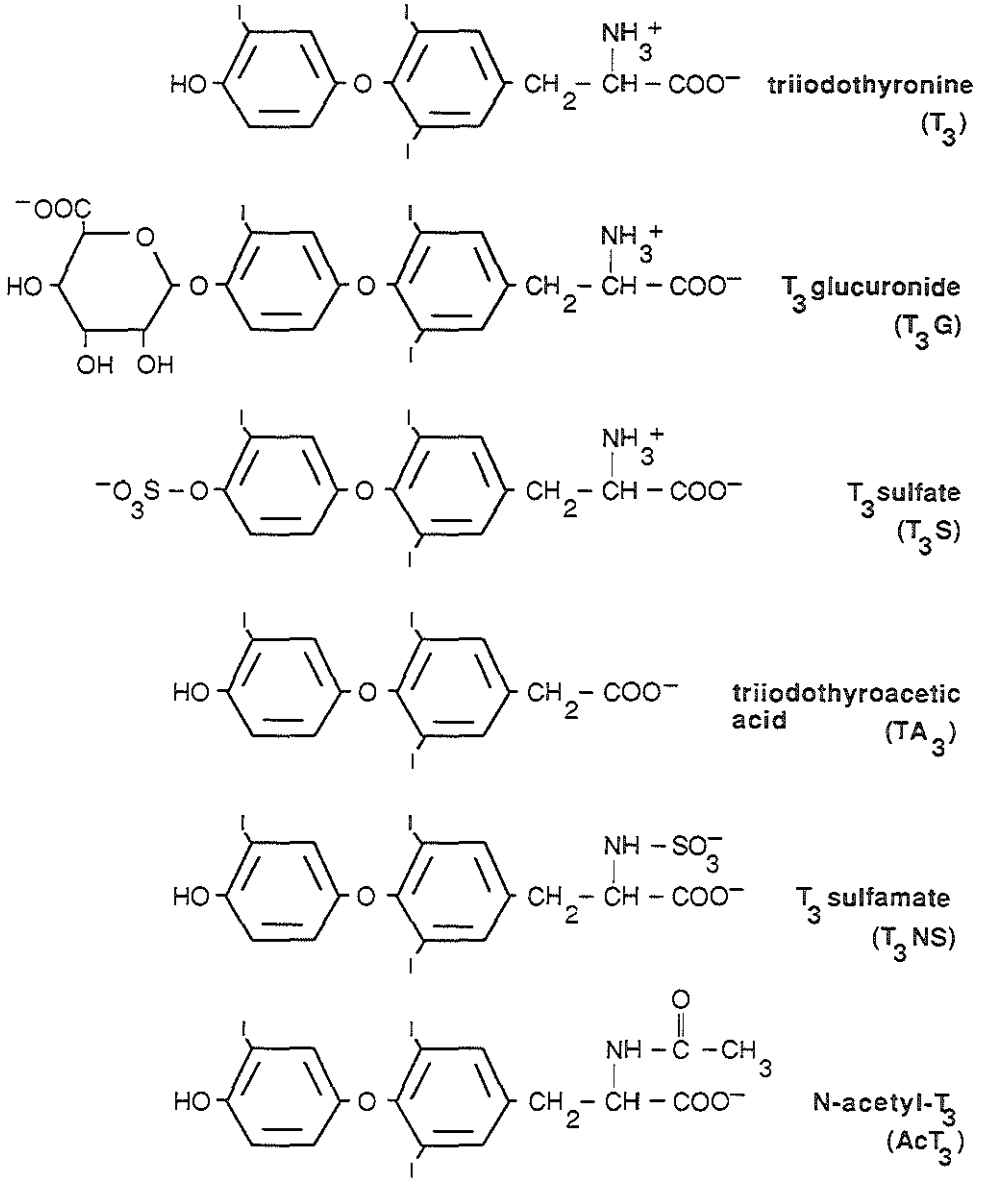

Figure 4. Structures of T_3 and derivatives

phenol sulfotransferase in rats.

Factors affecting conjugation

Normally, the plasma SO_4^{2-} level is not rate-limiting for *in vivo* sulfation (197,204). In contrast to starvation, protein-deficient diets that lower the intake of SH-containing amino acids, being SO_4^{2-} precursors, diminish the hepatic sulfation capacity (73). The latter also holds if generation of the cofactor PAPS is impaired due to a fructose-induced decrease in tissue ATP levels (128).

Glucuronidation is apparently dependent on glycogen-derived glucose-1-phosphate levels. Diminution of UDP-glucuronic acid supply, for instance induced by fasting or fructose treatment, will therefore inhibit glucuronidation (51,197). In general, expression of various UDPGTs is enhanced by microsomal enzyme inducers, which can also stimulate the glucuronidation of iodothyronines in rats (8). Such inducers are found amongst environmental pollutants, like benzpyrene (74) and polychlorobiphenyls (7), as well as drugs like barbiturates. This is probably also how antiepileptic drugs, for instance carbamazepine, lead to an increased metabolic clearance rate of thyroid hormones (197). UDPGT activities of liver microsomes isolated from Wistar rats treated with 3,3',4,4'-tetrachlorobiphenyl are significantly increased for both T_4 and p-nitrophenol, but are normal for T_3 and androsterone. This suggest the existence of different isozymes for T_4 and T_3 glucuronidation. This hypothesis is supported by the selective increase in the biliary excretion of T_4G, but not T_3G, following treatment with 3,3',4,4'-tetrachlorobiphenyl (11), which has also been documented for 2,3,7,8-tetrachlorodibenzo-p-dioxin (9) and nafenopin (97). A selective increase of the metabolic clearance of T_4 over T_3 in case of hexachlorobenzene intoxicated rats, points to a similar phenomenon (103). Selective induction of T_4 glucuronidation together with bilirubin UDPGT has also been observed in Fischer rats treated with ciprofibrate (200).

Phenol sulfotransferases and UDPGTs may compete for a common substrate. Sulfation is usually more important at low substrate levels, due to the high substrate affinity but low capacity of the sulfotransferases compared with the much lower affinity but higher capacity of the UDPGTs (11,51,127). Therefore, glucuronidation usually predominates at increased substrate availability. Cofactor supply from the cytosol, however, may become rate-limiting at high substrate levels, as the active center of a UDPGT is located on the lumenal surface of the endoplasmic reticulum (181,197). Administration of a high dose of, for instance, acetaminophen or salicylamide, which undergo extensive conjugation, could theoretically limit the conjugation of iodothyronines due to depletion of required cofactor (52,56,197).

It should be mentioned, that ether is known to deplete tissue UDP-glucuronic acid levels (202) and, therefore, observations of glucuronidation in ether-anesthetized animals should be interpreted with caution.

Occurence of iodothyronine conjugates

In general, administration to rats, dogs ands humans of radioiodinated T_4 or T_3 gives rise to a variety of conjugates appearing predominantly in the bile, but also in plasma and urine depending upon the experimental or pathophysiological conditions (for reviews, see Refs. 16,36,43,57,197,198). This section highlights some major findings concerning iodothyronine conjugation in rats and humans. For reasons of clarity, findings in dogs are not included although thyroid hormone and its derivatives are even more extensively sulfated in this species than in rats (16,33,62,64).

Bile. Since the early fifties, glucuronides of T_4, rT_3, T_3 and 3,3'-T_2 as well as sulfates of T_4, T_3, and 3,3'-T_2 have been demonstrated in bile of several animal species and humans (16,40,69,81,90,113,129,132,152,157,177,178,198).

Studies in normal rats, equilibrated with radioiodinated T_4 or T_3 have shown that at least 50 % of the administered radioactivity is ultimately excreted in urine as iodide, while the other half is cleared as 'free' iodothyronines with feces (45,126). The latter are likely derived from biliary-excreted conjugates, which are hydrolysed during intestinal transit (see below). Appearance of nonconjugated forms in the bile is negligible if low doses of iodothyronines are administered (40,111,178).

The biliary clearance rate of plasma T_3 is much more rapid than that of T_4 as deduced after iv injection of the radioiodinated hormone, and summarized in Table 1. Thyroid hormones are disposed with bile predominantly as glucuronides, while only small amounts of their sulfate conjugates are excreted because these normally undergo rapid hepatic deiodination. However, elimination of biliary iodothyronine sulfates is highly stimulated if the type I deiodination pathway is diminished, which is discussed in detail in Chapter IX, section 3.

Quantitative information regarding the biliary clearance of T_4 and T_3 in humans is limited, but glucuronidation seems less important than in rats (60,69,113,129,130,197).

Urine. Normally, conjugated iodothyronines are not excreted in urine of rats (16,45,46) or humans, although hypo- and hyperthyroid subjects may excrete some conjugates (89). Furthermore, as hepatectomized rats do excrete T_4G in their urine following iv administration of radioiodinated T_4, it is clear that other tissues than liver are able to conjugate iodothyronines (16,61,62,64).

Plasma. Iodothyronine glucuronides do not occur in plasma of normal rats (46,54), although conflicting results have been reported (81), and only minor amounts of T_3S have been detected in plasma of normal rats and humans (16,28,54,55,64). Following hepatectomy or ligation of the common bile duct of rats, however, T_4G and T_3G are present in the circulation together with considerable amounts of T_3S (16,29,64,149-152).

Table 1. Biliary excretion of thyroid hormones in rats

Hormone	% of injected dose excreted in bile	Major product	% of biliary radioactivity	Reference
iv T_4	20 (0-24 h)	T_4G	43	65,67,178
	10 (0- 6 h)	T_4G	42	65
	15 (0- 8 h)	T_4G	47	166
iv T_3	33 (0-24 h)	T_3G	45	64,67
	22 (0- 4 h)	T_3G	74	40

See also references 16 and 36.

An extraordinary accumulation of [^{125}I]T_3G in plasma of [^{125}I]T_3-injected, mutant TR$^-$ rats was recently demonstrated, which is explained by their defective hepatobiliary excretion of organic anions (see below; Ref. 42). Likewise, T_4G appears in plasma of patients suffering from obstructive biliary disease (188).

Iodothyronine glucuronidation and biliary clearance in mutant rats

In the Gunn rat, a mutant Wistar rat with hereditary jaundice (21,197), T_4 glucuronidation is impaired because of defective bilirubin and p-nitrophenol UDPGT activity, resulting in an 80 % decrease of biliary T_4G disposal after T_4 administration (6,16,68). In addition, these Gunn rats show a 60 % decrease in biliary T_3G clearance (16). However, direct measurement of T_4 and T_3 glucuronidation by liver microsomes has shown that T_4 UDPGT activity is decreased, while T_3 UDPGT activity is normal in Gunn rats (T.J. Visser, unpublished observations). The diminished metabolic clearance of T_3 in these Gunn rats may therefore be indirectly due to the disturbed bilirubin clearance.

As first shown by Matsui and Hakozaki (119), a genetic defect in androsterone UDPGT is frequently observed in Wistar rats. In these mutant animals, the hepatic glucuronidation of T_3, but not T_4, is significantly lower than in normal Wistar rats, expressing high androsterone UDPGT activity (11). This suggests that androsterone and T_3 are glucuronidated by a common UDPGT or by different isozymes encoded by a single gene (11). Consistent observations have been made in Fischer rats, which show a constitutive defect in androsterone UDPTG associated with low T_3 UDPGT activity and almost "normal" T_4 UDPGT activity (200).

Mutant TR$^-$ Wistar rats have an impaired hepatobiliary excretion pathway for organic

anions such as bilirubin, but not for bile acids, resembling the human Dubin-Johnson syndrome. This autosomal, recessive defect causes conjugated hyperbilirubinemia (93,94) which coincides with, amongst others, a diminished biliary clearance of T_3 (42). These animals can bearly excrete T_3G with bile, whereas biliary excretion of T_3S seems less severly disturbed (42).

Occurence of conjugates of iodothyroacetic acids

The metabolic clearance of iodothyroacetic acids closely resembles that of the corresponding iodothyronines as they also undergo conjugation of the 4'-OH group with sulfate and glucuronic acid (16,22,66). A limited number of papers have shown that iv administered TA_4 or TA_3 is excreted predominantly as the glucuronide in bile of rats and dogs (16,66-68,115) as well as humans (75). TA_3S is only observed in bile of rats with diminished type I activity (67,115,154).

In hepatectomized animals, iodothyroacetic acid sulfates and glucuronides appear in urine and some also in plasma, which demonstrates that conjugation of these iodothyronine analogs can occur in other tissues than the liver (16). This is furter illustrated by the production of TA_3S in addition to TA_3 during T_3 perfusion of the dog kidney *in vitro* (66,159). However, no extrahepatic sulfation of TA_4 was observed (66).

Intestinal hydrolysis of conjugates and fecal excretion of thyroid hormones

Various radiolabeled iodothyronine conjugates are completely hydrolysed in incubations with fecal suspensions (38,43). Recently, several strains of obligately anaerobic bacteria have been isolated from the microflora of rat and human, which can effectively deconjugate iodothyronine glucuronides and sulfates (43,82). After biliary clearance of iodothyronines as conjugates, they normally appear in feces in the nonconjugated form, due to the abundance of these bacterial hydrolases in the intestines of normal rats and humans (77,87,100). Although a direct mesenteric secretion of T_3 and T_4 from the blood to the intestinal lumen is not excluded (43,46,48), it does not appear to contribute significantly to iodothyronine disposal with feces (131).

Various *in vivo* experiments have shown effective intestinal deconjugation in intact rats, since they excrete only 'free' iodothyronines in feces after administration of T_3 conjugates by gastric tube (41), or after iv injection of T_3, which is excreted largely in the bile as T_3G (41,45). Oral treatment of rats with antibiotics drastically diminishes the intestinal microflora as confirmed by negligible numbers of obligately anaerobic bacteria present in cultures of fecal dilutions (5,41,59,82). As a consequence, the hydrolytic capacity is effectively abolished in these intestine-decontaminated (ID) rats. This results in an increased fecal loss with both abovementioned routes of T_3 administration (41).

The ß-glucuronidases and arylsulfatases involved are ubiquitous enzymes, not only in bacteria but also in plants and animals. They are associated with the lysosomal and

microsomal fraction of various tissues, although their physiological relevance is unknown (51,127,134,161,201). In contrast to arylsulfatase, mammalian ß-glucuronidase activity is found in saliva, bile, intestinal juice and mucosa cells of the proximal intestinal tract. The bacterial glucuronidase activity, however, is located in the distal part of the gut.

Under certain extreme experimental conditions, desulfation can take place outside the intestine. For instance, 24 h after administration of T_3S to bile-diverted rats with suppressed type I deiodinase activity, some T_3G is excreted in bile (158). Although T_3S can be hydrolysed by microsomal sulfatase activity of rat and human liver at supraphysiological T_3S concentration, this is unlikely to occur *in vivo* due to the rapid deiodinative clearance of T_3S in normal liver (56,197).

Enterohepatic circulation of iodothyronines

Iodothyronines excreted as conjugates with the bile are not definitely removed from the body, but may undergo enterohepatic circulation (EHC). Because conjugated compounds are in general poorly absorbed by the rat and human intestine (50,51,127), hydrolysis of the iodothyronine conjugates during intestinal transit is a prerequisite for resorption of the 'free' hormones in the portal blood (41,82). Part of the resorbed hormone is extracted by the liver and may start another enterohepatic cycle. However, a proportion of the liberated iodothyronines may escape this EHC, either by entering the systemic circulation or by fecal excretion (43,46,112,195,197).

Apart from various endogenous compounds (50,59,102,109,186), some food additives, drugs and pollutants are also engaged in an EHC (77,148), resulting in a prolonged body residence time. The enteral absorption of iodothyronines occurs apparently in the proximal part of the colon (80,112) like other compounds undergoing EHC (77,148), which is understandable in the light of the abundance of, especially, cecal bacteria (82,87).

3 OUTLINE OF THE THESIS

Extrathyroidal deiodination of T_4 is the most important route for production of bioactive T_3. This occurs predominantly in the liver, catalyzed by the microsomal type I deiodinase, which is also responsible for most of the degradation of the inactive rT_3. The liver is furthermore essential for the conjugation of iodothyronines with sulfate and glucuronic acid. This thesis deals with the connection between the various biochemical pathways of thyroid hormone metabolism. A better understanding of those metabolic routes involved in the regulation of T_3 bioavailability helps to clarify the changes in the thyroid hormone economy induced by pathophysiological conditions. The successive Chapters II-VIII roughly reflect the chronological order of my experimental studies.

The initial investigations were focused on the mechanism underlying the accelerated type I deiodination of iodothyronines due to sulfate conjugation. Therefore, these 4'-0-sulfonated iodothyronines were compared with N-sulfonated iodothyronines (Fig. 4) as substrates for the type I deiodinase of rat liver, to evaluate the importance of the site of sulfonation for efficient deiodination (Chapter II).

To further elucidate the structural requirements for optimal type I deiodination, we analyzed the impact of different iodothyronine side chain modifications with different effects on charge distribution and size. Besides the synthetic N-sulfonated and N-acetylated iodothyronines (Chapter II), we studied in more detail the naturally occurring iodothyroacetic acids, which also have a negatively charged side chain (Chapter IV). Their substrate behavior was compared with that of unmodified iodothyronines and their 4'-sulfated derivatives, possessing the native alanine side chain with the original zwitter ionic moiety (Fig. 4). The effects of sulfation on the type I deiodination of the iodothyroacetic acids TA_3 and 3,3'-TA_2 has also been investigated (Chapter IV). Because TA_3S appeared to be an extreme good type I substrate, extensive studies of the metabolic pathways of TA_3, including glucuronidation, were performed in cultured rat hepatocytes as well as in rats *in vivo* (Chapter V).

Meanwhile, the relevance of sulfation for the *in vivo* metabolism of thyroid hormones was further investigated. First, we analyzed plasma T_3S in PTU-treated rats and evaluated its role in the T_3 metabolism as described in Chapter III and elsewhere (40,54).

Over the years, HPLC methods were developed to analyze the various iodothyronine derivatives (Chapters II-V). This enabled us to identify 3,3'-TA_2S as an important metabolite of T_3 in rats treated with deiodinase inhibitors (40,53,54). Chapter VI is devoted to the identification of this compound and the speculations concerning its origin.

The final investigations of the importance of iodothyronine conjugation, especially sulfation, were confined to the biliary excretion of iodothyronine metabolites in rats. The studies with T_4 (Chapter VII) are focussed on the occurence and biological relevance of T_4S formation, which had not been quantified previously. Chapter VII further concerns the complex metabolism of T_4 and deals with the changes in biliary T_4 metabolites induced by PTU as well as prolonged anesthesia. Knowing that biliary-excreted iodothyronine conjugates undergo bacterial hydrolysis in the intestine, the study described in Chapter VIII deals with the fate of biliary T_3 conjugates, especially T_3G. Reabsorption of T_3 after intestinal hydrolysis of its conjugates was estimated to investigate the putative enterohepatic circulation of T_3 in rats (also Ref. 41).

Chapter IX reviews the interdependency of the major pathways of thyroid hormone metabolism.

Chapter II

DEIODINATION OF IODOTHYRONINE SULFAMATES BY TYPE I IODOTHYRONINE DEIODINASE OF RAT LIVER

Deiodination of Iodothyronine Sulfamates by Type I Iodothyronine Deiodinase of Rat Liver*

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ABSTRACT. The substrate behavior of synthetic *N*-sulfonated iodothyronines (iodothyronine sulfamates, T_4NS) for the type I deiodinase was compared with that of the naturally occurring 4′-*O*-sulfonated iodothyronines (iodothyronine sulfates, T4S), which have been shown to be deiodinated 40–200 times more efficiently than the native iodothyronines. Deiodination was studied in incubations of rat liver microsomes with unlabeled or 3′ (5′)-^{125}I-labeled T_4NS, rT_3NS, T_3NS, and 3,3′-T_2NS at 37 C and pH 7.2 in the presence of 5 mM dithiothreitol. Reaction products were analyzed by RIA or Sephadex LH-20 and HPLC. Kinetic studies were performed under initial reaction rate conditions to determine the apparent Michaelis Menten (K_m) constants and maximum velocity values. In contrast to T_4S, which is converted only by inner ring deiodination (IRD), T_4NS underwent both IRD and outer ring deiodination (ORD), similar to T_4, but more rapidly. At 10 nM T_4NS substrate, T_3NS was the major product observed, while no rT_3NS accumulated due to its rapid conversion to 3,3′-T_2NS. At least one third of the 3,3′-T_2NS was converted by IRD, unlike 3,3′-T_2 which is a pure ORD substrate. The type I deiodination efficiencies of T_4NS IRD and ORD were 17-fold higher than with T_4, mainly due to approximately 22-fold lower apparent K_m values. Deiodination of rT_3, the preferred type I substrate, was not improved by sulfamation. T_3NS and 3,3′-T_2NS were deiodinated 4–10 times more efficiently than T_3 and 3,3′-T_2, respectively, due to 2- to 4-fold decreases in apparent K_m values with a concomitant doubling of maximum velocity values. *N*-Sulfonation stimulates type I deiodination to a similar extent as other side-chain modifications that eliminate the positive charge of the nitrogen (*e.g.* iodothyroacetic acids). However, the effects are less dramatic than those induced by 4′-sulfation with respect to both efficiency and specificity of the catalytic process. (*Endocrinology* **129**: 1375–1381, 1991)

IN NORMAL humans, the thyroid is the unique source of T_4, but it secretes only about 20% of all T_3 (1). Most circulating T_3 is generated by outer ring deiodination (ORD), also called 5′-deiodination, of the prohormone T_4. Both T_4 and T_3 are inactivated by inner ring deiodination (IRD), also called 5-deiodination, resulting in rT_3 and 3,3′-diiodothyronine (3,3′-T_2), respectively (1–3). It is now well established that the type I iodothyronine deiodinase catalyzes the IRD and ORD of different iodothyronines (2–4). This enzyme is primarily associated with the microsomal fractions of liver, kidney, and thyroid, and rT_3 is the preferred substrate (2–5). In normal subjects, the type I deiodinase of liver (and kidney) is largely responsible for the production of plasma T_3 and the clearance of plasma rT_3. The enzyme, therefore, has an important role in the regulation of thyroid hormone bioactivity (6, 7).

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The type I deiodinase also acts on modified iodothyronine derivatives. The naturally occurring iodothyronine sulfates (1, 8, 9) and iodothyroacetic acid analogs (10–12) are even better substrates than the parent iodothyronines. Previous studies from our laboratory have shown that deiodination efficiency is increased at least 40 times by 4′-sulfation of iodothyronines, except for rT_3, for which deiodination is already optimal (2, 5, 9). Substitution of the alanine side-chain of T_3 and 3,3′-T_2 with acetic acid, resulting in 3,3′,5-triiodothyroacetic acid (TA_3) and 3,3′-diiodothyroacetic acid (3,3′-TA_2), enhances deiodination efficiency 16- and 3-fold, respectively (12). The IRD of TA_3 and the ORD of 3,3′-TA_2 are further stimulated about 50 times by 4′-sulfation, and TA_3S is the best known substrate for IRD due to its very high affinity for the type I deiodinase (12).

Iodothyronines and their 4′-sulfate conjugates have a zwitterionic alanine side-chain, in contrast to the iodothyroacetic acid analogs which possess a negatively charged side-chain. To further elucidate the structural requirements for efficient type I deiodination, additional enzyme kinetic studies were performed with iodothyronine sulfamates that have a *N*-sulfonated side-chain with a double negative charge. We compared the substrate behavior of synthetic sulfamates with that of the 4′-sulfated iodo-

thyronines as well as derivatives with other side-chain modifications (9, 12).

Materials and Methods

Chemicals

Iodothyronines were obtained from Henning Berlin GmbH (Berlin, Germany). [3′,5′-^{125}I]T_4 was purchased from Amersham (Amersham, Aylesbury, Buckinghamshire, United Kingdom; SA, ~1160 Ci/mmmol). Outer ring ^{125}I-labeled rT_3, T_3, and 3,3′-T_2 (~1700 Ci/mmol) were prepared in our laboratory using the chloramine-T method (Visser, T. J., and M. Rutgers, unpublished work). Unlabeled or ^{125}I-labeled iodothyronine sulfamates were prepared according to the method of Mol and Visser (13). All products were purified on Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and purity was checked by reverse phase HPLC (13–15). Free I^- was eliminated from [^{125}I]rT_3, [^{125}I]T_4, and their sulfamates on Sephadex LH-20 immediately before each experiment. All other reagents were of analytical grade.

Microsomal deiodinase assay

Rat liver microsomes were prepared as described previously (5, 16). The method of Bradford (17) was used to determine protein concentrations, with BSA as standard.

Deiodinase assay mixtures contained various concentrations of microsomal protein with unlabeled or 3′,5′-^{125}I-labeled substrate (~20 nCi) in 200 μl 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA, and 5 mM dithiothreitol (DTT). Incubations were carried out in duplicate or triplicate for different time periods at 37 C. Reactions were stopped by the addition of 200 μl 0.2 M NaOH, and the resultant mixtures were stored at −20 C until analysis. In control incubations, microsomes were added after the addition of NaOH.

Analysis of samples

The radioactive mixtures were completed with 0.4 ml 0.5 M HCl and 0.2 ml ethanol, and subsequently applied to Sephadex LH-20 columns (0.9-ml bed volume) equilibrated in 0.1 M HCl. Iodide and iodothyronine derivatives were separated by successive elution with 0.1 M HCl and 0.1 M ammonia in ethanol, as described previously (12), with 97% and 96% recovery, respectively.

Deiodination products from unlabeled substrate were measured in duplicate in the 0.1-M NaOH extracts of the reaction mixtures by specific RIAs (5, 16, 18). rT_3 sulfamate (rT_3NS), T_3 sulfamate (T_3NS), and 3,3′-T_2 sulfamate (3,3′-T_2NS) were determined using antisera for the corresponding iodothyronine and the proper sulfamate standards. The cross-reactivities of rT_3NS, T_3NS, and 3,3′-T_2NS with these antisera amounted to 189%, 114%, and 105%, respectively.

Reverse phase HPLC

Deiodinase assay mixtures with radioactive iodothyronine sulfamates were prepurified on Sephadex LH-20 (see above). The ammoniacal ethanol fractions were pooled and dried under a stream of N_2 at 50 C. The residues were reconstituted in HPLC mobile phase and injected onto a Chromspher C_{18} column (10 × 0.3 cm; Chrompack International BV, Middelburg, The Netherlands). A Waters HPLC system (Waters, Milford, MA) (14, 15) was used, and isocratic elution was performed with mixtures of 42–44% (vol/vol) methanol in 0.02 M ammonium acetate (pH 4) at a flow of 0.8 ml/min. Columns were calibrated with unlabeled synthetic reference compounds by monitoring the eluate absorbance at 254 nm. Radioactive samples were analyzed by collecting 0.3-min fractions for γ-counting. The recovery of applied radioactivity amounted to 92.5 ± 4.1% (mean ± SD; n = 34).

Data analysis

Iodide production was corrected for nonenzymatic release of I^- in the control incubations, which amounted to an average of 4% for T_4 (T_4NS), 2% for rT_3 (rT_3NS), and 0.3% for both T_3 (T_3NS) and 3,3′-T_2 (3,3′-T_2NS). To account for random deiodination at the 3′ or 5′ position of T_4, T_4NS, rT_3, and rT_3NS, I^- production from these substrates was multiplied by 2. Corrections were made for the slight cross-reactivities (<1%) of the substrates in the RIAs of the various deiodination products.

Double reciprocal plots of deiodination rate *vs.* substrate concentration were drawn by unweighted linear regression analysis. Maximum velocity (V_{max}) and K_m values were determined from the intercepts with the ordinate and abscissa, respectively (19).

All data are given as the mean ± SD (unless the SD is smaller than the figure symbol).

Results

The stepwise deiodination of T_4NS by rat liver microsomes was studied in parallel with that of T_4. Products from unlabeled T_4NS or T_4 were determined by RIA, and $^{125}I^-$ release from [3′,5′-^{125}I]T_4NS or [3′,5′-^{125}I]T_4 was quantified by Sephadex LH-20 chromatography. The *upper part* of Fig. 1 shows the reaction time and microsomal protein dependence of the conversion of 10 nM T_4NS. The major metabolite, T_3NS, was generated at about twice the rate of 3,3′-T_2NS. Production of rT_3NS was undetectable. Radioiodide release was equal to the sum of T_3NS and 3,3′-T_2NS generation. Product formations were roughly linear with microsomal protein up to 50 μg/ml and with incubation time up to 30 min.

T_3NS was also the major metabolite produced in incubations with 1 μM T_4NS, as illustrated in the *lower right panel* of Fig. 1. However, under these conditions considerable amounts of rT_3NS accumulated, which increased with the microsomal protein concentration up to 100 μg/ml. At lower protein concentrations, little 3,3′-T_2NS formation was detected, but more than proportional increments in 3,3′-T_2NS production were observed at increasing protein levels. A similar sequence of rT_3NS and 3,3′-T_2NS formation was observed when deiodination of 1 μM T_4NS was studied as a function of incubation time (not shown), suggesting that 3,3′-T_2NS was generated largely via deiodination of rT_3NS.

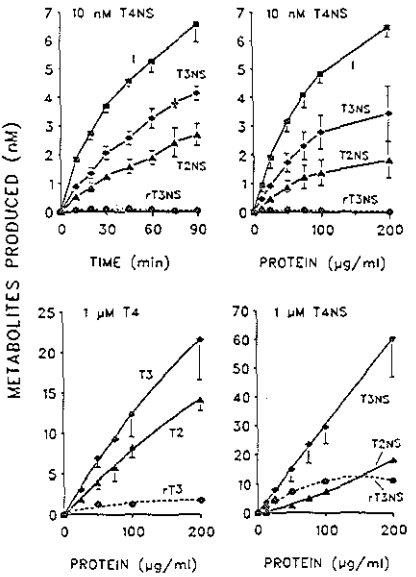

FIG. 1. Deiodination of T_4 and T_4NS as a function of enzyme concentration or reaction time. *Upper panels*, T_4NS (10 nM) was incubated for different time periods with 50 µg microsomal protein/ml (*left*) or for 20 min with varying microsomal protein concentrations (*right*). *Lower panels*, T_4 (1 µM; *left*) or T_4NS (1 µM; *right*) was incubated for 20 min with varying microsomal protein concentrations. Products from unlabeled substrates were estimated in three separate experiments by specific RIAs. The generation of radioiodide from [^{125}I]T_4NS was quantified on Sephadex LH-20.

The *lower left panel* of Fig. 1 illustrates the deiodination of 1 µM T_4 in relation to microsomal protein concentration. The production of T_3 exceeded that of 3,3′-T_2, and only little rT_3 accumulated, resembling the relative proportions of metabolites observed in incubations with 10 nM T_4NS. Comparison of their metabolism at equimolar (1 µM) substrate concentrations shows that conversion of T_4NS proceeded more rapidly than that of T_4. As previously demonstrated (16, 18), negligible amounts of rT_3 accumulate in incubations of T_4 with rat liver type I deiodinase, due to the very rapid ORD of rT_3 to 3,3′-T_2. The similar rapid deiodinative breakdown of rT_3NS (see below) explains the significant accumulation of 3,3′-T_2NS in the absence of detectable rT_3NS formation at low T_4NS substrate concentrations. Also, in the incubation with 1 µM T_4 or T_4NS, radioiodide production was equal to the sum of T_3 (T_3NS) plus 3,3′-T_2 (3,3′-T_2NS). This indicates that under these conditions the latter metabolites do not undergo significant further deiodination.

To fully comprehend the effect of sulfamation on the stepwise T_4 deiodination, T_3NS, rT_3NS, and 3,3′-T_2NS were tested as substrates for the type I deiodinase in relation to enzyme concentration and incubation time, and the results were compared with those obtained using the corresponding iodothyronines. It was found that, like rT_3, rT_3NS was very rapidly deiodinated, *e.g.* 10 nM rT_3NS was quantitatively converted to radioiodide and 3,3′-T_2NS within 20 min at 12.5 µg microsomal protein/ml.

Incubations of 1 µM T_3NS or T_3 with increasing microsomal protein concentrations (Fig. 2) clearly showed that the IRD of T_3NS proceeded much more rapidly than that of T_3, because at least 5 times more 3,3′-T_2NS than 3,3′-T_2 was produced. These data obtained by RIA were confirmed by HPLC analysis of radiolabeled metabolites in incubations with either 1 µM or 10 nM 3′-^{125}I-labeled T_3NS or T_3 (not shown). HPLC revealed, furthermore, the generation of small amounts of 3′-T_1NS in addition to the 3,3′-T_2NS.

Figure 3 shows an example of the HPLC analysis of the deiodination of 1 µM 3,[3′-^{125}I]T_2NS at different times of incubation. Apart from the major product radioiodide, considerable amounts of 3′-T_1NS were generated, but HPLC failed to detect any 3′-T_1 formation in incubations with 3,[3′-^{125}I]T_2 (not shown). Figure 4 shows the deiodination of 10 nM 3,3′-T_2NS and 3,3′-T_2 as a function of microsomal protein concentration. Similar proportions of I^- and 3′-T_1NS were produced from 1

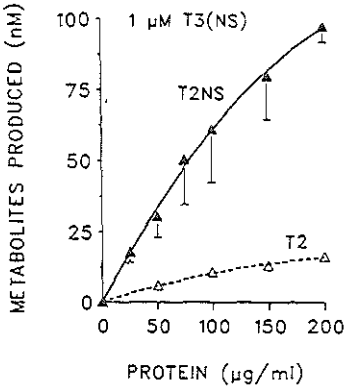

FIG. 2. Deiodination of 1 µM T_3 (△) and T_3NS (▲) by rat liver microsomes as a function of enzyme concentration. The reaction time was 30 min. Production of 3,3′-T_2 (two experiments) and 3,3′-T_2NS (five experiments) were determined by RIA. Coefficients of variation between the two experiments with T_3 amounted to less than 5–16% at the different protein concentrations.

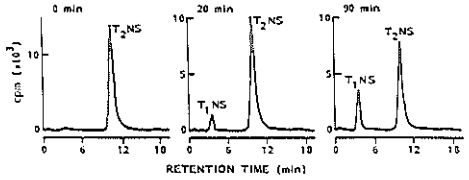

FIG. 3. HPLC analysis of the deiodination of 1 μM 3,[3′-^{125}I]T_2NS incubated for 20 (*middle*) and 90 (*right*) min with 75 μg microsomal protein/ml. Reaction mixtures were prepurified on Sephadex LH-20, resulting in elimination of 14% (20 min) and 28% (90 min) of the ^{125}I. The isolated sulfamates were separated by C_{18} HPLC, using 42% methanol in ammonium acetate (pH 4) as the mobile phase (see *Materials and Methods*).

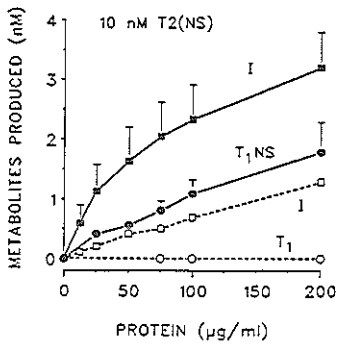

FIG. 4. Deiodination of 10 nM 3,[3′-^{125}I]T_2 (□ and ○) and 3,[3′-^{125}I]T_2NS (▣ and ●) with varying microsomal protein concentrations. Data are derived from three experiments; the incubation time was 30 min. Production of [3′-^{125}I]T_1 and [3′-^{125}I]T_1NS was measured by HPLC after prepurification of the assay mixtures on Sephadex LH-20 (see Fig. 3).

μM 3,3′-T_2NS (not shown). Sulfamation of 3,3′-T_2 greatly increased the rate of ORD, as indicated by the 4 times greater release of radioiodide from 3,3′-T_2NS than from 3,3′-T_2. Even more dramatic was the stimulation of IRD, considering the significant production of 3′-T_1NS compared with the absence of 3′-T_1 formation.

Finally, the kinetic parameters of the type I deiodination of iodothyronine sulfamates were determined under initial reaction rate conditions, in parallel with those of the corresponding iodothyronines. These conditions were chosen such that 1) product formation increased linearly with incubation time and enzyme concentration, and 2) less than 20% of the substrate was consumed during the reaction. Kinetics of T_4NS IRD were analyzed by summation of rT_3NS and 3,3′-T_2NS formation (Fig. 5, A and

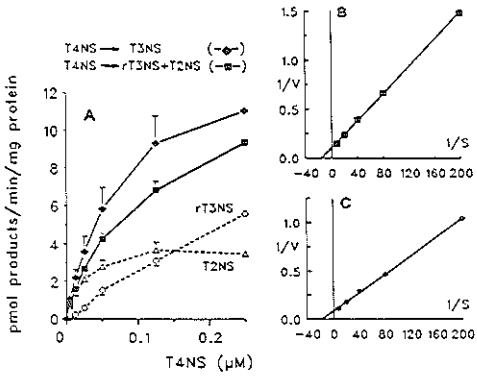

FIG. 5. Deiodination of T_4NS by rat liver microsomes as a function of substrate concentration. T_4NS (0.005–0.25 μM) was incubated for 20 min with 25 μg microsomal protein/ml. Production of T_3NS, rT_3NS, and 3,3′-T_2NS was measured by RIA (four to six experiments). The panels on the *right* show the double reciprocal plots of the rates of IRD (B) and ORD (C) of T_4NS.

B). This procedure is based on the assumption that virtually all 3,3′-T_2NS is produced via rT_3NS and not via T_3NS. This assumption was validated by calculating the 3,3′-T_2NS produced from the deiodination of rT_3NS and T_3NS using their apparent K_m and V_{max} values (see below). Double reciprocal plots of rT_3NS plus 3,3′-T_2NS production *vs.* T_4NS concentration were linear (Fig. 5B), from which K_m and V_{max} values were calculated (Table 1). Kinetic parameters were also determined from the linear Lineweaver-Burk plots for the ORD of T_4NS (Fig. 5C), the IRD of T_3NS (Fig. 6), and the ORD of rT_3NS and 3,3′-T_2NS (not shown). The K_m and V_{max} values of the different reactions are listed in Table 1, which also provides the V_{max}/K_m ratios as a measure of deiodination efficiency. The kinetic parameters determined previously for the deiodination of native iodothyronines as well as their 4′-sulfate conjugates are included for comparison. In the present study similar data for the deiodination of native iodothyronines were obtained in experiments performed in parallel with those using the sulfamated analogs (not shown).

T_4NS is converted, like T_4, to similar extents by IRD and ORD (Table 1). Apart from rT_3NS, the iodothyronine sulfamates are better substrates for the type I deiodinase of rat liver than the corresponding iodothyronines. Enhanced deiodination of T_4, T_3, and 3,3′-T_2 sulfamates is predominantly due to a considerable reduction in K_m values, which for T_4NS is offset by modest decreases in V_{max} values. As a result, V_{max}/K_m ratios for the sulfamates are increased 4- to 17-fold compared with those for native iodothyronines.

TABLE 1. Kinetic parameters for the deiodination of iodothyronine sulfamates by rat liver microsomes compared with the corresponding iodothyronines and 4′-sulfate conjugates

Substrate	Reaction	n	K_m (μM)	V_{max} (pmol/min·mg protein)	V_{max}/K_m
T_4[a]	ORD	5	2.3 ± 0.5	30 ± 17	13
T_4S[b]	ORD		ND	ND	
T_4NS	ORD	5	0.067 ± 0.029[c]	13.7 ± 6.9	204
T_4[a]	IRD	3	1.9 ± 0.4	18 ± 5	9
T_4S[b]	IRD	5	0.29 ± 0.044[c]	527 ± 203[d]	1,817
T_4NS	IRD	6	0.060 ± 0.021[c]	9.3 ± 2.6[d]	155
rT_3[e]	ORD	4	0.064 ± 0.008	559 ± 230	8,730
rT_3S[e]	ORD	6	0.06 ± 0.024	516 ± 171	8,600
rT_3NS	ORD	4	0.061 ± 0.021	631 ± 168	10,344
T_3[a]	IRD	5	6.2 ± 0.2	36 ± 7	6
T_3S[e]	IRD	4	4.6 ± 1.3[f]	1,050 ± 190[c]	230
T_3NS	IRD	6	1.4 ± 0.6[c]	79.4 ± 27.4[d]	57
3,3′-T_2[g]	ORD	4	8.9 ± 3.9	188 ± 94	21
3,3′-T_2S[g]	ORD	3	0.34 ± 0.07[h]	353 ± 137	1,040
3,3′-T_2NS	ORD	3	4.8 ± 1.4	415 ± 103[f]	87

Studies were carried out at 37 C in 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA, and 3–5 mM DTT. ND, Not detectable. *P* values are *vs.* underivatized iodothyronine (by Student's *t* test).

[a] Ref. 16.
[b] Ref. 21.
[c] $P < 0.001$.
[d] $P < 0.01$.
[e] Ref. 24.
[f] $P < 0.05$.
[g] Ref. 25.
[h] $P < 0.025$.

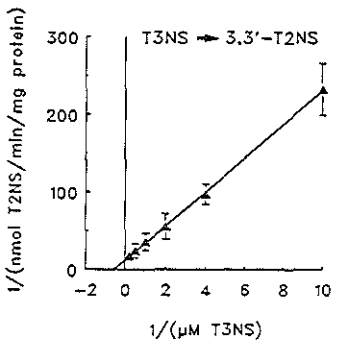

FIG. 6. Double reciprocal plot of the rate of IRD of T_3NS. Incubation of 0.1–5 μM T_3NS was carried out for 30 min with 75 μg microsomal protein/ml (six experiments). Production of 3,3′-T_2NS was quantified by RIA.

Discussion

The type I iodothyronine deiodinase of liver plays a central role in peripheral T_3 production from T_4, but also catalyzes the IRD and ORD of other iodothyronines and derivatives (2, 4, 6, 7, 20). Previous studies using rat and human liver microsomes as well as intact hepatocytes have shown that 4′-sulfation of iodothyronines greatly enhances their deiodination by the type I enzyme (for review, see Ref. 9). The purpose of the present study was to determine whether the observed facilitation of type I deiodination is due to the mere addition of the sulfonate group, or if this is also dependent on the site of its introduction, *i.e.* the amino group of the alanine side-chain in the case of sulfamates or the phenolic hydroxyl group in the case of sulfates.

We here report that, except for the optimal substrate rT_3, *N*-sulfonation results in a marked stimulation of the deiodination efficiency of different iodothyronines. This is, in general, due to a decrease in the apparent K_m value rather than an increase in the V_{max}. Clearly, the specificity of the deiodination of T_4 is not influenced by *N*-sulfonation, since there is an identical increase in V_{max}/K_m ratios for the IRD and ORD of T_4NS. However, the relatively greater increase in IRD *vs.* ORD by *N*-sulfonation of 3,3′-T_2 indicates that this modification can change the proportion of substrate undergoing IRD or ORD.

Figure 7 is a summary of the effects of *N*-sulfonation or 4′-sulfation on the deiodination of iodothyronines by

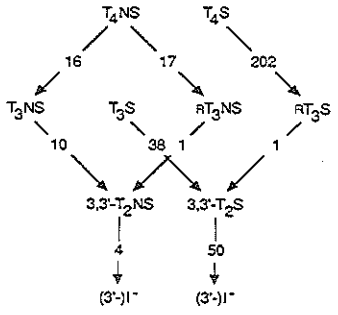

FIG. 7. Stepwise type I deiodination of iodothyronine sulfates and sulfamates. Numbers represent the factors by which the V_{max}/K_m ratio of the deiodination of the native iodothyronines is increased due to *N*-sulfonation (NS) or 4′-sulfation (S).

the type I deiodinase of rat liver microsomes. There are three major differences between the effects of these modifications on enzymatic deiodination. First, there is a difference in magnitude, insofar as 4′-sulfation induces a 4- to 12-fold greater increase in deiodination of the different substrates (except rT_3) compared with *N*-sulfonation. Second, it is remarkable that in contrast to the equal stimulation of IRD and ORD of T_4 by *N*-sulfonation, 4′-sulfation induces a far greater increase in IRD, whereas it fully blocks the ORD of T_4 (21). Third, the mechanisms by which 4′-sulfation and *N*-sulfonation influence the type I deiodination of iodothyronines differ, since 4′-sulfation also induces substantial increases in V_{max} values in addition to a decrease in apparent K_m values.

It is interesting to compare these effects of *N*-sulfonation with those of other side-chain modifications on the type I iodothyronine deiodination. Koehrle *et al.* (11, 20) have demonstrated that *N*-acetylalanine and acetic acid analogs of T_4 are better type I deiodinase substrates than T_4 due to roughly 10-fold lower K_m values (11). Furthermore, such side-chain modifications of iodothyronine derivatives have been shown to generate more potent inhibitors of the type I deiodination of T_4 (20). We have shown that TA_3 and 3,3′-TA_2 are deiodinated by the type I enzyme more efficiently than T_3 and 3,3′-T_2, with, in both cases, a marked decrease in the apparent K_m value (12). We have also found that *N*-acetyl-T_3 and *N*-acetyl-3,3′-T_2 are more effective substrates for the type I deiodinase than T_3 and 3,3′-T_2, respectively[1]. In both cases a substantial decrease in the apparent K_m value is offset by a smaller decrease in the V_{max} value.

A corrolary of these studies is that modification of the iodothyronine structure, such that the net negative charge in the 4′-position or the side-chain is increased, results in higher efficiencies of type I deiodination. With iodothyronine sulfamate, *N*-acetylalanine, and acetic acid derivatives, with net side-chain charges of −2, −1, and −1, respectively, the effect is largely associated with a decrease in the apparent K_m value. However, in the case of iodothyronine sulfates, where the negative charge is located at the 4′-position and the side-chain exists as the zwitterion, distinct increases up to 30-fold in V_{max} values occur as well. The effects of 4′-sulfation of T_4 indicate that modification of this site of the substrate molecule changes not only the rate, but also the specificity of its deiodination (*i.e.* inner *vs.* outer ring) by the type I enzyme. Another example of the dramatic effect of conjugation of the 4′-OH group is the observation that glucuronidation completely inhibits type I deiodination of different iodothyronines (9, 22, 23).

As mentioned above, the mechanism by which *N*-sulfonation increases the type I deiodination of iodothyronines by rat liver microsomes remains unknown, but appears to be different from that of 4′-sulfation. The decrease in the apparent K_m value may point to changes in substrate availability in this *in vitro* system. Both 4′-*O*- and *N*-sulfonation drastically increase the water solubility of iodothyronines. This may result in a significant decrease in the sequestration of these substrates in the microsomal membranes. Our kinetic studies were performed under conditions that provided reaction rates linear to the concentration of microsomal protein in the mixtures, suggesting that binding of substrate to nonenzyme microsomal constituents is negligible. However, it is not excluded that sulfonated iodothyronines have greater access to the enzyme-active center than the native iodothyronines due to changes in the interaction with the lipid environment of the enzyme. Of course, the effects of sulfonation may also reflect a decrease in the true K_m value due to an increased affinity of the substrate for the enzyme. We have previously postulated that this may be explained by the favored interactions of these negatively charged substrates with basic residues of the deiodinase (2, 12).

We have recently shown that the effect of 4′-sulfation on the deiodination of iodothyronines by the type I enzyme is physiologically relevant (for review, see Ref. 9). Strong evidence has been put forward that *in vivo* sulfation of T_4, T_3, and other metabolites strongly accelerates their hepatic deiodination (9, 23). Moreover, in the case of T_3, its deiodinative clearance highly depends on prior sulfate conjugation. T_4 is activated by ORD to T_3, and both T_4 and T_3 are inactivated by IRD to rT_3 and 3,3′-T_2, respectively. Since it accelerates the IRD of

[1] IRD of *N*-acetyl-T_3 and ORD of *N*-acetyl-3,3′-T_2 by rat liver microsomes and 5 mM DTT are characterized by apparent K_m values of 1.1 ± 0.3 and 0.8 ± 0.1 μM and V_{max} values of 12.1 ± 3.3 and 60 ± 20 pmol/min·mg protein, respectively (n = 3) (Rutgers, M., F. A. Heusdens, and T. J. Visser, unpublished work).

both T_4 and T_3 and blocks the ORD of T_4, 4′-sulfation is believed to be an important step leading to the irreversible inactivation of thyroid hormone. The present findings with synthetic iodothyronine sulfamates, which are probably not produced *in vivo*, indicate that although *N*-sulfonation also stimulates the IRD of T_4 and T_3, the effects are less dramatic than with 4′-*O*-sulfonation. Therefore, the effect of conjugation on the deiodination and, consequently, the bioactivity of thyroid hormone not only critically depends on the type (sulfonation *vs.* glucuronidation), but also on the site (4′-hydroxyl group *vs.* alanine side-chain) of the modification.

Acknowledgments

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References

1. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5:151–184
2. Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RJB, Van der Molen HJ (eds) Hormones and Their Action, Part I. Elsevier, Amsterdam, pp 81–103
3. Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, pp 189–229
4. Hesch RD, Koehrle J 1986 Intracellular pathways of iodothyronine metabolism. In: Ingbar SH, Braverman LE (eds) The Thyroid. Lippincott, Philadelphia, pp 154–200
5. Visser TJ, Kaptein E, Terpstra OT, Krenning EP 1988 Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 67:17–24
6. Visser TJ, Docter R, Krenning EP, Hennemann G 1986 Regulation of thyroid hormone bioactivity. J Endocrinol Invest [Suppl 4] 9:17–26
7. Hennemann G 1986 Thyroid Hormone Metabolism. Marcel Dekker, New York
8. Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford, pp 345–365
9. Visser TJ, Van Buuren JCJ, Rutgers M, Eelkman Rooda SJ, De Herder WW 1990 The role of sulfation in thyroid hormone metabolism. Trends Endocrinol Metab 1:211–218
10. Burger A 1986 Nondeiodinative pathways of thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, pp 255–276
11. Koehrle J, Hesch RD 1984 Biochemical characteristics of iodothyronine monodeiodination by rat liver microsomes: the interaction between iodothyronine substrate analogs and the ligand binding site of the iodothyronine deiodinase resembles that of the TBPA-iodothyronine ligand binding. Horm Metab Res [Suppl] 14:42–55
12. Rutgers M, Heusdens FA, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. I. Deiodination of TA_3 and TA_3 sulfate by microsomes. Endocrinology 125:424–432
13. Mol JA, Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. Endocrinology 117:1–7
14. Eelkman Rooda SJ, Van Loon MAC, Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. J Clin Invest 79:1740–1748
15. Rutgers M, Bonthuis F, De Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758–762
16. Visser TJ, Fekkes D, Docter R, Hennemann G 1979 Kinetics of enzymatic reductive deiodination of iodothyronines. Effect of pH. Biochem J 179:489–495
17. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 115:398–402
18. Visser TJ, Fekkes D, Docter R, Hennemann G 1978 Sequential deiodination of thyroxine in rat liver homogenate. Biochem J 174:221–229
19. Segel IH 1975 Enzyme Kinetics. Wiley and Sons, New York
20. Koehrle J, Auf'mkolk M, Rokos H, Hesch RD, Cody V 1986 Rat liver iodothyronine monodeiodinase. Evaluation of the iodothyronine ligand binding site. J Biol Chem 261:11613–11622
21. Mol JA, Visser TJ 1986 Rapid and selective inner ring deiodination of T_4 sulfate by rat liver deiodinase. Endocrinology 117:8–12
22. Eelkman Rooda SJ, Otten MH, Van Loon MAC, Kaptein E, Visser TJ 1989 Metabolism of triiodothyronine in rat hepatocytes. Endocrinology 125:2187–2197
23. Rutgers M, Heusdens FA, Bonthuis F, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. II. Deiodination and conjugation of TA_3 by rat hepatocytes and in rats *in vivo*. Endocrinology 125:433–443
24. Visser TJ, Mol JA, Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112:1547–1549
25. Otten MH, Mol JA, Visser TJ 1983 Sulfation preceeding deiodination of iodothyronines in rat hepatocytes. Science 221:81–83

Chapter III

ACCUMULATION OF PLASMA TRIIODOTHYRONINE SULFATE IN RATS TREATED WITH PROPYLTHIOURACIL

Accumulation of Plasma Triiodothyronine Sulfate in Rats Treated with Propylthiouracil

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Abstract

Triiodothyronine sulfate (T_3S) is rapidly deiodinated by the propylthiouracil (PTU)-sensitive type I deiodinase. Here we examined the effects of PTU on plasma T_3S levels in rats after intravenous administration of radiolabeled T_3 or T_3S. Sephadex LH-20 chromatography and high-performance liquid chromatography were used to quantify conjugated and nonconjugated iodothyronines, and iodide was measured as the TCA-soluble radioactivity. In control rats, radioiodide was the main metabolite of both T_3 and T_3S. Plasma T_3S was cleared more rapidly than plasma T_3 despite increased binding to plasma proteins. PTU reduced plasma iodide levels by 66 and 78% after T_3 and T_3S, respectively, and decreased plasma clearance of T_3S by 81%. However, PTU had no effect on plasma T_3 clearance but increased plasma T_3S from injected T_3 4.2 times. Biliary excretion of injected T_3S was < 20% in normal rats, in contrast to 70% within 4 h in PTU-treated rats. In conclusion, T_3S is an important intermediate in the in vivo metabolism of T_3 in rats and accumulates in plasma if type I deiodination is inhibited.

Introduction

Stepwise monodeiodination plays a central role in the metabolism of thyroid hormone in peripheral organs such as liver and kidney (1, 2). After the bioactivation of thyroxine (T_4)[1] to 3,3',5-triiodothyronine (T_3) by outer ring deiodination, T_3 is further converted to biologically inactive iodothyronines by successive deiodination in inner and outer rings (3). In humans, metabolic pathways other than deiodination seem equally important for the inactivation and elimination of T_3 (1). Conjugates of T_3 with glucuronic acid or sulfate have been detected by Bollman and Flock (4) in bile and urine of rats and dogs.

In vitro, enzyme kinetic studies with liver type I deiodinase have shown that, in contrast to T_3 itself, T_3 sulfate (T_3S) is rapidly deiodinated in the inner ring (5). The 3,3'-diiodothyronine sulfate (T_2S) produced is also a much better substrate for outer ring deiodination than nonconjugated T_2 (5, 6). Under normal conditions primary cultures of rat hepatocytes metabolize added [^{125}I]T_3 to iodide and T_3 glucuronide (T_3G). Addition of the type I deiodinase inhibitor 6-propyl-2-thiouracil (PTU) results in accumulation of the T_3S intermediate without affecting T_3 clearance (6). Iodide production is also decreased in sulfate-depiete cells or by inhibitors of phenol sulfotransferase, but under these conditions T_3 clearance is diminished as well (6). It was subsequently shown that PTU treatment resulted in a fivefold increase in biliary T_3S after administration of [^{125}I]T_3 to rats without affecting excretion of T_3G[2] (7). Therefore, sulfation and subsequent deiodination is an important metabolic pathway for T_3 in rat liver. To further investigate the physiological relevance of this pathway we analyzed plasma T_3S in rats with impaired type I deiodinase activity.

Methods

Materials. [^{125}I]T_3 was synthesized by radioiodination of 3,5-diiodothyronine (Henning GmbH, Berlin, FRG) with carrier-free Na^{125}I (Amersham Corp., Amersham, UK) using the chloramine T method. The sulfate conjugate of [^{125}I]T_3 was prepared with chlorosulfonic acid in dimethylformamide and purified on Sephadex LH-20 (8). PTU was purchased from Sigma Chemical Co., St Louis, MO. All other chemicals were of analytical grade.

Experimental procedures. Male Wistar rats, 230–350 g body weight (BW), were anesthetized by injection of 5 mg i.p. pentobarbital sodium per 100 g BW. Additional injections of 2–5 mg pentobarbital were administered during the experiment if necessary. Body temperature was maintained by placing the animals under an infrared lamp. A 100-mM PTU solution in 0.1 M NaOH was diluted five times in phosphate-buffered saline. Rats were injected with 220 μl i.v. (0.75 mg PTU) per 100 g BW of this mixture. Controls were studied in parallel and received the same volume of vehicle. 30 min later ~ 10 μCi [^{125}I]T_3 or [^{125}I]T_3S in 500 μl 0.01 M NaOH in saline was injected intravenously (t = 0). Blood samples (0.75 ml) were taken from the tail vein at 0.5, 1, and 2 h, and after cervical dislocation at 4 h the animals were bled by heart puncture.

Biliary excretion of intravenously injected [^{125}I]T_3S was studied as follows. The biliary duct of pentobarbital-anesthetized rats (200 g BW) was cannulated[2] and the animals were injected with PTU or vehicle followed by 10 μCi [^{125}I]T_3S as described above. Blood samples (0.3 ml) were obtained in heparinized vials after 0.5, 1, 2, and 4 h, and bile was collected in 10–20 min periods.

In a parallel experiment PTU was administered 2 h before the animals were anesthetized whereupon the bile duct was cannulated and [^{125}I]T_3 injected, i.e., 2.5 h after PTU. Bile and plasma were collected until 4 h after T_3 injection.

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1. *Abbreviations used in this paper:* BW, body weight; G, glucuronide; HPLC, high-performance liquid chromatography; PTU, 6-propyl-2-thiouracil; S, sulfate; SPE, solid-phase extraction; T_2, 3,3'-diiodothyronine; T_3, 3,3',5-triiodothyronine; T_4, thyroxine.

2. De Herder, W. W., F. Bonthuis, M. Rutgers, M. H. Otten, M. P. Hazenberg, and T. J. Visser. Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Submitted for publication.

Analysis of samples. Serum and bile were kept at −20°C until further analysis. Plasma $^{125}I^-$ was measured as trichloroacetic acid (TCA)–soluble radioactivity. For this purpose, 50–100 μl serum was mixed with 10% (wt/vol) ice-cold TCA to a final volume of 500 μl. After 10 min at 0°C, mixtures were centrifuged and radioactivity was determined in the supernatant. Radioiodide in bile was estimated similarly by addition of 100 μl pooled human serum to 10–25 μl bile followed by 400 μl 10% TCA. > 95% of T_3 or T_3S added to plasma or bile was precipitated by TCA while on average 97% of added $^{125}I^-$ remained in solution.

For analysis of other plasma metabolites, mixtures were prepared consisting of 250 μl serum, 500 μl 0.2 M HCl, and 250 μl ethanol. These were applied to small Sephadex LH-20 columns (bed vol, 1.3 ml) equilibrated in 0.1 M HCl. After rinsing the columns with 0.1 M HCl, conjugated and nonconjugated iodothyronines were eluted successively with 20% ethanol in water and 0.1 M ammonia in ethanol. Fractions of 1 ml were collected and counted for radioactivity. Recovery of T_3S and T_3 added to rat plasma amounted to 91 and 94%, respectively. For further identification of the isolated products by high-performance liquid chromatography (HPLC), peak fractions of corresponding time points were combined within each experimental group. The conjugate pool was lyophylized and the iodothyronine pool was evaporated under a stream of nitrogen at 50°C.

Iodothyronines and their conjugates were isolated from the 4-h plasma samples by solid-phase extraction (SPE) for subsequent analysis by HPLC. In short, 500 μl serum was mixed with an equal volume 0.25 M NaOH and applied to a C_{18}-SPE column (500 mg, J. T. Baker Chemical Co., Phillipsburg, NJ). Columns were washed successively with 2 × 1 ml of each 0.1 M NaOH, H_2O, 0.1 M HCl, and H_2O before elution of both conjugates and iodothyronines with 1 ml methanol. The recovery of T_3 and T_3S added to rat plasma was 90 and 96%, respectively.

HPLC analysis. Reversed-phase HPLC was done on a 10 × 0.3–cm Chromspher C_{18} analytical column in combination with a 10 × 2.1–mm guard column (Chrompack International BV, Middelburg, Netherlands). Elution was performed with a 20-min gradient of 18–40% acetonitrile in 0.02 M ammonium acetate (pH 4). Solvent flow was 0.8 ml/min and fractions of 0.5 min were collected. A nonlinear gradient (No. 7) as programmed by the automated gradient controller (model 680, Waters Associates, Milford, MA) was used. The residues of the plasma extracts were dissolved in mobile phase and the gradient was started at the time of injection. Retention times of possible products were determined using synthetic and biosynthetic reference compounds (9). HPLC analysis of biliary products was performed on 5–25–μl aliquots of bile diluted with 4 vol of mobile phase. Analysis, especially of glucuronide conjugates in bile, was more accurate if a 25-min gradient of 16–40% acetonitrile was used.

Results

Identification of metabolites. All metabolites of interest were well separated using reversed-phase HPLC, and a typical chromatogram is shown in Fig. 1. Neither serum residues nor small amounts of bile in the HPLC samples affected the elution profile seen with pure tracers.

Analysis of plasma T_3 and T_3S metabolites. Fig. 2 shows the distribution of the major radioactive compounds in plasma 2 h after administration of the tracers as determined by LH-20 chromatography. Further analysis of the obtained conjugate and iodothyronine fractions by reversed-phase HPLC is illustrated in Fig. 3. HPLC of the LH-20 fractions of plasma obtained after 0.5, 1, and 4 h indicated similar compositions. On average, 78% of the radioactivity in the conjugate fraction coeluted with T_3S on HPLC, while 84% of the iodothyronine fraction eluted as T_3. If plasma was spiked with labeled T_3 or T_3S, in both cases 89% of the radioactivity in the respective

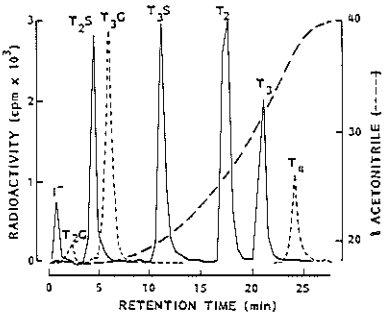

Figure 1. Separation by reversed-phase HPLC of a mixture of ^{125}I-labeled sulfates (*S*), glucuronides (*G*), and nonconjugated iodothyronines. The C_{18} column was eluted at 0.8 ml/min using a 20-min gradient of 18–40% acetonitrile in 0.02 M ammonium acetate (pH 4). (*Dashed curve*) Actual composition of the mobile phase.

LH-20 fractions eluted as T_3 or T_3S on HPLC. Therefore, plasma radioactivity other than iodide as fractionated on LH-20 consisted predominantly of T_3S or T_3. Nonconjugated T_2 was never observed, but T_2S was detected in T_3-injected rats where it comprised on average 10% of the radioactivity in the

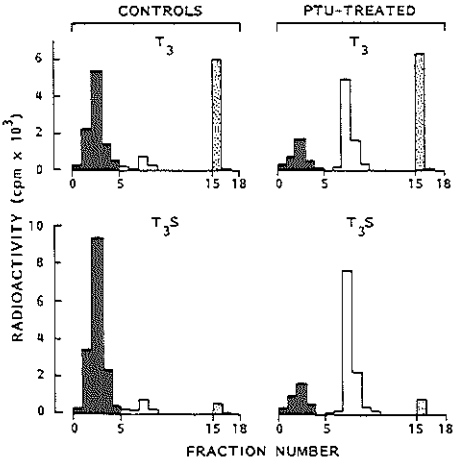

Figure 2. Sephadex LH-20 chromatography of serum from rats injected with T_3 or T_3S. Rats received saline (*left*) or PTU (*right*) 30 min prior to 10 μCi [^{125}I]T_3 (*upper*) or [^{125}I]T_3S (*lower*) by intravenous injections. Serum (0.25 ml) was acidified and applied to Sephadex LH-20 columns as described in Methods. Free iodide, conjugated and nonconjugated iodothyronines were successively eluted with 0.1 M HCl (fractions 1–5), 20% ethanol in water (fractions 6–14), and 0.1 M ammonia in ethanol (fractions 16–18) with a recovery of 95% for I^-, 91% for T_3S, and 94% for T_3. Mean values for each experimental group are given (T_3, n = 5; T_3S, n = 4). Total radioactivity in the samples amounted to 0.39 (T_3), 0.34 (T_3 + PTU), 0.42 (T_3S), and 0.31 (T_3S + PTU) % dose/ml plasma.

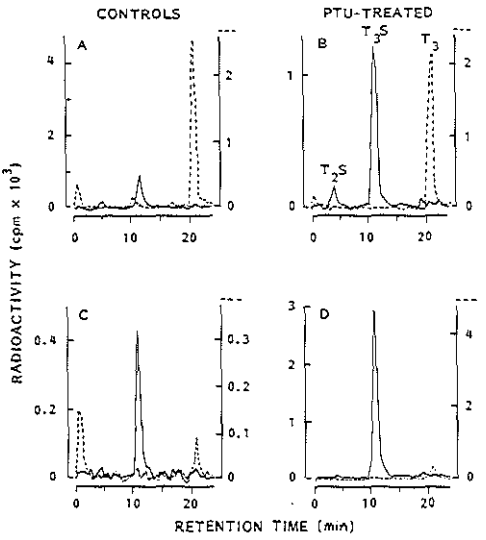

Figure 3. HPLC analysis of serum T_3 and T_3S fractions. Serum was obtained from control and PTU-treated rats 2 h after injection of [^{125}I]T_3 (*A* and *B*) or [^{125}I]T_3S (*C* and *D*) and chromatographed on Sephadex LH-20 (Fig. 2). Corresponding conjugate (*solid line*) or iodothyronine (*dashed line*) fractions were pooled for each experimental group and further analyzed by HPLC (Fig. 1). Chromatograms represent ~ 49, 80, 25, and 78% of total plasma radioactivity due to removal of mainly $^{125}I^-$ by LH-20 from samples *A*, *B*, *C*, and *D*, respectively. Note the smaller scale in *C*, where the first peak represents only 2% of plasma iodide.

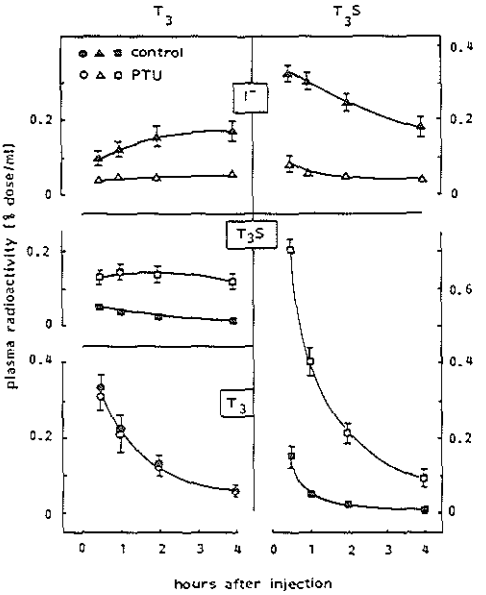

Figure 4. Effect of PTU on plasma T_3 and T_3S metabolites. Serum was analyzed from rats injected with [^{125}I]T_3 (*left*) or [^{125}I]T_3S (*right*) after pretreatment with saline (*solid symbols*) or PTU (*open symbols*). Plasma iodide (*triangles*) was estimated as the TCA-soluble radioactivity, whereas T_3S (*squares*) and T_3 (*circles*) were quantified by Sephadex LH-20 fractionation (Fig. 2). In [^{125}I]T_3-injected rats roughly 10% of the radioactivity in the "T_3S fraction" consisted of T_2S. Radioactivity of compounds is expressed as mean percent dose per milliliter plasma (±SD unless smaller than symbol) and plotted as a function of time after tracer injection. Total amount of plasma radioactivity decreased over the 4-h period from 0.52±0.07 to 0.33±0.05 after T_3 (n = 5), from 0.50±0.08 to 0.26±0.04 after T_3 + PTU (n = 5), from 0.68±0.04 to 0.29±0.04 after T_3S (n = 4), and from 0.82±0.03 to 0.17±0.03 after T_3S + PTU (n = 4). Except for plasma disappearance of T_3, differences between controls and PTU rats were highly significant ($P < 0.001$) as estimated by Student's unpaired t test.

conjugate fraction. After T_3S injection, plasma T_3 was negligible in control rats and represented < 4% of plasma radioactivity in PTU-treated rats. Neither T_4 nor the glucuronides of T_3 and T_2 were observed in any of the samples. HPLC of solid-phase extracts of 4-h plasma samples were in close agreement with analysis of the LH-20 fractions.

> 95% of radioiodide added to plasma eluted in the first five fractions of the LH-20 chromatography. However, coelution of some protein-bound radioactivity or unknown metabolites could not be excluded. Therefore, accurate measurements of free iodide in plasma was performed by TCA precipitation. The radioactivity in the HCl fractions after LH-20 correlated well with the amount of TCA-soluble radioactivity as shown by linear regression analysis. For the means of these parameters in the different experimental groups the following function was derived: y (HCl) = 1.3 x (TCA) + 0.1 (r = 0.997, n = 16) with x and y expressed as percentage of plasma radioactivity. Apparently, the LH-20 method overestimated plasma iodide levels and was not used for calculation of the results.

Effect of PTU on the metabolism of T_3 and T_3S. Results of the measurement of plasma T_3 and T_3S by Sephadex LH-20 and of iodide by TCA precipitation are summarized in Fig. 4. Radioiodide was the main plasma metabolite of both T_3 and T_3S in control rats. PTU did not affect plasma T_3 clearance but decreased T_3S clearance by 81%, as estimated from the area under the plasma T_3S concentration curve. In control rats clearance of plasma T_3S was faster than that of T_3, but the reverse was true after PTU treatment. The administration of PTU diminished plasma iodide levels by 60–71% after T_3 injection and by 74–80% after T_3S. PTU increased plasma T_3S 2.7 times at 0.5 h to 7.5 times at 4 h after T_3 injection, with an average of 4.2 times. This resulted in T_3S being the major radioactive compound in plasma from 2 h after T_3 injection onwards. Similar results were obtained in rats with bile cannules, where T_3 injection was delayed until 2.5 h as opposed to 0.5 h after PTU administration.

Biliary clearance of T_3S (Fig. 5). In control rats < 20% of radioactivity injected as T_3S was excreted in the bile, occurring predominantly during the first 30 min. In PTU-treated rats biliary excretion was greatly increased up to 70% of the dose after 4 h. HPLC analysis demonstrated that T_3S was the only

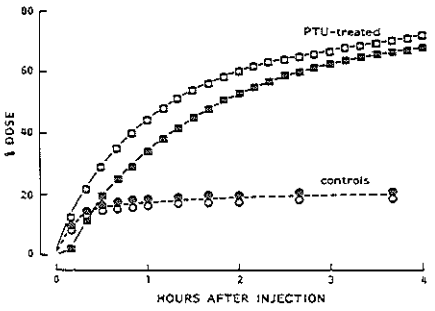

Figure 5. Biliary clearance of T_3S. Rats with bile cannules under pentobarbital anesthesia received intravenous injections of PTU or saline 30 min before 10 μCi [^{125}I]T_3S. Cumulative excretion was estimated by summation of radioactivity in successive 10-min bile aliquots and expressed as percent dose. Different symbols represent individual rats. Total bile volumes collected over 4 h were 3.5 (*open circle*), 4.4 (*solid circle*), 3.7 (*open square*), and 4.2 (*solid square*) ml. Bile flow was low in one of the PTU-treated rats (*solid square*) during the first 10 min.

significant compound in bile. Iodide was excreted only in bile of control rats and amounted to 2.5% of the administered dose. T_2S was observed in bile of PTU-treated rats but accounted for < 3% of the biliary radioactivity. In these cannulated rats distribution of plasma radioactivity was the same as in intact rats and pretreatment with PTU resulted in a 57–84% decrease in plasma iodide over the 4-h period.

Plasma free T_3 and T_3S fractions. The non-protein-bound fractions of T_3 and T_3S in rat plasma were determined in duplicate by equilibrium dialysis. The free fraction was 0.35±0.03 (mean±SD, n = 6) for T_3 and 0.20±0.03 (n = 4) for T_3S, and both were unaffected by 1 mM PTU.

Discussion

The role of conjugation in the metabolism of thyroid hormone, especially in humans, has received little attention in the literature. Extensive conjugation of thyroid hormone has been demonstrated in experimental animals. Bollman and Flock (4) have identified glucuronides as the main excretory products of various iodothyronines in the bile of normal rats. However, the sum of T_2S and T_3S excreted in bile equalled or exceeded that of T_3G after administration of T_3 to dogs (10, 11). Studying the biliary clearance of T_4 in rats, Flock and Bollman (12) observed that thiouracil treatment increased the excretion of an acid-hydrolyzable T_4 conjugate which perhaps represented T_4 sulfate (8). A similar effect was also observed with butyl 4-hydroxy-3,5-diiodobenzoate, which is also an inhibitor of type I deiodinase activity. Treatment of rats with this compound in addition to labeled T_3 or T_4 led to a far greater increase in biliary sulfates compared with glucuronides (13).

Roche et al. (14) reported on the presence of radioactive T_3S in bile and plasma of thyroidectomized rats after injection with labeled T_3. We previously observed an increase in biliary T_3S from exogenous T_3 in rats treated with PTU[2] (7). We have now shown that the same treatment results in a marked accumulation of plasma T_3S. In retrospect, it is possible to explain the findings of Roche et al. (14) as it has become evident that hypothyroidism in rats is associated with an impaired hepatic deiodinase activity (15). Although these investigators did not study euthyroid rats, their results agree with ours, indicating that T_3S accumulates if subsequent deiodination is inhibited.

In control rats, injected T_3S is metabolized more rapidly than T_3 although it binds with higher affinity to plasma proteins, and only radioiodide was detected as a metabolite. Similar rapid deiodinative clearance of T_3S in humans has been reported recently (16). Clearance of T_3S is strongly inhibited by PTU, indicating that it is largely metabolized by type I deiodination. In contrast, T_3 disposition is not affected by PTU as also observed by others (17), illustrating that direct inner ring deiodination of T_3 by the liver type I deiodinase is a negligible metabolic pathway. The slower metabolic clearance rate of T_3S compared with T_3 in PTU-treated rats has also been observed in thyroidectomized rats (18) and entirely explains the increase in plasma T_3S from exogenous T_3. The plasma T_3S levels thus obtained underscore the importance of sulfation as metabolic pathway of T_3.

In PTU-treated rats plasma T_3S is cleared predominantly by biliary excretion. It is possible, therefore, that also after injection of T_3 most T_3S in bile is derived from plasma T_3S. Although T_3 is sulfated and glucuronidated in rat hepatocytes (6), it is not excluded that part of plasma T_3S originates by sulfation of T_3 in other tissues as was observed in hepatectomized dogs (4, 10).

Plasma T_2S was observed in both normal and PTU-treated rats after injection of T_3 but not after T_3S. Therefore, it is probably derived from sulfation of T_2 that is produced by PTU-insensitive (type III) inner ring deiodination of T_3 (2). This would also explain the marked biliary T_2S excretion after T_3 injection to PTU-treated rats[2] (7) in contrast to the negligible amounts of T_2S in bile of T_3S-injected rats (this study).

In conclusion, the present study of plasma T_3S formation in T_3-injected rats extends previous observations in bile, indicating that sulfation is an important pathway of T_3 metabolism in rats. However, unless subsequent type I deiodination of the conjugate is prevented, little T_3S is observed in both body fluids. The finding of significant amounts of T_3S in rat plasma opens the perspectives of studying the importance of T_3 sulfation in humans. The recent development of a radioimmunoassay for T_3S in our laboratory should facilitate such investigations (19). The findings that in human liver T_3 is a substrate for phenol sulfotransferase (20) and that T_3S is rapidly deiodinated (21) suggest that successive sulfation and deiodination of T_3 indeed occurs in humans.

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References

1. Engler, D., and A. G. Burger. 1984. The deiodination of the iodothyronines and their derivatives in man. *Endocr. Rev.* 5:151–184.

2. Leonard, J. L., and T. J. Visser. 1986. Biochemistry of deiodina-

tion. *In* Thyroid Hormone Metabolism. G. Hennemann, editor. Marcel Dekker, New York. 189–229.

3. Visser, T. J., D. Fekkes, M. H. Otten, J. A. Mol, R. Docter, and G. Hennemann. 1984. Deiodination and conjugation of thyroid hormone in rat liver. *In* Hormones and Cell Regulation. Vol. 8. J. E. Dumont and J. Nunez, editors. Elsevier/North-Holland, Amsterdam. 179–191.

4. Bollman, J. L., and E. V. Flock. 1965. The role of the liver in the metabolism of I^{131}-thyroid hormones and analogues. *In* The Biliary System. W. Taylor, editor. Blackwell, Oxford. 345–365.

5. Visser, T. J., J. A. Mol, and M. H. Otten. 1983. Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. *Endocrinology.* 112:1547–1549.

6. Otten, M. H., J. A. Mol, and T. J. Visser. 1983. Sulfation preceeding deiodination of iodothyronines in rat hepatocytes. *Science (Wash. DC).* 221:81–83.

7. De Herder, W. W., F. Bonthuis, M. P. Hazenberg, and T. J. Visser. 1986. Biliary excretion and reabsorption of T_3 and its metabolites. *Ann. Endocrinol.* 47:23. (Abstr.)

8. Mol, J. A., and T. J. Visser. 1985. Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. *Endocrinology.* 117:1–7.

9. De Herder, W. W., M. P. Hazenberg, A. M. Pennock-Schröder, G. Hennemann, and T. J. Visser. 1986. Hydrolysis of iodothyronine glucuronides by obligately anaerobic bacteria isolated from human faecal flora. *FEMS (Fed. Eur. Microbiol. Soc.) Microbial. Lett.* 35:249–253.

10. Flock, E. V., J. L. Bollman, and J. H. Grindlay. 1960. Conjugates of triiodothyronine and its metabolites. *Endocrinology.* 67:419–429.

11. Flock, E. V., J. L. Bollman, and J. H. Grindlay. 1960. 3,3'-Diiodothyronine, a metabolite of 3,5,3'-triiodothyronine. *Proc. Mayo Clin.* 35:75–81.

12. Flock, E. V., and J. L. Bollman. 1962. The effect of thiouracil on the metabolism of L-thyroxine. *Biochem. J.* 84:621–626.

13. Flock, E. V., J. L. Bollman, C. A. Owen, Jr., and P. E. Zollman. 1965. Conjugation of thyroid hormones and analogs by the Gunn rat. *Endocrinology.* 77:303–314.

14. Roche, J., R. Michel, J. Closon, and O. Michel. 1959. Sur la sulfoconjugaison hépatique de la 3,5,3'-triiodothyronine et la présence d'un ester sulfurique de cette hormone dans la bile et la plasma. *Biochim. Biophys. Acta.* 33:461–468.

15. Kaplan, M. M. 1986. Regulatory influences on iodothyronine deiodination in animal tissues. *In* Thyroid Hormone Metabolism. G. Hennemann, editor. Marcel Dekker, New York. 231–254.

16. Nimalasuriya, A., L. Y. Mizuno, C. A. Spencer, A. Marcus, and J. T. Nicoloff. 1984. Program of the 60th Annual Meeting of the American Thyroid Association, New York, NY. 57. (Abstr.)

17. Silva, J. E., M. B. Gordon, F. R. Crantz, J. L. Leonard, and P. R. Larsen. 1984. Qualitative and quantitative differences in the pathways of extrathyroidal triiodothyronine generation between euthyroid and hypothyroid rats. *J. Clin. Invest.* 73:898–907.

18. Roche, J., R. Michel, J. Closon, and O. Michel. 1960. Sur le métabolisme du sulfoconjugué de la 3,5,3'-triiodothyronine chez le rat. *Biochim. Biophys. Acta.* 38:325–332.

19. Eelkman Rooda, S. J., E. Kaptein, M. A. C. Van Loon, and T. J. Visser. 1986. Sulfate conjugation of T_3 by rat hepatocytes as analysed by a T_3 sulfate radioimmunoassay. *Ann. Endocrinol.* 47:24. (Abstr.)

20. Young, W. F., C. A. Gorman, and R. M. Weinshilboum. 1986. Triiodothyronine (T_3): a substrate for two forms of human phenol sulfotransferase (PST). Program of the 61st Annual Meeting of the American Thyroid Association, Phoenix, AZ. 45. (Abstr.)

21. Visser, T. J., and E. P. Krenning. 1983. Deiodination of iodothyronines in human liver. Program of the 59th Annual Meeting of the American Thyroid Association, New Orleans, LA. 31. (Abstr.)

Chapter IV

METABOLISM OF TRIIODOTHYROACETIC ACID (TA_3) IN RAT LIVER. I: DEIODINATION OF TA_3 AND TA_3 SULFATE BY MICROSOMES

Metabolism of Triiodothyroacetic Acid (TA_3) in Rat Liver. I. Deiodination of TA_3 and TA_3 Sulfate by Microsomes*

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ABSTRACT. The deiodination of the acetic acid side-chain analogs of T_3 as well as 3,3′-diiodothyronine (3,3′-T_2) was investigated by incubating ^{125}I-labeled 3,3′,5-triiodothyroacetic acid (TA_3) and 3,3′-diiodothyroacetic acid (3,3′-TA_2) with rat liver microsomes at 37 C and pH 7.2 in the presence of 5 mM dithiothreitol. TA_3 sulfate (TA_3S) and 3,3′-TA_2S were also tested as substrate since sulfation is known to accelerate T_3 and 3,3′-T_2 conversion. Reaction products were analyzed on Sephadex LH-20 and HPLC. TA_3 underwent only inner ring deiodination (IRD), but 3,3′-TA_2 was equally converted by IRD and outer ring deiodination (ORD). TA_3S was metabolized very rapidly by IRD to 3,3′-TA_2S which was only observed transiently due to its rapid deiodination predominantly in the outer ring. Kinetic studies under initial reaction rate conditions yielded apparent Michaelis-Menten (K_m) values (micromolar) of 1.3 for TA_3, 0.8 for 3,3′-TA_2, and 0.004 for TA_3S, and 0.02 for 3,3′-TA_2S and V_{max} values (picomoles per min/mg protein) of 174 for TA_3, 49 for 3,3′-TA_2, 21 for TA_3S, and 63 for 3,3′-TA_2S. The V_{max}/K_m ratios for the IRD of TA_3 and TA_3S were 16 and 930 times higher, respectively, relative to T_3. Deiodinations were sensitive to propylthiouracil inhibition, indicating the involvement of the type I iodothyronine deiodinase. Furthermore, the iodothyroacetic acid derivatives competitively inhibited the ORD of rT_3 with apparent inhibition constant (K_i) values (0.45 μM for TA_3, 4 nM for TA_3S, and 0.04 μM for 3,3′-TA_2S) in agreement with corresponding K_m values. We conclude that 1) TA_3 and 3,3′-TA_2 are better substrates than T_3 and 3,3′-T_2 for the type I deiodinase of rat liver; 2) the IRD of TA_3 and ORD of 3,3′-TA_2 are markedly enhanced by sulfation similar to the parent iodothyronines; and 3) TA_3S in the best known substrate for IRD due to its very high affinity for the type I deiodinase. (*Endocrinology* 125: 424–432, 1989)

THYROXINE is secreted entirely by the thyroid, but only approximately 20% of the production of the bioactive hormone T_3 originates from this gland. The remaining T_3 is produced in peripheral tissues by outer ring deiodination (ORD) of T_4 (1). Both T_4 and T_3 are inactivated by inner ring deiodination (IRD) to rT_3 and 3,3′-diiodothyronine (3,3′-T_2), respectively, and the latter is also produced by ORD of rT_3 (2). The liver is an important organ for the ORD as well as for IRD of iodothyronines (3). Both reactions are catalyzed by the type I iodothyronine deiodinase which is associated with the endoplasmic reticulum of hepatocytes and inhibited by 6-propyl-2-thiouracil [PTU (2)].

Tetraiodothyroacetic acid (TA_4) and 3,3′,5-triiodothyroacetic acid (TA_3) are naturally occurring side-chain analogs of T_4 and T_3. They derive from the parent thyroid hormones by deamination and decarboxylation as has been demonstrated *in vitro* as well as *in vivo* (1).

T_3 exerts its physiological function by binding to nuclear T_3 receptors, thereby regulating the expression of specific genes (4). TA_3 binds more strongly than T_3 to these receptors, but the *in vivo* bioactivity of TA_3 is considerably lower than that of T_3 (4–6).

Apparently, TA_3 and TA_4 are metabolized in rats, dogs, and humans via similar pathways as the parent hormones, *i.e.* by stepwise deiodination, resulting in urinary I^- excretion (7–11), and by conjugation predominantly with glucuronic acid but also with sulfate (7, 10). Normally, the conjugates are excreted with bile, but they may appear also in plasma and urine (7). Despite an increased binding to plasma proteins (10, 12), TA_3 is cleared at least as rapidly as T_3 in humans and rats (9, 11, 13). This suggests a rapid cellular metabolism of the acetic acid analog and explains the low *in vivo* bioactivity of TA_3 (6, 12).

In consideration of the central role of the liver in thyroid hormone metabolism (3), we have investigated

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the possible differences between the hepatic metabolism of TA_3 and T_3. In the present study, TA_3 was tested as a substrate for the type I deiodinase of rat liver microsomes. In the accompanying paper, the metabolism of TA_3 in isolated rat hepatocytes and in rats *in vivo* is reported (14). In contrast to the zwitterionic nature of the alanine side chain of T_3, the acetic acid moiety of TA_3 possesses a negative charge. Previous studies have shown that introduction of a negative charge at the opposite site of the T_3 molecule by sulfation of the 4'-hydroxyl group facilitates the IRD of T_3 and the subsequent ORD of 3,3'-T_2 (2, 15). Since TA_3 is known to undergo sulfation *in vivo* (7, 10), we have also studied the type I deiodination of TA_3 sulfate (TA_3S). The results demonstrate that TA_3 is a better substrate for type I deodinase than T_3 and that sulfation accelerates the IRD of TA_3 to an even greater extent than is the case with T_3.

Materials and Methods

Chemicals

All iodothyronines, 3,5-TA_2, and TA_3 were obtained from Henning Berlin GmbH (Berlin, West Germany). T_3 and TA_3 were also purchased from Sigma Chemical Co. (St. Louis, MO). 3,3'-TA_2 was kindly donated by Dr. H. J. Cahnmann (NIH, Bethesda, MD) and provided to us by Dr. A. G. Burger (University of Geneva, Geneva, Switzerland). Carrier-free $Na^{125}I$ (Amersham, Amersham, England) was used to prepare outer ring ^{125}I-labeled rT_3, T_3, 3,3'-T_2, TA_3 (specific radioactivity ≈1700 Ci/mmol) and 3,3'-TA_2 (≈90 Ci/mmol) from 3,3'-T_2, 3,5-T_2, 3-iodothyronine(3-T_1), 3,5-TA_2, and 3,3'-TA_2, respectively, using the chloramine T method (Visser, T. J., and M. Rutgers, unpublished work). The 4'-sulfate esters of both radiolabeled and unlabeled 3,3'-TA_2, TA_3, 3,3'-T_2, and T_3 were synthesized using chlorosulfonic acid in dimethylformamide (16). The products were isolated from the reaction mixtures by Sephadex LH-20 chromatography, and purity was checked by reverse-phase HPLC. Sephadex LH-20 and G-10 were purchased from Pharmacia (Uppsala, Sweden); all the other reagents were of analytical grade.

Microsomal deiodinase assay

Microsomes were prepared from livers of male Wistar rats (≈200 g body wt) in 50 mM Tris/HCl (pH 7.4), 3 mM EDTA, and 3 mM dithiothreitol (DTT) as previously described (17, 18) and stored at −80 C. Protein concentration was determined by the method of Bradford (19) using BSA as standard.

Deiodinase assay mixtures contained various concentrations of microsomal protein and substrate, with or without inhibitor, in usually 200 μl 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA, and 5 mM DTT. Incubations were done in duplicate or triplicate in a shaking waterbath at 37 C in the presence of 10–50 nCi ^{125}I-labeled substrate, representing ≤10% of total substrate concentration.

Inhibition of rT_3 deiodination

The effect of increasing concentrations of iodothyroacetic acid analogs (10^{-10} to 10^{-3} M) on the production of $^{125}I^-$ (see below) from 0.05 μM [^{125}I]rT_3 (30 nCi) was assessed in 15 min incubations with 3.5 μg microsomal protein/ml. [^{125}I]rT_3 was purified on Sephadex LH-20 to eliminate I^- immediately before use. Inhibition constants (K_i) were determined in triplicate incubations of 0.02–0.5 μM rT_3 for 15 min with 2 μg microsomal protein/ml in the absence or presence of a fixed concentration of TA_3, TA_3S, or 3,3'-TA_2.

Analysis of products

Reactions with radioactive substrate were stopped by addition of 200 μl 0.2 M NaOH, and the resultant mixtures were stored at −20 C, or by addition of 600 μl 0.5 M HCl and processed immediately. Samples were made up in 1 ml 0.20–0.33 M HCl and 20% (vol/vol) ethanol and applied to Sephadex LH-20 columns (0.9 ml bed volume), equilibrated in 0.1 M HCl. Iodide was eluted with 2 × 1 ml 0.1 M HCl (recovery ≈98%), and columns were rinsed with 1 ml water and 0.6 ml 0.1 M aqueous ammonia/ethanol(1:1, vol/vol). The iodothyroacetic derivatives were subsequently eluted with 1.1 ml 0.1 M ammonia in ethanol with a recovery of 94–97% for synthetic TA_3, 3,3'-TA_2, and their sulfates.

Since 3'-T_1S partly elutes with 0.1 M HCl from Sephadex LH-20, Sephadex G-10 columns (bed volume 1 ml, equilibrated in 1.0 M HCl) were used to analyze the deiodination products of ^{125}I-labeled T_2S or 3,3'-T_2S. Elution was performed similarly as described above with a recovery of 96% for T_3S and 3,3'-T_2S.

Production of $^{125}I^-$ from [^{125}I]rT_3 was estimated as trichloroacetic acid (TCA)-soluble radioactivity. For this purpose the reaction was stopped with 100 μl of an ice-cold mixture of human serum and 10 mM PTU in 0.1 M NaOH (1:1, vol/vol). Protein-bound radioactivity was precipitated with 500 μl ice-cold 10% (wt/vol) TCA. After 10 min at 0 C, mixtures were centrifuged, and radioactivity in the supernatant was determined. The mean recovery of $^{125}I^-$ was 96%, and on average 96% of rT_3 and 90% of 3,3'-T_2 were precipitated.

Production of 3,3'-T_2 from unlabeled rT_3 was measured in 0.1 M NaOH extracts of the reaction mixtures by specific RIA (17, 18, 20). In two experiments, production of 3,3'-TA_2 from 0.1–5.0 μM unlabeled TA_3 was estimated similarly using 3,3'-T_2 antiserum and a 3,3'-TA_2 standard curve. Cross-reactivity of 3,3'-TA_2 in the 3,3'-T_2 RIA amounted to 7.0%.

Reversed-phase HPLC

Solvent of samples prepurified on Sephadex was evaporated under a stream of N_2 at 50 C. The residue was redissolved in HPLC mobile phase and injected onto CP Spher or Chromspher C_{18} columns (10 × 0.3 cm; Chrompack International BV, Middelburg, The Netherlands) fitted in a Waters HPLC system [Waters, Milford, MA (21, 22)]. Isocratic or gradient elution was performed with mixtures of methanol or acetonitrile in 0.02 M ammonium acetate (pH 4) at a flow of 0.5–0.8 ml/min, programmed by a model 680 Gradient Controller (Waters). Fractions of 0.3 min were collected and counted for radioactiv-

ity. Further details, depending on the analogs to be separated, are given in the legends to the figures. Retention times of unlabeled, synthetic compounds were determined by monitoring the absorbance of the eluate at 254 nm. The mean recovery of applied radioactivity was greater than 96%.

Data analysis

Product formation in the deiodinase assay was corrected for nonenzymatic deiodination as determined in control incubations without microsomes, *i.e.* 0.9 ± 0.2% for TA_3 or 3,3′-TA_2 (n = 15), and 1.3 ± 0.1% for all sulfated analogs (mean ± SE, n = 49). The slight cross-reactivity of the substrates rT_3 (in the absence or presence of the inhibitors TA_4 or 3,3′-TA_2S) and TA_3 was taken into account when deiodination products 3,3′-T_2 and 3,3′-TA_2 were determined by RIA. TA_3S itself and the deiodination products of TA_4, TA_4S, and 3,3′-TA_2S did not interfere in the 3,3′-T_2 RIA under the conditions of the rT_3 ORD assay. The amount of $^{125}I^-$ generated from labeled rT_3 was multiplied by 2 to account for random deiodination at the 3′ or 5′ position.

Double reciprocal plots of initial velocities *vs.* substrate concentration were drawn by unweighted linear regression analysis. V_{max} and apparent K_m values were determined from the intercepts with the ordinate and abscissa, respectively (23). Apparent K_i values were calculated using the equation: $K'_m = K_m (1 + [\text{inhibitor}]/K_i)$.

Results

Deiodination of 3,3′-TA_2 and TA_3

The conversion of [^{125}I]TA_3 or 3,[3′-^{125}I]TA_2 was studied in relation to microsomal protein concentration and incubation time. Radioiodide and iodothyroacetic acids were quantified by Sephadex LH-20 chromatography and HPLC analysis, respectively. A typical example of the HPLC analysis of the sequential deiodination of TA_3 to 3,3′-TA_2 and 3′-TA_1 is illustrated in Fig. 1. A summary of the results obtained with TA_3 and 3,3′-TA_2 as substrates is given in Fig. 2. These data indicate that deiodination of TA_3 was linear with protein up to 50 μg/ml and with time for at least 30 min (Fig. 2). In addition to I^-, deiodination of 3,3′-TA_2 produced a metabolite eluting on HPLC before 3,3′-TA_2. Most likely this represented 3′-TA_1 (Fig. 1), but no synthetic 3′-TA_1 was available for validation. Because the I^- and 3′-TA_1 production curves were similar, 3,3′-TA_2 was probably deiodinated at random in the inner and outer ring. The results demonstrate that TA_3 is deiodinated only in the inner ring, and the small amounts of I^- are produced indirectly via ORD of 3,3′-TA_2.

Deiodination of TA_3S and 3,3′-TA_2S

We have demonstrated previously that the IRD of T_3 and the subsequent ORD of 3,3′-T_2 are greatly stimulated by sulfate conjugation (2). We have, therefore, also studied the microsomal deiodination of [^{125}I]TA_3S and 3,[3′-^{125}I]TA_2S using low enzyme concentrations and also short incubation periods. A representative set of HPLC analyses of incubations with TA_3S (Fig. 3) shows the sequential production of 3,3′-TA_2S and 3′-TA_1S in relation to remaining TA_3S. The time and protein dependence of product formation, including I^-, from TA_3S is depicted in Fig. 4, A and B. The results demonstrate a distinct, but transient, accumulation of 3,3′-TA_2S and the subsequent generation of predominantly I^- and smaller amounts of 3′-TA_1S. Direct ORD of TA_3S was probably negligible because I^- was not an initial reaction product. That I^- originated from ORD of 3,3′-TA_2S was further substantiated in studies of the deiodination of this intermediate as a function of protein concentration (Fig. 4C) and incubation time (not shown). It is shown that indeed 3,3′-TA_2S was rapidly degraded by ORD to I^- and, to some extent also, by IRD to 3′-TA_1S.

TA_3S and 3,3′-TA_2S were deiodinated by the type I enzyme much more efficiently than the nonsulfated compounds (Fig. 2). Although equal amounts of 3,3′-TA_2 were converted via ORD to I^- and via IRD to 3′-TA_1, 3,3′-TA_2S was preferentially deiodinated in the outer ring (Fig. 4C).

Deiodination of T_3S and 3,3′-T_2S

In previous studies of the type I deiodination of T_3S, its IRD product 3,3′-T_2S was measured by RIA of the 3,3′-T_2 liberated by acid hydrolysis (24). For comparison with the above analysis of TA_3S deiodination we reinvestigated the deiodination products of [^{125}I]T_3S by HPLC (Fig. 5). In agreement with earlier findings using unlabeled T_3S (24) and similar to the above results with TA_3S, transient formation of 3,3′-T_2S was observed which preceded the extensive release of I^-. However, conversion rates were considerably lower with T_3S and 3,3′-T_2S (Fig. 5) than with TA_3S and 3,3′-TA_2S (Fig. 4). In addition, conversion of 3,3′-T_2S to 3′-T_1S was low compared with IRD of 3,3′-TA_2S.

Deiodination kinetics

The effect of sulfation on the deiodination of iodothyroacetic acids was further characterized by determination of the kinetic parameters under initial reaction rate conditions. Circumstances were chosen such that 1) deiodination rates were linear with incubation time and microsomal protein concentration, 2) less than 20% of the substrate was consumed during the reaction, and 3) further deiodination of initial products (*e.g.* 3,3′-TA_2S from TA_3S) was negligible. Linear Linewaever-Burk plots were drawn as shown in Fig. 6, and the thus obtained apparent K_m and V_{max} values are presented in Table 1. As a measure of the efficiency of the different

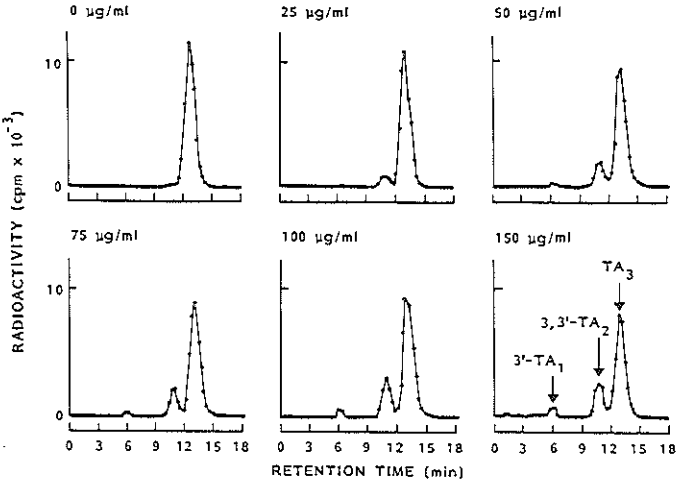

FIG. 1. HPLC analysis of the deiodination of 0.01 µM [^{125}I]TA_3 as a function of microsomal protein. After incubation for 30 min, all radioactivity except I^- was isolated on Sephadex LH-20 and eluted with 0.1 M ammonia in ethanol. The solvent was evaporated and the residue redissolved in mobile phase. The samples were applied to a Cp-tm-Spher C_{18} column which was eluted with a 58:42 (vol/vol) mixture of methanol and 0.02 M ammonium acetate (pH 4) at 0.6 ml/min.

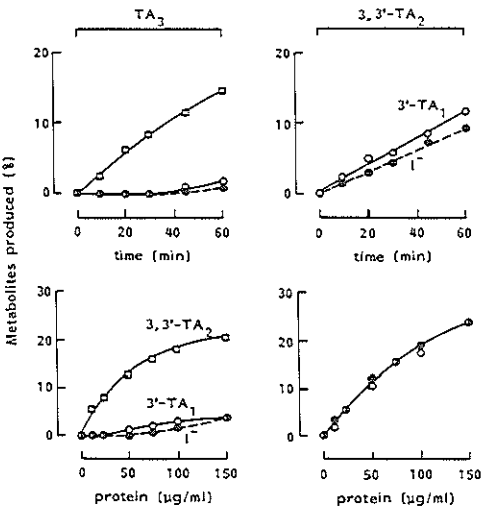

FIG. 2. Deiodination of 10 nM [^{125}I]TA_3 (*left*) or 3,3'-TA_2 (*right*) during incubation for 30 min with varying microsomal protein concentrations (*lower panels*) or for varying time periods with 50 µg microsomal protein/ml (*upper panels*). Production of outer ring labeled 3,3'-TA_2 (□) and/or 3'-TA_1 (○) was quantified by HPLC after isolation of I^- (●) during prepurification of the assay mixture on Sephadex LH-20. The data are the results from one of two (TA_3) or three (3,3'-TA_2) closely agreeing experiments performed under slightly different conditions.

deiodinations the V_{max}/K_m ratios are also given in Table 1. The kinetic parameters of the deiodination of T_3, 3,3'-T_2, and their sulfates determined previously (2) are included for comparison (Table 1). In this study, similar data for T_3 and 3,3'-T_2 deiodination were obtained in experiments performed in parallel with those of the acetic acid analogs (not shown).

Irrespective of the sulfation of the 4'-hydroxyl group, the iodothyroacetic acid derivatives were better substrates for the type I deiodinase than corresponding iodothyronine analogs. In general, this was due to a decrease in K_m value which was most dramatic in the case of TA_3S *vs.* T_3S. The replacement of the alanine side chain by acetic acid resulted in a higher V_{max} value for TA_3 relative to T_3 but decreased the V_{max} of the other analogs, which was again most substantial comparing TA_3S with T_3S.

Sulfation of TA_3 and 3,3'-TA_2 caused a marked decrease in apparent K_m values resulting in high V_{max}/K_m ratios despite a decrease in the V_{max} for TA_3S. The facilitory effect of sulfation on the deiodination of TA_3 and 3,3'-TA_2 was similar or even more pronounced than that for the deiodination of T_3 and 3,3'-T_2, although in case of the IRD of T_3S this was due to an increase in V_{max}. Only the parameters for the ORD of 3,3'-TA_2 were estimated, but the results depicted in Fig. 2 indicate similar rates of IRD and ORD.

Inhibition by PTU

The effect of increasing levels of PTU (0–100 µM) was assessed on the microsomal deiodination of 10 nM ^{125}I-

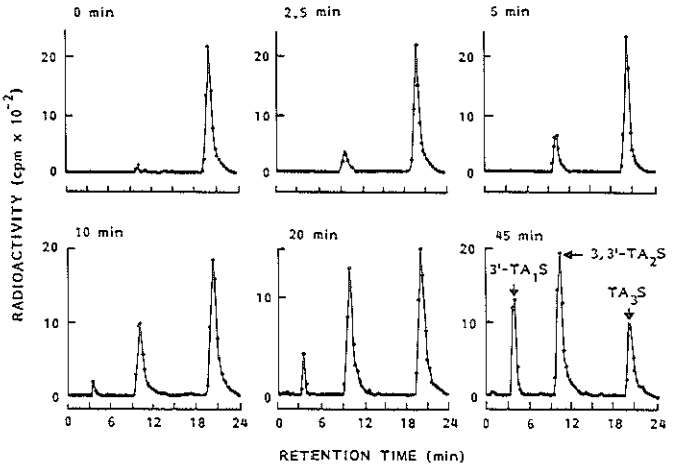

FIG. 3. HPLC analysis of the deiodination of 1 nM [^{125}I]TA_3S incubated for different time periods with 15 μg microsomal protein/ml. Reaction mixtures were precleaned on Sephadex LH-20, and the thus eliminated I^- amounted to 0, 0.8, 2.6, 6.3, 20.0, and 47.7% for the increasing reaction times shown, respectively. The noniodide compounds were applied to a Cp-tm-Spher C_{18} column (t = 0) which was eluted at a flow of 0.8 ml/min with 25% acetonitrile in 0.02 M ammonium acetate (pH 4) until 12.5 min. The proportion of acetonitrile was increased linearly to 30% between 12.5–15 min followed by isocratic elution.

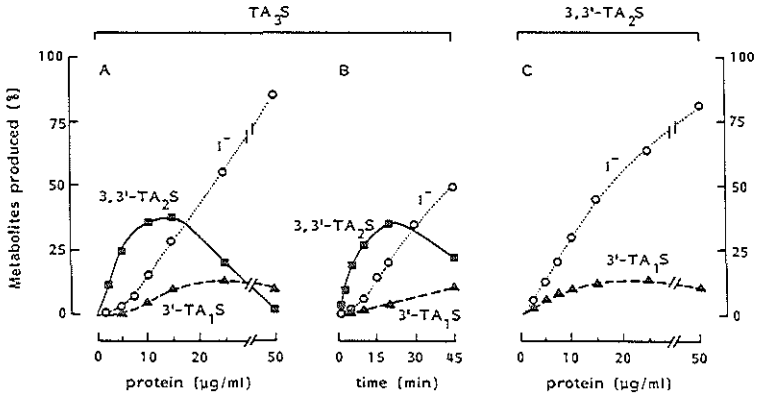

FIG. 4. Deiodination of 1 nM [^{125}I]TA_3S (A and B) or 3,[3'-^{125}I]TA_2S (C) during incubation for 20 min (A) or 15 min (C) with varying enzyme concentrations or for different incubation periods with 15 μg microsomal protein/ml (B). Production of I^- was determined on Sephadex LH-20 and that of 3,3'-TA_2S and 3'-TA_1S by HPLC (Fig. 3). Data are representative (A) or the means from two to four experiments (B and C). The coefficient of variation between experiments amounted to 12–17% for production of I^- and to 5–13% for production of 3,3'-TA_2S or 3'-TA_1S.

labeled iodothyroacetic acid derivatives, and the results with TA_3 and TA_3S are shown in Fig. 7. Under the conditions employed, 20% of TA_3, 31% of 3,3'-TA_2 (not shown), 93% of TA_3S, and 96% of 3,3'-TA_2S (not shown) were deiodinated in control incubations without PTU; 1 μM PTU had little effect on the different deiodinations, and progressive inhibition was observed with 10 and 100 μM of the inhibitor. At the highest dose, IRD of TA_3S and ORD of 3,3'-TA_2 and 3,3'-TA_2S were inhibited by 93–97%, but the IRD of TA_3 was inhibited by only 61%.

Inhibition of rT_3 deiodination

The effect of increasing concentrations of T_3, 3,3'-T_2, TA_3, 3,3'-TA_2, and their sulfates on the production of $^{125}I^-$ from [^{125}I]rT_3 is depicted in Fig. 8. The inhibitor concentrations which resulted in half-maximal inhibition (IC_{50}) were: 2.4 nM TA_3S, 42 nM 3,3'-TA_2S, 0.19 μM 3,3'-TA_2, 0.31 μM TA_3, 0.47 μM 3,3'-T_2S, 1.2 μM T_3S, 4.5 μM 3,3'-T_2, and 5.0 μM T_3. These values are in reasonable agreement with the K_m values for the different analogs.

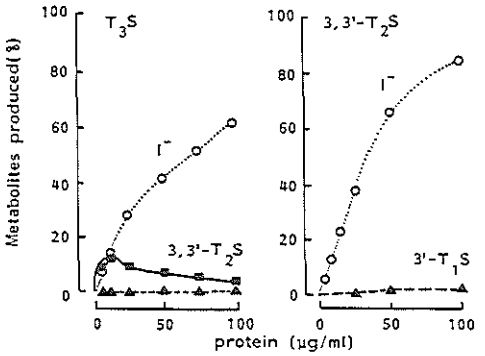

FIG. 5. Deiodination of 10 nM [^{125}I]T_3S (*left*) or 3,3′-T_2S (*right*) for 30 min (T_3S) or 15 min (T_2S) as a function of microsomal protein concentration. The sulfated derivatives were isolated from I^- on Sephadex G-10 and analyzed by HPLC using a Cp-tm-Spher C_{18} column. Elution was performed at a flow of 0.7 ml/min with a concave gradient (no. 8) of 15–25% acetonitrile in 0.02 M ammonium acetate (pH 4) over the first 8 min, and isocratic elution was continued with the 25:75 mixture. Retention times (minutes) were 4.1 for 3′-T_1S, 13.7 for 3,3′-T_2S, and 16.1 for T_3S. Parts of the experiments were repeated on different occasions with closely agreeing results.

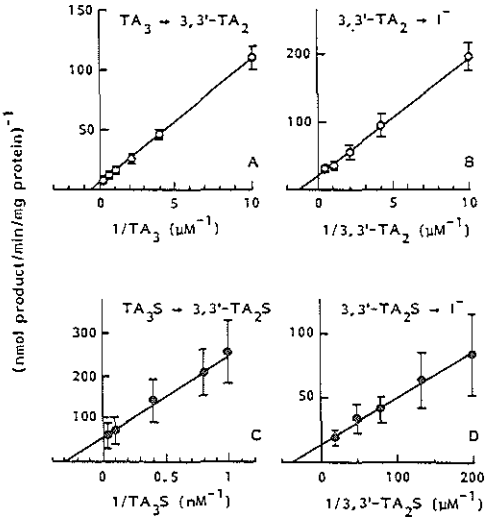

FIG. 6. Double-reciprocal plots of the rate of the IRD of TA_3 and TA_3S (*left*) or the ORD of 3,3′-TA_2 and 3,3′-TA_2S (*right*) *vs.* [^{125}I]substrate concentration. Incubations of 0.1–5.0 µM TA_3 (A) were performed for 30 min with 50 µg microsomal protein/ml and those of 1–25 nM TA_3S (C) for 10 min with 5 µg protein/ml. 3,3′-TA_2 (A) or 3,3′-TA_2S (C) was quantified on HPLC after precleaning of reaction mixtures on Sephadex LH-20. Production of 3,3′-TA_2 from TA_3 was estimated twice by RIA with similar results as those obtained by HPLC analysis. 3,3′-TA_2 (0.1–2.0 µM) (B) was incubated for 30 min with 75 µg protein/ml and 5–50 nM 3,3′-TA_2S (D) for 10 min with 7.5 µg protein/ml. Production of 3′-I^- was determined by Sephadex LH-20 chromatography. Results are the mean ± SD of four (C and D) or five (A and B) experiments.

showing a higher inhibitory potency for the acetic acid derivatives than for the corresponding iodothyronines. However, IC_{50} values were considerably lower than K_m values for TA_3 and 3,3′-TA_2. Lineweaver-Burk analysis of the deiodination of varying rT_3 concentrations in the absence or presence of 1 or 3 µM TA_3, 5 or 10 nM TA_3S, or 0.1 µM 3,3′-TA_2S demonstrated that inhibition was competitive. This provided apparent K_i values (mean ± SD) of 3.7 ± 2.1 nM for TA_3S (n = 6), 0.043 ± 0.16 µM for 3,3′-TA_2S (n = 4), and 0.45 ± 0.30 µM for TA_3 (n = 3). The K_i value of 3,3′-TA_2 could not be determined because of the high cross-reactivity in the 3,3′-T_2 RIA used to measure rT_3 conversion.

Discussion

The present study concerns the further investigation of the structure-activity relationship of substrates for the type I iodothyronine deiodinase. Furthermore, it was examined at the subcellular level whether deiodination of TA_3 by this enzyme contributes to the short half-life of this metabolite in the body relative to T_3. Few reports have appeared about the effects of side-chain modification on the deiodination of thyroid hormone. Extensive studies by Köhrle *et al.* (25, 26) have demonstrated that 1) T_4 analogs with an *N*-acetylalanine or acetic acid side chain are better substrates for the type I deiodinase with approximately 10-fold lower K_m values than T_4 (25), and 2) acetylation of alanine or replacement with acetic acid

TABLE 1. Kinetic parameters for the deiodination of TA_3 and 3,3′-TA_2 and sulfate conjugates by rat liver microsomes in comparison with those of the corresponding iodothyronines

Substrate	Reaction	n	K_m (µM)	V_{max} (pmol/min·mg protein)	V_{max}/K_m
TA_3	IRD	5	1.79 ± 0.53	174 ± 45	97
TA_3S	IRD	5	0.004 ± 0.001	21 ± 8	5568
3,3′-TA_2	ORD	5	0.75 ± 0.27	49 ± 14	65
3,3′-TA_2S	ORD	4	0.023 ± 0.011	64 ± 14	2783
T_3[a]	IRD	5	6.2 ± 0.2	36 ± 7	6
T_3S[a]	IRD	4	4.6 ± 1.3	1050 ± 190	230
3,3′-T_2[b]	ORD	4	8.9 ± 3.9	188 ± 94	21
3,3′-T_2S[c]	ORD	3	0.34 ± 0.07	353 ± 137	1040

Studies were carried out at 37 C in 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA, and 5 mM DTT. Data are the mean ± SD.

[a] See Ref. 17.

[b] See Ref. 24.

[c] See Ref. 35.

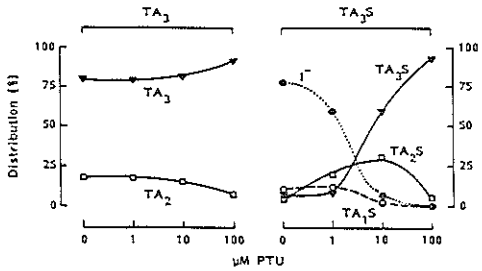

FIG. 7. The effect of PTU on the sequential deiodination of 10 nM [^{125}I]TA_3 (*left*) or [^{125}I]TA_3S (*right*). TA_3 was incubated for 30 min with 100 μg protein/ml and TA_3S for 20 min with 50 μg protein/ml in the presence of 0–100 μM PTU. Before HPLC analysis, reaction mixtures were processed on Sephadex LH-20, resulting in the separation of I^- from the iodothyroacetic acid derivatives. The latter fraction was applied to a Chromspher C_{18} column (t = 0), which was eluted with acetonitrile in 0.02 M ammonium acetate (pH 4). The iodothyroacetic acids were separated by isocratic elution with 38% acetonitrile at a flow of 0.6 ml/min, resulting in retention times (minutes) of 3.6 for 3′-TA_1, 7.3 for 3,3′-TA_2, and 9.8 for TA_3. Elution of sulfated analogs was performed at a flow of 0.8 ml/min initially with 23% acetonitrile, but the proportion of organic solvent was increased linearly from 23–25% between 5–6 min and 25–30% between 10–12 min. 3′-TA_1S, 3,3′-TA_2S, and TA_3S eluted at 3.6, 10.5, and 17.1 min, respectively. Results are the means of duplicate determinations in one experiment, and several conditions were retested on different occasions with similar results.

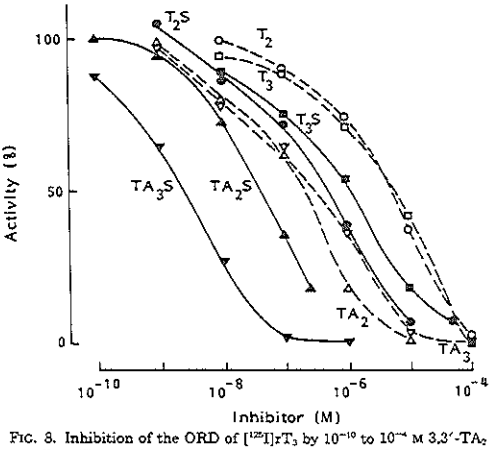

FIG. 8. Inhibition of the ORD of [^{125}I]rT_3 by 10^{-10} to 10^{-4} M 3,3′-TA_2 (△), TA_3 (▽), 3,3′-T_2 (○), T_3 (□), 3,3′-TA_2S (▲), TA_3S (▼), 3,3′-T_2S (●), and T_3S (■). Production of $^{125}I^-$ was determined as TCA-soluble radioactivity. Data are expressed as percent activity of control (without inhibitor) and are the means of four experiments each performed in triplicate.

markedly increases the potency of thyronine analogs with different ring substituents to inhibit the type I deiodination of T_4 (25, 26). Likewise, *N*-acetyl T_3 (27) and TA_3 (28, 29) are more potent inhibitors than T_3 of the ORD of rT_3 by the type I enzyme. An extremely potent inhibitor in this category is *N*-bromoacetyl-T_3 which at subnanomolar levels reacts covalently with the type I deiodinase (27). It has also been shown that TA_3 effectively inhibits the IRD of T_3 by rat brain microsomes (30) or monkey hepatocarcinoma cell preparations (28). However, we are not aware of studies that have tested TA_3 as a substrate for the different deiodinases.

TA_4 and TA_3 are normal constituents of human plasma albeit in low concentration (1, 11). The formation of TA_3 may contribute up to 14% of the metabolism of T_3 in humans (13) in contrast to production of TA_4, which is only a very minor pathway in the metabolism of T_4 (1, 31). TA_3 has been shown to be sulfated in kidney slices (32) and *in vivo* in mammals (7, 10). TA_3 and TA_3S, therefore, represent physiological model compounds in studies of the enzymatic deiodination.

In this paper we present the characteristics of the *in vitro* conversion of the acetic acid analogs of T_3, 3,3′-T_2, and their sulfate esters by rat liver microsomes in the presence of DTT. The close correlation between the potency of the different iodothyroacetic acids as inhibitors of rT_3 deiodination and their corresponding K_m values as well as the inhibition of the deiodination of these side-chain analogs by PTU strongly suggest the involvement of the type I deiodinase. It is noted that the IRD of TA_3 was only partially abolished by 100 μM PTU. This may be explained by the uncompetitive nature of type I enzyme inhibition by PTU (2), although some IRD of TA_3 by a type III-like, PTU-insensitive deiodinase can not be excluded (Eelkman Rooda, S. J., M. H. Otten, M. A. C. van Loon, E. Kaptein, and T. J. Visser, submitted for publication).

Substitution of acetic acid for alanine increases the efficiency of the type I deiodination of T_3 and 3,3′-T_2 as well as their sulfates. The V_{max}/K_m ratios for the ORD of 3,3′-TA_2 and 3,3′-TA_2S are only 3 times higher than those of 3,3′-T_2 and 3,3′-T_2S, but the IRDs of TA_3 and TA_3S are increased about 20-fold compared with T_3 and T_3S. In all cases, side-chain modification results in a decrease in apparent K_m values, while V_{max} may either increase or even decrease. The lowering of the apparent K_m is in agreement with that previously reported for the IRD of TA_4 *vs.* T_4 [0.2 *vs.* 2 μM; (25)]. Apart from the stimulation of the type I deiodination of iodothyronines by replacement of alanine with acetic acid, the preference of the enzyme for the position of the iodine to be eliminated may also be changed. Whereas 3,3′-T_2 only undergoes ORD, 3,3′-TA_2 is deiodinated to similar extents in the outer and inner ring. The sequential deiodination of TA_3 and TA_3S by microsomes is summarized in Fig. 9.

Previous observations in our laboratory have shown

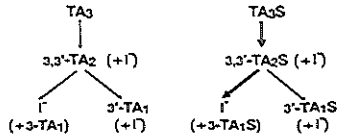

FIG. 9. Sequential deiodination of [3′-^{125}I]TA_3 and [3′-^{125}I]TA_3S by rat liver microsomes. Products within *parentheses* represent nonradiolabeled compounds.

that 4′-*O*-sulfation accelerates the deiodination of iodothyronines by the type I enzyme. Thus, the V_{max}/K_m ratios for the IRD of T_4 and T_3 as well as the ORD of 3,3′-T_2 are increased 40–200 times by sulfate conjugation (2). Sulfation prevents the ORD of T_4 but does not affect the ORD of rT_3 (2, 33). The results of the present study demonstrate that sulfated iodothyroacetic acid derivatives are much better substrates for the type I deiodinase than the nonsulfated analogs. Introduction of the negatively charged sulfate group at the 4′-position induces a dramatic decrease in apparent K_m values. Despite the decreased V_{max} for TA_3S deiodination, the V_{max}/K_m ratios are 40–60 times higher for the sulfates than for TA_3 and 3,3′-TA_2 themselves. In agreement with earlier findings using T_4S (33) and similar to the effects of side-chain modification discussed above, sulfation does not merely facilitate deiodination but it also influences the specificity with which a substrate undergoes ORD or IRD. While 3,3′-TA_2 is equally deiodinated in either ring, deiodination of 3,3′-TA_2S occurs preferentially in the outer ring. However, the specificity of the conversion of T_3 by IRD, as opposed to ORD, is not changed by sulfation or oxidative deamination of the side chain.

The mechanism by which sulfation or deamination of substrates alters the interaction with the type I enzyme remains unknown. Although both modifications usually result in substantially increased V_{max}/K_m ratios, the effect on the separate kinetic parameters varies considerably (present study and Ref. 2). It may be speculated that the introduction of a net negative charge at either site of the substrate molecule favors the interaction with the type I deiodinase of rat liver, which is a basic protein with an isoelectric point (pI) value of 9.3 (34). It is also interesting that sulfation and deamination have additive effects on the type I deiodination of T_3, culminating in the 3 orders of magnitude more rapid IRD of TA_3S.

The preference of the type I enzyme for TA_3 over T_3 as the substrate is in complete correspondence with the higher potency of TA_3 when tested as a deiodinase inhibitor, mentioned above (28, 29). We observed that TA_3 and 3,3′-TA_2 are more effective inhibitors of rT_3 ORD than expected from their apparent K_m values. This difference may be explained by variations in the actual free hormone concentration due to the different protein concentrations used in the microsomal incubations.

On the basis of comparison of the structure-activity relationship of substrates for the type I enzyme and of ligands for T_4-binding prealbumin (TBPA), Köhrle (25, 26) suggested a homology between the binding sites of these proteins (see also Ref. 5). These studies demonstrated that the interaction of iodothyronine derivatives with these proteins was favored by a negatively charged side chain as well as an ionized 4′-substituent. The fact that in comparison with T_3, TA_3 is both a better substrate for the deiodinase and an improved ligand for TBPA is in agreement with this hypothesis. Further support is provided by the high affinity of TA_3S for the type I deiodinase, although its binding characteristics to TBPA remain to be investigated.

Despite an increased binding to plasma proteins (10, 12) the TA_3 half-life is decreased in rats (11, 12) or roughly the same in humans (3, 10) compared with T_3. This discrepancy may be explained by a rapid turnover of TA_3 in the tissues (12). The present results suggest that the efficient deiodination of TA_3 by the type I enzyme, directly or via sulfation, may contribute to its relatively short *in vivo* half-life. However, results obtained with rat hepatocytes and intact rats presented in the accompanying paper (14) indicate that effective glucuronidation and subsequent biliary excretion is an even more important pathway for the *in vivo* metabolism of TA_3 than sulfation and/or deiodination.

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References

1. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5:151
2. Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 189
3. Hennemann G 1986 Thyroid hormone deiodination in healthy man. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 277
4. Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NCW, Freake HC 1987 Advances in our understanding of thyroid hormone action at the cellular level. Endocr Rev 8:288
5. Cody V 1980 Thyroid hormone interactions: molecular conformation, protein binding, and hormone action. Endocr Rev 1:140
6. Jorgensen EC 1978 Thyroid hormones and analogs. II. Structure-activity relationships. In: Li Ch (ed) Hormonal Proteins and Peptides. Academic Press, New York, vol 6:106
7. Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford, p 345
8. Flock EV, Bollman JL, Stobie GHC 1962 Metabolic pathways of tetraiodothyroacetic acid, triiodothyroacetic acid, tetraiodothyropropionic acid and triiodothyropropionic acid. Biochem Pharmacol 11:627
9. Flock EV, Bollman JL 1964 Effect of butyl-4-hydroxy-3,5-diiodobenzoate on the metabolism of L-thyroxine and related compounds.

Endocrinology 75:721
10. Green WL, Ingbar SH 1961 The peripheral metabolism of tri- and tetraiodothyroacetic acids in man. J Clin Endocrinol Metab 21:1548
11. Burger A 1986 Nondeiodinative pathways of thyroid hormone metabolism: In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 255
12. Goslings B, Schwartz HL, Dillmann W, Surks MI, Oppenheimer JH 1976 Comparison of the metabolism and distribution of L-triiodothyronine and triiodothyroacetic acid in the rat: a possible explanation of differential hormonal potency. Endocrinology 98:666
13. Gavin LA, Livermore BM, Cavalieri RR, Hammond ME, Castle JN 1980 Serum concentration, metabolic clearance, and production rates of 3,5,3′-triiodothyroacetic acid in normal and athyreotic man. J Clin Endocrinol Metab 51:529
14. Rutgers M, Heusdens FA, Bonthuis F, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. II. Deiodination and conjugation of TA_3 by rat hepatocytes and in rats *in vivo*. Endocrinology 125:433
15. Rutgers M, Bonthuis F, de Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758
16. Mol JA, Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. Endocrinology 117:1
17. Visser TJ, Fekkes D, Docter R, Hennemann G 1979 Kinetics of enzymic reductive deiodination of iodothyronines. Effect of pH. Biochem J 179:489
18. Visser TJ, Kaptein E, Terpstra OT, Krenning EP 1988 Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 67:17
19. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 115:398
20. Visser TJ, Fekkes D, Docter R, Hennemann G 1978 Sequential deiodination of thyroxine in rat liver homogenates. Biochem J 174:221
21. Eelkman Rooda SJ, Van Loon MAC, Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. J Clin Invest 79:1740
22. De Herder WW, Bonthuis F, Rutgers M, Hazenberg MH, Visser TJ 1988 Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Endocrinology 122:153
23. Segel IH 1975 Enzyme Kinetics. John Wiley & Sons, New York
24. Visser TJ, Mol JA, Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112:1547
25. Köhrle J, Hesch RD 1984 Biochemical characteristics of iodothyronine monodeiodination by rat liver microsomes: the interaction between iodothyronine substrate analogs and the ligand binding site of the iodothyronine deiodinase resembles that of the TBPA-iodothyronine ligand binding. Horm Metab Res 14 [Suppl]:42
26. Köhrle J, Auf'mkolk M, Rokos H, Hesch RD, Cody V 1986 Rat liver iodothyronine monodeiodinase. Evaluation of the iodothyronine ligand binding site. J Biol Chem 261:11613
27. Mol JA, Docter R, Kaptein E, Jansen G, Hennemann G, Visser TJ 1984 Inactivation and affinity-labeling of rat liver iodothyronine-deiodinase with *N*-bromoacetyl-3,3′,-5-triiodothyronine. Biochem Biophys Res Commun 124:475
28. Sorimachi K, Yasumura Y 1981 High affinity of triiodothyronine (T_3) for nonphenolic ring deiodinase and high affinity of tetraiodothyroacetic acid (TETRAC) for phenolic ring deiodinase in cultured monkey hepatocarcinoma cells and in rat liver homogenates. Endocrinol Jpn 28:775
29. Sorimachi K, Niwa A, Yasumura Y 1980 Phenolic ring deiodination in cultured rat hepatoma cells, and subcellular localization of deiodinases in cultured rat hepatoma, monkey hepatocarcinoma cells and normal rat liver homogenates. Biochim Biophys Acta 630:469
30. Kaplan MM, Visser TJ, Yaskoski KA, Leonard JL 1983 Characteristics of iodothyronine tyrosyl ring deiodination by rat cerebral cortical microsomes. Endocrinology 112:35
31. Pittman CS, Shimuzu T, Burger A, Chambers JB 1980 The non-deiodinative pathways of thyroxine metabolism: 3,5,3′,5′-tetraiodothyroacetic acid turnover in normal and fasting subjects. J Clin Endocrinol Metab 50:712
32. Braverman LE, Ingbar SH 1962 Effects of propylthiouracil and thiouracil on the metabolism of thyroxine and several of its derivatives by rat kidney slices *in vitro*. Endocrinology 71:701
33. Mol JA, Visser TJ 1986 Rapid and selective inner ring deiodination of T_4 sulfate by rat liver deiodinase. Endocrinology 117:8
34. Mol JA, van den Berg TP, Visser TJ 1988 Partial purification of the microsomal rat liver iodothyronine deiodinase. I. Solubilization and ion-exchange chromatography. Mol Cell Endocrinol 55:149
35. Otten MH, Mol JA, Visser TJ 1983 Sulfation preceding deiodination of iodothyronines in rat hepatocytes. Science 221:81

Chapter V

METABOLISM OF TRIIODOTHYROACETIC ACID (TA_3) IN RAT LIVER. II: DEIODINATION AND CONJUGATION OF TA_3 BY RAT HEPATOCYTES AND IN RATS IN VIVO

Metabolism of Triiodothyroacetic Acid (TA_3) in Rat Liver. II. Deiodination and Conjugation of TA_3 by Rat Hepatocytes and in Rats *in Vivo**

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ABSTRACT. The hepatic metabolism of 3,3′,5-triiodothyroacetic acid (TA_3), a naturally occurring side-chain analog of T_3, was studied *in vitro* and *in vivo*. Metabolites were quantified by HPLC after Sephadex LH-20 prepurification of samples obtained after incubation of [^{125}I]TA_3 or 3,[3′-^{125}I]diiodothyroacetic acid (3,[3′-^{125}I]TA_2) with isolated rat hepatocytes under various conditions or after iv administration of [^{125}I]TA_3 to normal or 6-propyl-2-thiouracil (PTU)-treated rats. In protein-free incubations with hepatocytes, TA_3 glucuronide (TA_3G) and I^- were normally the main TA_3 products, *i.e.* 44% and 49%, respectively. In the presence of the type I deiodinase inhibitor PTU, the I^- production from added TA_3 decreased to 3%, and TA_3 sulfate (TA_3S) increased from 2–14%. Normally, 3,3′-TA_2 was converted to I^-, but in the presence of PTU 3,3′-TA_2S was produced. In SO_4^{2-}-depleted cultures incubated with TA_3 or 3,3′-TA_2, production of I^- was diminished, and the glucuronides of the substrates and the deiodinated products were generated. If both sulfation and deiodination were inhibited, TA_3 and 3,3′-TA_2 were cleared completely via glucuronidation. The metabolism of TA_3 and especially 3,3′-TA_2 was greatly retarded in cultures with 0.1% BSA. PTU treatment of TA_3-injected rats reduced plasma I^- levels 6-fold, increased plasma sulfates 2.6-fold, but did not affect plasma TA_3 clearance. Biliary excretion of radioactivity until 4 h after [^{125}I]TA_3 injection amounted to 55% of the dose in controls *vs.* 85% in PTU-treated rats. In both groups, an unknown metabolite X was detected in serum and its sulfate conjugate XS in bile. The mean percent distribution of TA_3G/TA_3S/XS in bile amounted to 70:8:13 in control and 57:22:12 in PTU rats. In conclusion, TA_3 is effectively metabolized in rat liver by glucuronidation and subsequent biliary excretion of TA_3G, which may explain its rapid *in vivo* clearance relative to T_3. Furthermore, a significant proportion of TA_3 is deiodinated by the type I deiodinase, either directly or after prior sulfation. (*Endocrinology* 125: 433–443, 1989)

DEIODINATION and conjugation are major pathways in the peripheral metabolism of different iodothyronines (1, 2). Deiodination is catalyzed by membrane-bound enzymes that differ in substrate specificity, reaction kinetics, tissue location, and regulation by thyroid hormone status. The type I deiodinase is predominantly located in liver, kidney, and thyroid and is capable of both inner ring deiodination (IRD) and outer ring deiodination (ORD) (3). The type II deiodinase of brain, pituitary, and brown adipose tissue is a specific ORDase, while the type III enzyme of brain, placenta, and skin is a pure IRDase. Both are insensitive to inhibition by 6-propyl-2-thiouracil (PTU) in contrast to the type I enzyme (3). The liver is also a major site for conjugation of endogenous or exogenous substrates with glucuronic acid or sulfate due to the abundance of uridine diphosphate-glucuronyltransferases in the endoplasmic reticulum and phenol sulfotransferases in the cytoplasma (for reviews see Refs. 4 and 5).

Early *in vitro* studies with homogenates of liver, kidney, and brain demonstrated that extrathyroidal tissues also convert iodothyronines by oxidative deamination and decarboxylation to the corresponding iodothyroacetic acids (1, 6, 7). Free as well as conjugated acetic acid analogs were observed in tissues and body fluids of experimental animals after administration of radiolabeled T_4, T_3, or 3,3′-T_2 (8–13). With the use of specific RIAs tetraiodothyroacetic acid (TA_4) and 3,3′,5-triiodothyroacetic acid (TA_3) were shown to be present in normal human plasma at concentrations of 0.6–1.6 nM (2, 14) and 0.03–0.24 nM (2, 15, 16), respectively. However, lower values of 0.06–0.16 nM TA_4 were obtained using gas chromatography-mass spectrometry (17). In

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man, less than 2% of T_4 is metabolized to TA_4 [daily production ≈1.6 nmol, (2, 14)], and less than 3% of administered TA_4 is converted to TA_3 (2). The approximately 8 nmol TA_3 produced daily in humans (15) is largely derived from side-chain alteration of T_3, and Gavin *et al.* (15) showed that up to 14% of T_3 is metabolized via this route. A daily urinary excretion of 30 nmol thyroacetic acid (TA_0) was estimated in man after [^{14}C]T_4 administration (18). This suggests that side-chain transformation of especially lower iodothyronines is a significant pathway in total T_4 metabolism (2, 19).

TA_3 displays relatively little hormonal activity although it binds more firmly to the nuclear thyroid hormone receptor than T_3 (20–22). Although little is known about the *in vivo* production of iodothyroacetic acids, even less is reported on the metabolic pathways of these thyroid hormone analogs in humans or rats. Despite increased plasma binding, the short half-life in both species of TA_3 relative to T_3 indicates a rapid metabolic turnover and/or effective excretion from the body (2, 21, 23–25).

In rat liver T_3 is conjugated with glucuronic acid as well as sulfate. In contrast to T_3 glucuronide (T_3G), T_3 sulfate (T_3S) is normally rapidly deiodinated[1] (3, 26). Previous studies have shown that after administration of T_3, T_3S accumulates in rat hepatocyte cultures (see Footnote 1) or in bile and plasma of rats if the type I deiodinase is inhibited by PTU (26, 27) or iopanoic acid (28). In the preceding paper (29) we demonstrated that IRD of TA_3 and subsequent ORD of 3,3′-TA_2 by the type I enzyme of rat liver microsomes are facilitated by sulfate conjugation to similar extents as previously observed with T_3 and 3,3′-T_2 (3). The purpose of this study was to investigate the possible significance of these *in vitro* findings for the *in vivo* metabolism of TA_3. We show that in isolated rat hepatocytes and in intact rats TA_3 is metabolized predominantly by glucuronidation and to some extent by successive sulfation and deiodination.

Materials and Methods

Chemicals

Unlabeled or 3′-^{125}I-labeled iodothyronines, iodothyroacetic acids, and their sulfate conjugates were obtained as described elsewhere (29). HEPES, insulin, D-saccharic acid 1,4-lactone, PTU, BSA, sulfatase type VIII, and β-glucuronidase type IX were purchased from Sigma Chemical Co. (St. Louis, MO), and salicylamide (SAM) from Riedel-de Haen AG (Hannover, West Germany). All other reagents were of the highest quality commercially available.

[1] Eelkman Rooda, S. J., M. H. Otten, M. A. C. van Loon, E. Kaptein, and T. J. Visser, submitted for publication.

Experiments with rat hepatocytes

Hepatocytes were isolated from normally fed male Wistar rats (Harlan, Zeist, The Netherlands; 180–250 g body wt) by collagenase perfusion (30). Cells were seeded at a density of 10^6 per well (φ 3.5 cm), and 4 h after plating the medium with nonviable cells was aspirated (see Footnote 1). The monolayers were rinsed once and replenished with Hanks balanced salt solution supplemented with 25 mM HEPES (pH 7.4), 1 mM vitamin C, 2 mM glutamine, and 12 mU/liter insulin (Hanks medium). Incubations were performed (in duplicate) for 3 h at 37 C with 10 nM ^{125}I-labeled substrate (≤0.1 μCi) in 2 ml Hanks medium with or without 0.1% (wt/vol) BSA or 100 μM PTU, unless stated otherwise. After the incubation, the medium was collected and cells were extracted immediately with 1 ml 0.1 M NaOH. Medium and cell extracts were stored at −20 C until further analysis of radioactivity on Sephadex LH-20 and HPLC. The $^{125}I^-$ observed after control incubations of tracer in Hanks medium without hepatocytes amounted to only 0.35 ± 0.02% (mean ± SE, n = 61) and was neglected.

Cultures were depleted of SO_4^{2-} by a 40 min preincubation in Hanks medium containing 1 mM $MgCl_2$ instead of $MgSO_4$ and supplemented with 100 μM SAM (see Footnote 1). The monolayers were then washed and incubated with substrate and, if required, PTU in the same medium without SAM. Parallel controls were preincubated and incubated in SO_4^{2-}-complete Hanks medium without SAM.

Experiments with rats in vivo

Male Wistar rats weighing approximately 200 g were anesthetized with 6 mg pentobarbital/100 g body wt. A single injection of 1 mg PTU/100 g body wt was given ip, and controls received an equivalent amount of vehicle (30 mM NaOH in PBS). The common bile duct was cannulated (26) and approximately 1.5 h after PTU administration 10 μCi [^{125}I]TA_3 in about 500 μl 0.01 M NaOH in saline was injected in the penile vein (t = 0). Bile was collected over 15 or 30 min periods, and blood samples (≈800 μl) were taken from the tail vein at 0.5, 1, and 2 h. Body temperature (36–38 C), hydration, and anesthesia were maintained as previously described (26, 27). The animals were bled at 4 h by heart puncture, and radioactivity in serum and bile was analyzed by different chromatographic techniques.

Sephadex LH-20 chromatography

Prepurification of iodothyroacetic acid metabolites before HPLC analysis was performed on Sephadex LH-20. Hepatocyte incubation medium or cell extract was made up in 0.2–0.3 M HCl and 20% (vol/vol) ethanol and applied to small Sephadex LH-20 columns (29). Elution was performed as previously described (29), *i.e.* I^- was isolated in 0.1 M HCl, and conjugates plus nonconjugated compounds were collectively obtained in 0.1 M ammonia in ethanol. Alternatively, the elution of iodide was followed by a separation of conjugates from nonconjugated compounds. First, conjugates were eluted with 8 × 1 ml 20% (vol/vol) ethanol in water with a recovery of 95.4 ± 1.3% (±SE, n = 7) for synthetic 3,[3′-^{125}I]TA_3S or [^{125}I]TA_3S. Then, the nonconjugated compounds were isolated in 3 × 1 ml 0.1 N NaOH/ethanol (1:1, vol/vol) with a recovery of 98.7 ± 0.5%

(±SE, n = 40) for pure radiolabeled 3,3′-TA_2 and TA_3. Less than 0.2% of the applied 3,[3′-^{125}I]TA_2 or [^{125}I]TA_3 eluted in the conjugate fraction. Samples from parallel incubations with T_3 or 3,3′-T_2 were analyzed on Sephadex LH-20 in the same way.

Conjugates were concentrated by loading pooled 20% ethanol in water fractions, adjusted to 0.2 M HCl, on Sephadex LH-20 columns (0.9 ml bed volume) equilibrated in 0.1 M HCl. The columns were rinsed successively with 1 ml 0.1 M HCl, 0.6 ml water, and 0.6 ml 0.1 M ammonia in ethanol. The applied radioactivity was eluted quantitatively with 1.1 ml 0.1 M ammonia in ethanol.

Serum (≤100 μl) and bile (≤50 μl) were prepared in 0.1 M HCl with 25% (vol/vol) ethanol and applied to Sephadex LH-20 columns (1.3 ml bed volume) equilibrated in 0.1 M HCl. In succession, 1 ml aliquots of 0.1 M HCl (4×) and 25% ethanol in water (10×) were applied to isolate I^- and conjugates, respectively. The columns were washed with 750 μl 0.1 M ammonia in ethanol, and all retained radioactivity was eluted with 1.2 ml 0.1 M ammonia in ethanol (modified from Ref. 27). The recovery of [^{125}I]TA_3S, TA_3G, or TA_1 added to normal serum or bile amounted to on average 95%.

Solid-phase extraction (SPE)

Before HPLC analysis of radioactivity in serum, aliquots of 250–500 μl were precleaned on C_{18}-SPE columns (500 mg, J. T. Baker Chemical Co., Phillipsburg, NJ). Samples were processed as previously described (27), providing a methanol extract containing all serum radioactivity except I^-. The recovery of rat serum spiked with [^{125}I]TA_3 or [^{125}I]TA_3S was greater than 97%.

Reversed-phase HPLC

A Waters HPLC system (Waters Associates, Milford, MA; Refs. 26 and 31) was used with a 10 × 0.3 cm Chromspher C_{18} analytical column and a 10 × 2.1 mm reversed-phase guard column (Chrompack International BV, Middelburg, Netherlands). The column was equilibrated in 24% acetonitrile in 0.02 M sodium phosphate (pH 2.7) and eluted with a linear gradient of 24–28% acetonitrile over the first 12 min after sample injection. Between 12–20 min, the acetonitrile concentration was increased to 44% as programmed by the nonlinear gradient no. 7 of the model 680 Gradient Controller (Waters). Solvent composition was maintained at 44% acetonitrile until greater than 33 min. The flow was 0.8 ml/min, the radioactivity in the eluate was measured in 0.3 min fractions, and the absorbance at 254 nm was monitored continuously. Calibration was carried out using ^{125}I-labeled or unlabeled synthetic TA_3, TA_3S, 3,3′-TA_2, and 3,3′-TA_2S, biosynthetic 3′-TA_1 and 3′-TA_1S (29) as well as the different glucuronides (isolated from hepatocyte incubations, present study). All available compounds were clearly separated in a single run as is shown in Fig. 1.

Samples were prepared for HPLC by evaporation of the alcoholic solvents at 50 C under a stream of N_2, and the residues were redissolved in 24% acetonitrile in 0.02 M sodium phosphate (pH 2.7). Elution positions of the extracted radioactive substances were the same as that of the pure reference compounds. Metabolites in unprocessed bile (<25 μl) were quantified directly by application of a 1:5 dilution of bile in mobile phase to the HPLC system. The presence of 15–25 μl bile accelerated the elution of especially TA_3S by 0.5–1.2 min.

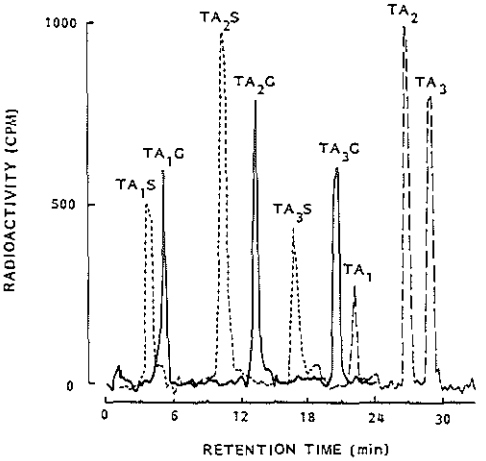

FIG. 1. Separation of 3′-^{125}I-labeled free and conjugated iodothyroacetic acids by reversed-phase C_{18} HPLC. Elution was performed at a flow of 0.8 ml/min, first with a linear gradient of 24–28% acetonitrile (0–12 min) in 0.02 M sodium phosphate (pH 2.7), followed by an increase to 44% acetonitrile (12–20 min). Fractions (0.3 min) were collected and counted for radioactivity. The elution profile was constructed from three runs, performed with several of the available ^{125}I-labeled compounds (see *Materials and Methods*).

Identification of iodothyroacetic acid metabolites

Conjugates were isolated from rat hepatocyte cultures or rat serum on Sephadex LH-20 and treated with sulfatase or β-glucuronidase. Incubations were performed for 18–20 h at 37 C with sulfatase type VIII (250 μg/ml) in 0.05 M sodium acetate (pH 5.0), or for 2 h with β-glucuronidase type IX (50 μg/ml) in 0.05 M sodium phosphate (pH 6.8) using a reaction volume of 500–750 μl. D-Saccharic acid 1,4-lactone (5–10 mM) was included in reaction mixtures with sulfatase to inhibit possible glucuronidase activity. Unprocessed bile (30–50 μl) or biliary radioactivity isolated on HPLC was treated similarly. After digestion, the mixtures were made up in 0.2–0.3 M HCl and 10–20% (vol/vol) ethanol and fractionated on Sephadex LH-20 (see above) to separate the liberated iodothyroacetic acids from the remaining conjugates before HPLC analysis.

Control incubations were done with pure ^{125}I-labeled synthetic T_3S which was fully resistant to the β-glucuronidase treatment, and biosynthetic T_3G was shown to be stable in incubations with sulfatase. Approximately 94% (n = 8) of each T_3 conjugate was hydrolyzed by the proper enzyme independent of the presence of up to 50 μl bile.

Statistical analysis

Data are given as means ± SE, and differences were considered to be significant for $P < 0.05$ as determined by Student's unpaired t test (32).

Results

Metabolism of TA_3 and 3,3′-TA_2 by rat hepatocytes

Time dependence (Fig. 2). Initially, monolayers of hepatocytes were incubated under standard conditions, *i.e.* using 10 nM substrate in SO_4^{2-}-replete Hanks medium with 0.1% BSA. Distribution of TA_3 and 3,3′-TA_2 metabolites was compared with that of metabolites produced from T_3 and 3,3′-T_2 in parallel experiments (Fig. 2). After 3 h the cell-associated radioactivity was 15% (T_3) or less. Production of I^- from TA_3, T_3, and 3,3′-TA_2 was linear with incubation time up to 3 h. In comparison with T_3, incubations with TA_3 yielded less I^- ($P < 0.05$) but more conjugates. Unlike 3,3′-T_2, which was largely converted to I^- within 1 h, metabolism of 3,3′-TA_2 was slow. Less than 7% conjugates were produced from either 3,3′-T_2 or 3,3′-TA_2.

Effect of TA_3 substrate concentration (Fig. 3). Hepatocyte cultures were incubated for 3 h with 1–10,000 nM [^{125}I]TA_3 in medium with 0.1% BSA and SO_4^{2-} with or without 100 μM PTU. The amounts of I^- and conjugates isolated from the medium on Sephadex LH-20 are presented in Fig. 3. Less than 10% of total radioactivity was associated with the cells. The percentage production of I^- was decreased due to saturation of the deiodinase at higher TA_3 concentration or by addition of PTU, and this was accompanied by an increase in conjugates. As shown by β-glucuronidase treatment and HPLC TA_3G represented 35.8 ± 1.2% of the radioactivity in the latter fraction (n = 10), independent of substrate concentration or addition of PTU. Thus, TA_3G was always the major metabolite observed. Small amounts of TA_3S were found mainly at high substrate concentrations or in incubations with PTU, amounting, at the most, to ≈10% of medium radioactivity. At the higher substrate concentrations, clearance of TA_3 from the medium increased from 48–61%, but these differences were not significant.

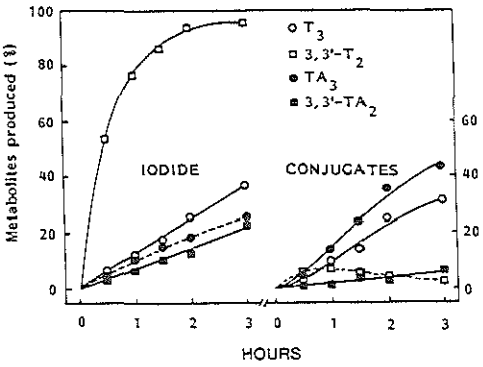

FIG. 2. Metabolism of TA_3, T_3, 3,3′-TA_2, and 3,3′-T_2 in primary cultures of rat hepatocytes that were incubated with 10 nM ^{125}I-labeled substrate in SO_4^{2-}-complete Hanks medium in the presence of 0.1% BSA. At the indicated time points, small aliquots (≤75 μl) of the medium were withdrawn, and the production of I^- (*left*) and conjugates (*right*) was estimated by Sephadex LH-20 chromatography. Results are the means of two to four experiments and expressed as percent of medium radioactivity. The coefficient of variation between experiments amounted to 4–23% for the production of I^- and to 27% for the different conjugates.

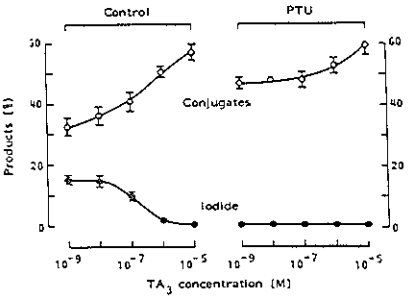

FIG. 3. Effects of substrate concentration and PTU on the metabolism of TA_3. Monolayers of rat hepatocytes were incubated for 3 h with 1 nM to 10 μM [^{125}I]TA_3 in SO_4^{2-}-complete Hanks medium with 0.1% BSA, in the absence (*left*) or presence (*right*) of 100 μM PTU. The I^- and conjugates in the medium were isolated on Sephadex LH-20 and expressed as percent of medium radioactivity. Results are the means ± SE from three to five experiments.

Effect of BSA (Table 1). The metabolism of TA_3 and 3,3′-TA_2 under different conditions was studied in detail by HPLC analysis (Fig. 1). The effect of the presence of BSA in SO_4^{2-}-replete incubations without PTU is illustrated in Table 1. These data clearly demonstrate that the metabolism of TA_3, and especially that of 3,3′-TA_2, was markedly enhanced in hepatocyte cultures without BSA. In BSA-containing cultures, SO_4^{2-}-depletion and/or PTU addition decreased, although not significantly, the clearance of TA_3 from 52% to 42–47% and that of 3,3′-TA_2 from 23% to 10–18% (not shown). In contrast, clearances of TA_3 or 3,3′-TA_2 were near-complete (>93%) in incubations without BSA even after depletion of SO_4^{2-} or addition of PTU (not shown). SO_4^{2-} depletion and/or addition of PTU produced similar effects on the proportions of sulfate and glucuronide conjugates generated from TA_3 or 3,3′-TA_2 if hepatocytes were incu-

TABLE 1. Effect of BSA on the *in vitro* metabolism of TA_3 and 3,3′-TA_2

	10 nM 3,5,[3′-^{125}I]TA_3				10 nM 3,[3′-^{125}I]TA_2			
	n	Products: Iodide (%)	Products: Conjugates (%)	Remaining substrate (%)	n	Products: Iodide (%)	Products: Conjugates (%)	Remaining substrate (%)
+BSA	5	12.6 ± 2.3	33.5 ± 5.0	48.4 ± 5.9	2	20.9/18.3	3.7/0.9	74.9/78.5
−BSA	6	48.8 ± 5.2	50.0 ± 8.0	1.8 ± 0.5	3	66.8 ± 7.2	28.8 ± 9.0	1.0 ± 0.5

Monolayers of rat hepatocytes were incubated for 3 h with radiolabeled TA_3 or 3,3′-TA_2 in Hanks medium containing SO_4^{2-} (without PTU) in the presence or absence of 0.1% BSA. Afterward, 97% (+BSA) or 92% (−BSA) of total radioactivity was recovered in the medium. The I^- produced was eliminated by prepurification of medium and of cell-associated radioactivity on Sephadex LH-20. Conjugates and nonmetabolized substrates were quantified by subsequent HPLC analysis. Data are given as percent (mean ± SE) of total radioactivity.

bated in the presence or absence of BSA. Therefore, data from BSA-free cultures incubated with [^{126}I]TA_3 and 3,[3′-^{125}I]TA_2 are presented in detail because of the higher yield of products.

Effect of PTU and SO_4^{2-} (Figs. 4 and 5). Figure 4 shows HPLC profiles representing typical composition of TA_3 and 3,3′-TA_2 metabolites after incubation in medium without BSA. Quantification of products, including I^-, under the various conditions tested is summarized in

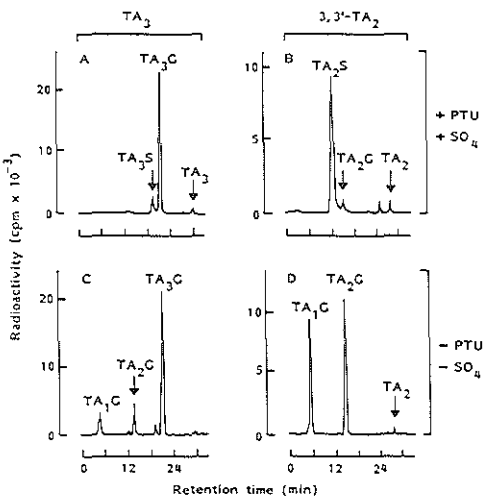

FIG. 4. HPLC analysis of metabolites produced by hepatocytes from 10 nM [^{126}I]TA_3 (A and C) and 3,[3′-^{125}I]TA_2 (B and D). Monolayers were preincubated and incubated in Hanks medium without BSA under SO_4^{2-}-complete (A and B) or SO_4^{2-}-deplete (C and D) conditions. After preincubation, 100 μM PTU was added in panels A and B. All radioactivity, except I^-, was extracted on Sephadex LH-20 (*Materials and Methods*) and analyzed by HPLC (Fig. 1). Chromatograms represent 97, 99, 87, and 58% of medium radioactivity due to removal of I^- from samples A, B, C, and D, respectively. Recovery of radioactivity applied to HPLC was 99.4 ± 0.5% (n = 99).

Fig. 5.

In SO_4^{2-}-complete cultures, TA_3 was equally converted to I^- and TA_3G (Table 1). Addition of PTU reduced production of I^- from 49% to 3% and increased TA_3S 7-fold from 2% to 14% (Fig. 4A). Generation of TA_3G was also stimulated from 44–62%, and only 7% of added TA_3 remained after 3 h.

In incubations without SO_4^{2-}, I^- production was markedly reduced, but not eliminated, amounting to 8%. In addition to 65% TA_3G, appreciable amounts of 3,3′-TA_2G (10%) and 3′-TA_1G (4%) were detected (Fig. 4C) in contrast to SO_4^{2-}-containing controls. It was noted that after incubation in the presence of BSA, up to 5% of only nonconjugated 3,3′-TA_2 accumulated in SO_4^{2-}-deplete cultures (not shown). TA_3G was virtually the only product formed when both sulfation and deiodination were prevented, and even under these conditions 95 ± 1% of added TA_3 was metabolized.

In SO_4^{2-}-replete cultures without PTU, the majority of the 3,[3′-^{125}I]TA_2 was deiodinated (Table 1). Some conjugates were also observed, mostly in the form of sulfates, *i.e.* 15% 3,3′-TA_2S and 6% 3′-TA_1S. Addition of PTU nullified I^- production (<1.5%), which was accompanied by a 4.8-fold increase in 3,3′-TA_2S from 15–72% (Fig. 4B). 3,3′-TA_2G was also higher in PTU *vs.* control incubations, *i.e.* 13% *vs.* 5.4%, and in these incubations with PTU 96 ± 1% of added 3,3′-TA_2 was still metabolized.

In SO_4^{2-}-deplete cultures without PTU a considerable amount of I^- (≈28%) was still generated. Glucuronides were most abundant as illustrated by the production of 50% 3,3′-TA_2G and 8% 3′-TA_1G (Fig. 4D) in the absence of PTU or 85% 3,3′-TA_2G in the presence of PTU. Therefore, SO_4^{2-}-depleted hepatocytes metabolized 3,3′-TA_2 largely via glucuronidation, being a minor pathway in SO_4^{2-}-replete cultures.

Metabolism of TA_3 in rats

Identification of conjugates (Fig. 6). In bile and plasma of [^{125}I]TA_3-injected rats several radioactive metabolites

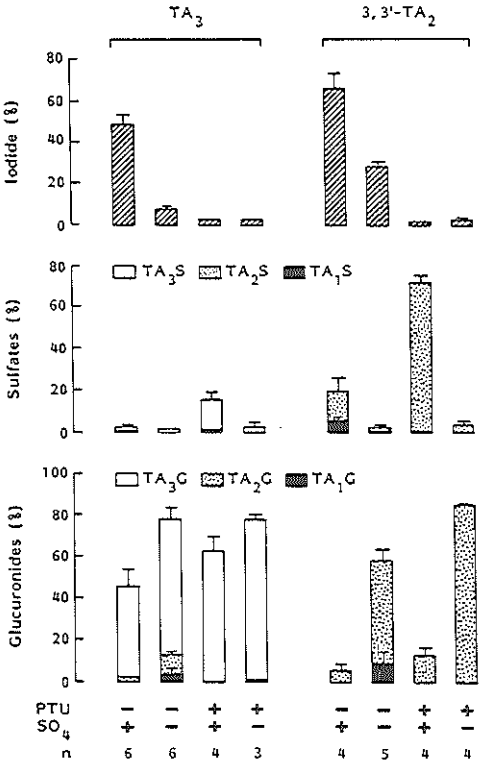

FIG. 5. Effects of SO_4^{2-} and PTU on the metabolism of 10 nM [^{125}I]TA_3 (*left*) and 3,[3′-^{125}I]TA_2 (*right*). Hepatocytes were incubated in the absence of BSA, essentially as described in the legend to Fig. 4. Radioactivity in medium and cells, representing on average 81% and 19% of total, was analyzed. Production of I^- (*upper panel*) was estimated on Sephadex LH-20, and sulfates (*middle panel*) and glucuronides (*bottom panel*) were quantified by HPLC. Data are expressed as percent (mean ± SE) of total radioactivity.

were identified. At least 91% of the sulfate and more than 95% of the glucuronide conjugates of the iodothyroacetic acids were hydrolyzed by specific treatment with either the sulfatase or β-glucuronidase, respectively. Four typical radioactive compounds in bile were characterized by HPLC and enzyme digestions as shown in Fig. 6. In addition to the 3,3′-TA_2S, TA_3S, and TA_3G also observed *in vitro* (Fig. 4), another conjugate XS was detected in bile and plasma of normal and PTU-treated rats. It was hydrolyzed by treatment with sulfatase (Fig. 6B), but the liberated compound X (Fig. 6C) was not further identified.

Biliary metabolites (Fig. 7). Radioactivity excreted in bile after iv administration of [^{125}I]TA_3 was 50% higher after PTU treatment of rats, amounting after 1 h to 60 ± 1% of the dose *vs.* 42 ± 4% in controls (Fig. 7). Between 0.5 and 4 h after TA_3 injection the proportion of excreted TA_3G decreased from more than 70% to 53% in controls and to 39% in PTU rats, but in all samples TA_3G remained the major metabolite. In 0–4 h bile pools the mean percent distribution of $TA_3S/TA_3G/XS$ was 8:70:13 in controls and 22:57:12 in PTU rats (Fig. 7). The remaining ≈10% consisted of I^- (<2.6%), 3,3′-TA_2 conjugates (<2%), TA_3 (<2.5%), and minor unidentified compounds. Only in the later bile samples from PTU-treated rats were considerable proportions of up to 11% 3,3′-TA_2S observed (Fig. 6A). PTU treatment significantly increased the total biliary disposition of TA_3G and TA_3S by 1.3- and 3.9-fold, respectively, but excretion of XS was not affected.

Plasma metabolites (Figs. 8 and 9). Distribution of non-iodide radioactivity in rat plasma 4 h after injection of [^{125}I]TA_3 is illustrated by typical HPLC elution profiles in Fig. 8. Time-dependent production of metabolites, including I^-, and clearance of injected tracer are depicted in Fig. 9. Total radioactivity expressed as percent dose per ml plasma was not different in control and PTU-treated rats. HPLC of the nonconjugate fraction isolated on Sephadex LH-20 demonstrated that the mean ratio of TA_3/X amounted to 80:17 in controls and to 88:12 in PTU rats, but 3,3′-TA_2 was not observed. At 2–4 h, plasma I^- in normal rats exceeded conjugates as well as injected TA_3. PTU had no effect on plasma TA_3 clearance, but reduced I^- levels (percent dose/ml) by 76–87% and increased plasma conjugates by 1.7- to 3.6-fold. The latter fraction predominated from 1 h onward and largely consisted of sulfates (HPLC). After 2 h 3,3′-TA_2S was the major radiolabeled metabolite in plasma of PTU-treated rats, while roughly similar amounts of XS were found in both groups. TA_3G was a minor plasma metabolite and was not affected by PTU (Fig. 9).

Miscellaneous. The nonprotein-bound fractions of iodothyronines and analogs in Hanks medium containing 0.1% BSA were determined in duplicate by equilibrium dialysis. The free fractions (n = 3–6) were: 0.6 ± 0.2% for 3,3′-TA_2, 1.7 ± 0.04% for TA_3, 8.9 ± 0.3% for 3,3′-T_2, and 6.4 ± 0.3% for T_3.

Biosynthetic ^{125}I-labeled TA_3G and 3,3′−TA_2G were tested twice as substrate for the type I deiodinase in incubations for 1 h at 37 C with 100 μg/ml rat liver microsomes and 5 mM dithiothreitol as described elsewhere (29). Analysis of reaction mixtures by HPLC established that deiodination of the glucuronides did not

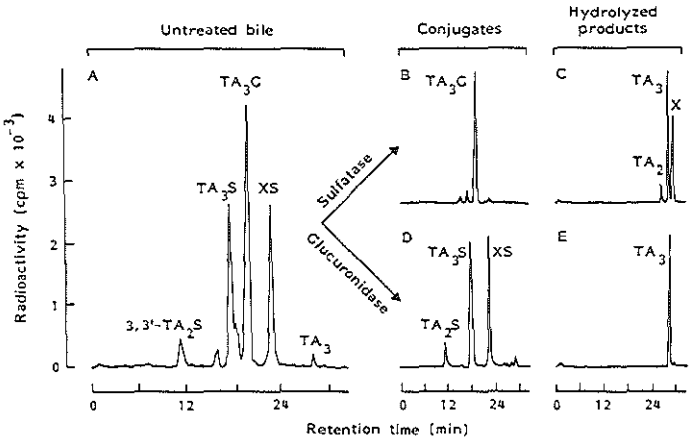

FIG. 6. Identification of TA_3 metabolites. Bile, collected 90–120 min after iv injection of [^{125}I]TA_3 to two PTU-treated rats, was combined and a 12 μl aliquot was analyzed by HPLC (A). After treatment of bile with sulfatase (B and C) or β-glucuronidase (D and E) the conjugated and nonconjugated iodothyroacetic acids were separated on Sephadex LH-20 and analyzed by HPLC (detailed in *Materials and Methods*). Elution profiles of the compounds liberated are depicted in panels C and E and those of the remaining conjugates in panels B and D. The radioactivity applied to HPLC was equivalent with 32–40 μl bile, and the recovery of radioactivity over HPLC amounted to 101 ± 1% (n = 41).

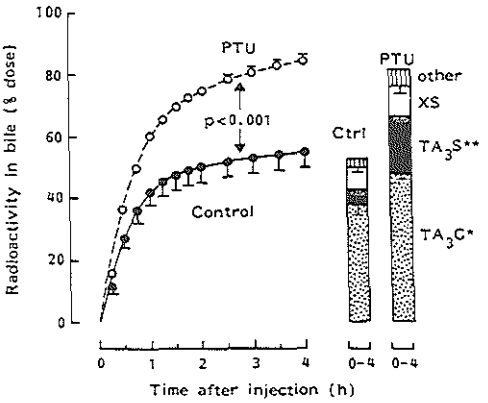

FIG. 7. Effect of PTU on the biliary excretion of TA_3 metabolites. Control and PTU-treated rats were injected iv with [^{125}I]TA_3. Cumulative excretion of radioactivity in bile is depicted on the *left* as a function of time after tracer administration to control (●) and PTU rats (○). *Bars* represent total excretion (0–4 h) of individual metabolites as determined by HPLC of bile pools. Data are expressed as mean percent of the injected dose (±SE unless smaller than symbol, n = 4). Bile flow amounted to 502 ± 28 μl/h·100 g body wt, independent of PTU. *, $P < 0.05$; **, $P < 0.001$.

occur in contrast to nonconjugated 3,3′-TA_2 and TA_3 and especially their sulfate conjugates (29).

Discussion

Glucuronidation is a significant pathway in the metabolism of T_3 in rat hepatocytes (see Footnote 1) as well as

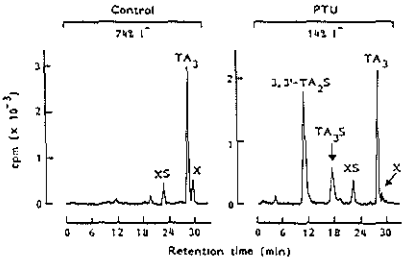

FIG. 8. HPLC analysis of plasma radioactivity after iv administration of [^{125}I]TA_3 to control (*left*) or PTU-treated rats (*right*). Serum obtained after 4 h was processed by SPE-C_{18} before HPLC analysis, and metabolites were identified by sulfatase and β-glucuronidase treatment. Chromatograms A and B represent 26% and 86% of total plasma radioactivity due to removal of mainly I^- by SPE, and recovery of radioactivity on HPLC was 95.4 ± 2.8% (n = 20).

in the metabolism of T_3 and T_4 in experimental animals (9, 26, 27, 33) and humans (34). We recently showed that biliary excretion of T_3S (26, 27) and also T_4S (33) in rats is greatly stimulated by PTU treatment due to the decreased deiodinative clearance of these sulfates by the type I enzyme. The half-life of circulating TA_3 in rats is substantially shorter than that of T_3 (2, 23, 25), and the same probably also holds for humans (15, 24, 35). TA_3 and 3,3′-TA_2 are more rapidly converted by the type I deiodinase of rat liver microsomes than T_3 and 3,3′-T_2, and the deiodination is further enhanced by 4′-OH sulfation of these compounds (29). The present study examines to what extent the conjugation and deiodination pathways contribute to the rapid metabolic clearance of TA_3.

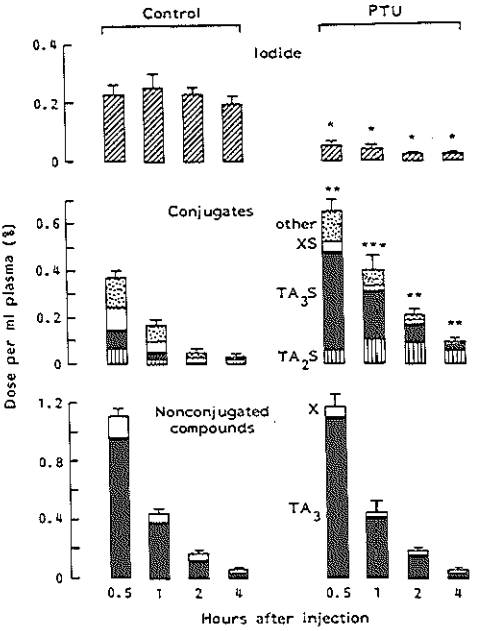

FIG. 9. Effect of PTU on plasma TA_3 and metabolites. Rats were injected iv with [^{125}I]TA_3 after pretreatment with saline (*left*) or PTU (*right*), and serum was obtained up to 4 h. Serum was analyzed for I^- (*upper*), conjugates (*middle*), and nonconjugated compounds (*bottom*) by Sephadex LH-20 chromatography. The conjugate and nonconjugate fractions were subdivided into the different components on the basis of HPLC. Radioactivity is given as percent (mean ± SE) dose per ml plasma (n = 4). Except for plasma disappearance of TA_3, differences between control and PTU rats are significant: ***, $P < 0.025$; **, $P < 0.01$; and *, $P < 0.001$.

Metabolism of TA_3 and 3,3′-TA_2 by rat hepatocytes

First, a detailed study was made of the metabolism of TA_3 in primary cultured rat hepatocytes, which have been shown to metabolize iodothyronines by glucuronidation, sulfation, and deiodination (see Footnote 1 and Refs. 30 and 31). Incubations of TA_3 or 3,3′-TA_2 with rat hepatocytes resulted in mixtures of deiodinated and/or conjugated products, which were separated by a modified HPLC system (29) using solvent buffered at pH 2.7. An accurate resolution of all metabolites was obtained, even for 3,3′-TA_2G and TA_3S, which coeluted at pH 4 used in previous experiments (29).

In the presence of BSA, hepatocytes metabolize TA_3 at rates similar to the metabolism of T_3 despite a 3.5-fold higher free fraction of T_3. TA_3 undergoes largely glucuronidation whereas T_3 is conjugated to similar extents with glucuronic acid and sulfate. Under normal conditions the T_3S is rapidly degraded by the PTU-sensitive type I deiodinase via 3,3′-T_2S to I^-. Irrespective of the presence of BSA, the amounts of TA_3S detected after incubation of TA_3 with hepatocytes treated with PTU is only one third of the proportion of T_3S accumulating from T_3 in the presence of this inhibitor (see Footnote 1). The low degree of TA_3 sulfation is further supported by the small amounts of TA_3S observed at high TA_3 substrate concentrations. In consideration of the low K_m value of TA_3S for the type I deiodinase [4 nM (29)], significant sulfation of TA_3 would have resulted in the ready saturation of the enzyme with TA_3S and the consequent accumulation of this conjugate.

The cellular metabolism of TA_3 proceeds more rapidly than that of T_3 in BSA-free cultures, since TA_3 is metabolized completely in 3 h (this study) whereas only approximately 60% of T_3 is cleared under comparable SO_4^{2-}-replete conditions (see Footnote 1). In the absence of BSA, roughly equal proportions of TA_3 are converted to TA_3G and I^-. In SO_4^{2-}-deplete monolayers the latter is significantly decreased, but not abolished, indicating that only part of the I^- production occurs via successive sulfation and deiodination. In general, under SO_4^{2-}-deplete conditions in presence of PTU, production of sulfates was negligible (<4%) thus excluding SO_4^{2-} contamination. Therefore, the I^- in the SO_4^{2-}-deplete cultures without PTU was generated by direct IRD of TA_3 and subsequent ORD of 3,3′-TA_2.

In contrast to T_3 (see Footnote 1), sulfation is not a rate-limiting step in the metabolism of TA_3, since the latter is not affected by SO_4^{2-}-depletion. Apparently, when the pathway via TA_3 sulfation is prevented this is compensated by an increased glucuronidative clearance as well as direct deiodination. The SO_4^{2-}-independent IRD of TA_3 is largely mediated by the PTU-sensitive type I deiodinase, because production of 3,3′-TA_2 (followed by glucuronidation) from 10 nM TA_3 was completely abolished by addition of PTU. This substantial direct deiodination of TA_3 by hepatocytes is in agreement with the efficient IRD of TA_3 by rat liver microsomes, *i.e.* approximately 16 times more rapid than IRD of T_3 (29). Recent data suggest a significant production of 3,3′-T_2 from ≤10 nM T_3 in hepatocyte cultures via direct IRD by a PTU-insensitive mechanism (see Footnote 1). However, no such type III-like deiodinase activity was detected in our study although we did not test TA_3 at very low concentrations.

Normally the metabolism of 3,3′-TA_2, added or generated from TA_3, proceeds predominantly via sulfation and subsequent ORD (like 3,3′-T_2, Refs. 30, 31, 36) as well as IRD (unlike 3,3′-T_2). Little 3,3′-TA_2G is observed, and 3,3′-TA_2S accumulates only if deiodination is inhibited. However, in BSA-free, SO_4^{2-}-deplete cul-

tures 3,3′-TA_2 is fully metabolized by ORD and direct IRD (29) as well as glucuronidation, resulting in the production of I^-, 3,3′-TA_2G, and 3′-TA_1G. The glucuronidation capacity of the cells for 3,3′-TA_2 appears sufficient to convert all 3,3′-TA_2 to 3,3′-TA_2G if both sulfation and deiodination are inhibited (*i.e.* in SO_4^{2-}-deplete hepatocytes incubated with PTU).

Our findings are in agreement with those reported by Braverman and Ingbar (37) on the conversion of TA_3 in rat kidney slices, in that they also found that the rate of TA_3 metabolism is not affected by PTU and that it is more rapid than the metabolism of T_3. Furthermore, an increase in sulfate conjugates was observed if deiodination was effectively inhibited with thiouracil. However, in contrast to our findings in hepatocytes, sulfation appeared an important pathway in the metabolism of TA_3 in kidney slices, but no glucuronides were observed (37).

The minor contribution of sulfation to the *in vitro* hepatic metabolism of TA_3 as compared with T_3 is surprising in the light of the finding that TA_3 is a better substrate for hepatic phenol sulfotransferase than T_3 (38). This may be explained by an even greater preference of the alternative pathways of glucuronidation and direct deiodination for TA_3 over T_3. 3,3′-TA_2 has not been tested as a substrate for hepatic phenol sulfotransferases but, in analogy with T_3 and 3,3′-T_2 (38), it is probably a much better substrate than TA_3, underlying the rapid sulfation of 3,3′-TA_2 in hepatocytes.

Metabolism of TA_3 in rats

The physiological relevance of the different metabolic pathways observed in hepatocytes was further studied by analysis of radioactivity in plasma and bile of rats injected with [^{125}I]TA_3. Acute PTU treatment was used to selectively eliminate the type I deiodinative pathway.

Previous studies in rats and dogs by Flock and coworkers (8, 39, 40) and in humans by Green and Ingbar (24) have demonstrated that TA_3G is normally the major TA_3 metabolite in bile. Flock *et al.* (8, 39) reported that within 6 h rats excreted 55% of injected TA_3 with bile. Treatment with butyl-4-hydroxy-3,5-diiodobenzoate (BHDB), another type I deiodinase inhibitor (8), increased the excretion of radioactivity in bile of [^{125}I]TA_3-injected rats by 25%, which is less than the PTU-induced stimulation of biliary TA_3 clearance we observed. In addition, urinary radioactivity was decreased 60% by BHDB treatment, (8, 39) a reduction similar to that of plasma I^- levels induced by PTU in the present study. Evidence for a significant role of the liver in the deiodinative clearance of TA_3 was provided by the findings of reduced plasma or urinary $^{125}I^-$ after administration of [^{125}I]TA_3 to hepatectomized rats or dogs, respectively (9).

The present results, when compared with those previously obtained in our laboratory with respect to the metabolism of T_3 in rats, show that despite initially higher plasma levels, disappearance of plasma TA_3 is much faster (27). These findings are compatible with the approximately 16-fold increased binding of TA_3 to rat serum proteins *in vitro* and a more rapid tissue metabolism of this compound in comparison with T_3. The rapid metabolism despite the strong plasma binding of TA_3 may be explained by a more active uptake than T_3 by liver and perhaps other tissues. However, we are not aware of evidence to support this hypothesis. After injection of [^{125}I]TA_3 the rate of biliary excretion of total radioactivity is more than twice that of T_3. Excretion of TA_3G amounted to approximately 29% of the dose in controls or approximately 37% in PTU-treated rats over the first hour after [^{125}I]TA_3 injection, which is much higher than the 4–6% T_3G disposition 1 h after T_3 administration (26, 28).

In analogy with the metabolism of T_3, the present findings are compatible with the view that under normal conditions any TA_3S generated in the liver is rapidly degraded by the type I deiodinase. If this enzyme is inhibited with PTU, TA_3S is released into the hepatic sinusoids as well as into the canaliculi. Biliary excretion of TA_3S may occur both directly and indirectly after reuptake of the conjugate from the circulation. The transient nature of plasma TA_3S in PTU-treated rats probably reflects this latter route. A very persistent metabolite in plasma of these animals is 3,3′-TA_2S, the production of which may involve IRD of TA_3 (or TA_3S) by a PTU-insensitive, type III-like deiodinase outside the liver. The persistence of plasma 3,3′-TA_2S may be explained by its extremely strong binding to plasma proteins, with a dialysable fraction of only 0.008% (our unpublished data).

Although TA_3 was sulfated in isolated hepatocytes to

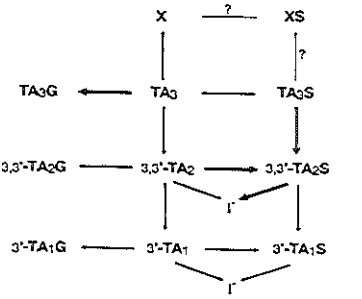

FIG. 10. Pathways for the metabolism of [3′-^{125}I]TA_3 in rats by sulfation (→), glucuronidation (⟵), IRD (↓), ORD (↙ ↘) and an unidentified reaction (↑). Only labeled products are shown.

a lesser extent than T_3, plasma sulfate levels were initially higher after TA_3 than after T_3 administration to PTU-treated rats (27). This may be explained by 1) a more extensive extrahepatic sulfation of TA_3, and/or 2) a more preferential release of TA_3S produced in hepatocytes from the sinusoidal cell surface as opposed to the canalicular membrane.

In contrast with our results, Flock *et al.* (8) did not detect sulfate conjugates in bile of normal TA_3-injected rats by paper chromatography, and after BHDB treatment only 8% of the radioactivity in bile consisted of sulfates. This was less than the proportion of TA_3S plus XS (≈34%) in the PTU-treated animals in our study. Flock *et al.* (40) observed 3,3′-TA_2S and smaller amounts of TA_3S and 3′-TA_1S in plasma of normal dogs, 5–12 h after iv [^{125}I]TA_3. The 3′-TA_1S was the predominant metabolite observed in urine of hepatectomized dogs, demonstrating considerable extrahepatic deiodination and sulfation in this species.

The unidentified metabolite X which, together with its sulfate conjugate XS, was detected in both control and PTU-treated animals, may be similar to 3,3′,5-triiodothyroformic acid (TF_3), according to its behavior on HPLC. However, X is probably not identical with TF_3, since Nakai *et al.* (41) reported that TF_3 is largely excreted in rat bile as the glucuronide conjugate while we only observed the sulfate conjugate of X.

The metabolic pathways of TA_3 observed in this study are summarized in Fig. 10. In conclusion, despite the fact that TA_3 is a better substrate for phenol sulfotransferases (38) and the fact that TA_3S is a very good substrate for the type I deiodinase (29), TA_3 is effectively cleared predominantly by glucuronidation, both in isolated rat hepatocytes and in intact rats. In addition, TA_3 is deiodinated directly by the type I enzyme, although most IRD of TA_3 in the liver occurs after sulfation. This explains the significant deiodinative clearance of TA_3 in normal rats and the accumulation of sulfate conjugates in plasma and bile induced by PTU. These findings are analogous to those previously reported for T_3 (see Footnote 1 and Refs. 26 and 27), although glucuronidation is far more extensive with TA_3 than with T_3, which explains the rapid clearance of the acetic acid derivative (2, 23, 24, 39).

Acknowledgments

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References

1. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5:151
2. Burger A 1986 Nondeiodinative pathways of thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 255
3. Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 189
4. Mulder GJ 1981 Sulfation of Drugs and Related Compounds. CRC Press, Boca Raton, FL
5. Dutton GJ 1980 Glucuronidation of Drugs and Other Compounds. CRC Press, Boca Raton, FL
6. Tomita K, Lardy HA 1960 Enzymic conversion of iodinated thyronines to iodinated thyroacetic acids. J Biol Chem 235:3292
7. Galton VA, Pitt-Rivers R 1959 The identification of the acetic acid analogues of thyroxine and tri-iodothyronine in mammalian tissues. Biochem J 72:319
8. Flock EV, Bollman JL, Owen CA, Zollman PE 1965 Conjugation of thyroid hormones and analogs by the Gunn rat. Endocrinology 77:303
9. Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford, UK, p 345
10. Roche J, Michel R, Jouan P, Wolf W 1956 The recovery of 3:5:3′-triiodothyroacetic acid and 3:3′-diiodothyronine from rat kidney after injection of 3:5:3′-triiodothyronine. Endocrinology 59:425
11. Roche J, Michel R, Nunez J, Jaquemin C 1959 On the metabolism of 3,3′-diiodothyronine and 3,3′,5′-triiodothyronine. Endocrinology 65:402
12. Roche J, Michel R 1960 On the peripheral metabolism of thyroid hormones. Ann NY Acad Sci 86:454
13. Takai NA, Rapoport B, Yamamoto M 1980 Biliary excretion of iodothyronines in rats as determined by high pressure liquid chromatography: effect of starvation. Endocrinology 107:176
14. Pittman CS, Shimuzu T, Burger A, Chambers JB 1980 The nondeiodinative pathways of thyroxine metabolism: 3,5,3′,5′-tetraiodothyroacetic acid turnover in normal and fasting subjects. J Clin Endocrinol Metab 50:712
15. Gavin LA, Livermore BM, Cavalieri RR, Hammond ME, Castle JN 1980 Serum concentration, metabolic clearance, and production rates of 3,5,3′-triiodothyroacetic acid in normal and athyreotic man. J Clin Endocrinol Metab 51:529
16. Nakamura Y, Chopra IJ, Solomon DH 1978 An assessment of the concentration of acetic acid and propionic acid derivatives of 3,5,3′-triiodothyronine in human serum. J Clin Endocrinol Metab 46:91
17. Ramsden DB, Crossley DN 1986 Serum concentrations of 3,5,3′,5′-tetraiodothyroacetate (T_4A) in subjects with hypo-, hyper- and euthyroidism. Acta Endocrinol (Copenh) 112:1912
18. Pittman CS, Buck MV, Chambers JB 1972 Urinary metabolites of ^{14}C-labeled thyroxine in man. J Clin Invest 51:1759
19. Chopra IJ, Boado RJ, Geffner DL, Solomon DH 1988 A radioimmunoassay for measurement of thyronine and its acetic acid analog in urine. J Clin Endocrinol Metab 67:480
20. Andrea TA, Cavalieri RR, Goldfine ID, Jorgensen EC 1980 Binding of thyroid hormone analogues to the human plasma protein prealbumin. Biochemistry 19:55
21. Jorgensen EC 1978 Thyroid hormones and analogs. II. Structure-activity relationships. In: Li CH (ed) Hormonal Proteins and Peptides. Academic Press, New York, vol 6:106
22. Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NCW, Freake HC 1987 Advances in our understanding of thyroid hormone action at the cellular level. Endocr Rev 8:288
23. Goslings B, Schwartz HL, Dillmann W, Surks MI, Oppenheimer JH 1976 Comparison of the metabolism and distribution of L-triiodothyronine and triiodothyroacetic acid in the rat: a possible explanation of differential hormonal potency. Endocrinology 98:666
24. Green WL, Ingbar SH 1961 The peripheral metabolism of tri- and tetraiodothyroacetic acids in man. J Clin Endocrinol Metab 21:1548
25. Larson FC, Albright EC 1958 Distribution of 3:5:3′-triiodothyroacetic acid in the rat. Endocrinology 63:183
26. De Herder WW, Bonthuis F, Rutgers M, Hazenberg MH, Visser TJ 1988 Effects of inhibition of type I iodothyronine deiodinase

and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Endocrinology 122:153
27. Rutgers M, Bonthuis F, de Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758
28. Eelkman Rooda SJ, van Loon MAC, Bonthuis F, Heusdens FA, Kaptein E, Rutgers M 1988 Effects of iopanoic acid (IOP) on the metabolism of T_3 in rats. Ann Endocrinol 49:182 (Abstract)
29. Rutgers M, Heusdens FA, Bonthuis F, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. I. Deiodination of TA_3 and TA_3 sulfate by microsomes. Endocrinology 125:424
30. Otten MH, Hennemann G, Docter R, Visser TJ 1984 Metabolism of 3,3′-diiodothyronine in rat hepatocytes: interaction of sulfation with deiodination. Endocrinology 115:887
31. Eelkman Rooda SJ, Van Loon MAC, Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. J Clin Invest 79:1740
32. Snedecor GW, Cochran WG 1967 Statistical Methods. The Iowa State University Press, Ames, IA, p 258
33. Rutgers M, Pigmans IGAJ, Bonthuis F, Visser TJ 1988 Effects of PTU on the biliary clearance of T_4 in rats. Ann Endocrinol 49:181 (Abstract)
34. Myant NB 1956 Biliary excretion of thyroxine in humans. Clin Sci 15:227
35. Hennemann G 1986 Thyroid hormone deiodination in healthy man. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 277
36. Otten MH, Mol JA, Visser TJ 1983 Sulfation preceding deiodination of iodothyronines in rat hepatocytes. Science 221:81
37. Braverman LE, Ingbar SH 1962 Effects of propylthiouracil and thiouracil on the metabolism of thyroxine and several of its derivatives by rat kidney slices *in vitro*. Endocrinology 71:701
38. Sekura RD, Sato K, Cahnmann HJ, Robbins J, Jakoby WB 1981 Sulfate transfer to thyroid hormones and their analogs by hepatic aryl sulfotransferases. Endocrinology 108:454
39. Flock EV, Bollman JL 1964 Effect of butyl-4-hydroxy-3,5-diiodobenzoate on the metabolism of L-thyroxine and related compounds. Endocrinology 75:721
40. Flock EV, Bollman JL, Stobie GHC 1962 Metabolic pathways of tetraiodothyroacetic acid, triiodothyroacetic acid, tetraiodothyropropionic acid and triiodothyropropionic acid. Biochem Pharmacol 11:627
41. Nakai Y, Shirakawa Y, Tanayama S, Fujita T, Suzuoki Z, Kato S, Hayashi N 1972 Metabolism of a thyroxine analogue, 3,5-diiodo-4-(4′-acetoxy-3′-iodophenoxy)benzoic acid in rats. Xenobiotica 2:277

Chapter VI

IDENTIFICATION OF 3,3'-DIIODOTHYROACETIC ACID SULFATE: A MAJOR METABOLITE OF 3,3',5-TRIIODOTHYRONINE IN PROPYLTHIOURACIL-TREATED RATS

Identification of 3,3′-Diiodothyroacetic Acid Sulfate: A Major Metabolite of 3,3′,5-Triiodothyronine in Propylthiouracil-Treated Rats*

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ABSTRACT. The sulfate conjugate 3, [3′-^{125}I] diiodothyroacetic acid (3,3′-TA_2S) was discovered in plasma, and occasionally in bile, of 6-propyl-2-thiouracil-treated rats after administration of [^{125}I]T_3. The identification of this T_3 metabolite was based on the following evidence: 1) the compound co-eluted in two different HPLC systems with synthetic 3,3′-TA_2S; 2) its chromatographic behavior on Sephadex LH-20 was characteristic for a conjugated iodothyronine derivative; and 3) the metabolite was hydrolyzed by arylsulfatase and the liberated product comigrated with synthetic 3,3′-TA_2 on HPLC. Marked accumulation of 3,3′-TA_2S was observed only in rats with impaired type I deiodinase activity but not in controls. Furthermore, plasma and biliary 3,3′-TA_2S levels varied with the experimental conditions such as anesthesia, i.e. both were increased in ketamine-anesthetized over pentobarbital-anesthetized animals. It was not possible to indicate the exact pathway through which 3,3′-TA_2S is generated from T_3; neither is it known how much of T_3 is acutally metabolized via 3,3′-TA_2S. However, the significant plasma 3,3′-TA_2S levels, even in ununesthetized animals, illustrate the physiological relevance of this T_3 metabolite. (*Endocrinology* **127**: 1617–1624, 1990)

THE MAJOR routes in the metabolism of iodothyronines in mammals are deiodination and conjugation of the phenolic hydroxyl group with glucuronic acid or sulfate (1, 2). Transformation of the thyronine skeleton of T_4 by side-chain modification or ether-link cleavage has been demonstrated *in vivo*, but these are generally regarded as minor metabolic pathways at least under physiological conditions (3). However, deamination and decarboxylation of the alanine side chain significantly contributes to the clearance of the active thyroid hormone 3,3′,5-triiodothyronine (T_3) in humans, resulting in detectable plasma levels of 3,3′,5-triiodothyroacetic acid (TA_3) (3, 4, 5). Furthermore, excretion of thyroacetic acid in urine of normal humans also shows significant metabolic breakdown of endogenous iodothyronines via the acetic acid pathway (6, 7).

Apart from direct inner ring deiodination, T_3 is converted in rat liver to T_3 glucuronide (T_3G) or T_3 sulfate (T_3S), as demonstrated *in vitro* with isolated hepatocytes (8) and *in vivo* with bile duct-cannulated or intact rats (1, 2, 9–11). The T_3G is rapidly eliminated with bile and hydrolyzed by intestinal β-glucuronidases of bacterial and mucosal origin. The liberated T_3 is either reabsorbed in the portal blood (enterohepatic circulation) or excreted with feces (12, 13). Because sulfated iodothyronine derivatives are much better substrates than the parent compounds for the type I iodothyronine deiodinase (14–17), the sulfation of T_3 results in an enhanced deiodinative breakdown of the hormone. If the type I enzyme is inhibited, for instance, by treatment with 6-propyl-2-thiouracil (PTU) (14, 15), the accumulation of T_3S from administered T_3 is strongly increased as has been demonstrated in hepatocyte cultures (8), bile and plasma of rats (9–11), and to a lesser extent also in human plasma (18).

We have recently demonstrated (19) that the metabolism of 3,3′,5-triiodothyroacetic acid (TA_3) in isolated rat hepatocytes is similar to that of T_3 (8, 16), involving 1) glucuronidation; 2) sulfation and subsequent deiodination; and 3) direct inner ring deiodination. However, unlike T_3, biliary excretion of TA_3 glucuronide (TA_3 G) is clearly more important than formation of TA_3 sulfate (TA_3S) as a metabolic pathway in rats (19).

Here we report on the isolation and identification of the sulfate conjugate of 3,[3′-^{125}I]diiodothyroacetic acid

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(3,3′-TA_2S), which was regularly observed in plasma and bile of [^{125}I]T_3-injected rats. Marked accumulation of this metabolite was observed in animals treated with PTU, and this was further influenced by experimental conditions such as anesthesia. These results provide more information about the interrelations between side-chain modification, sulfation, and deiodination in the metabolism of idothyronines.

Materials and Methods

Materials

Iodothyronines and iodothyroacetic acids were obtained from Henning GmbH (Berlin, West Germany) or Sigma Chemical Co. (St Louis, MO). In our laboratory, compounds were ^{125}I-labeled at the 3′-position using the chloramine T method and isolated on Sephadex LH-20. The purity of all tracers was checked by reversed-phase HPLC (13, 19). PTU, sulfatase type VIII, and β-glucuronidase type IX were purchased from Sigma; ketamine (Ketalar) was from Parke-Davis & Co (Barcelona, Spain).

Experimental procedures

Normally fed male Wistar rats weighing 190–280 g were used. PTU treatment was by ip injection of 1 mg PTU/100 g body wt dissolved in alkaline saline.

Experiment 1

Rats were anesthetized by ip administration of 8 mg ketamine/100 g body wt and received a single dose of PTU or vehicle (controls). The vena jugularis externa, the common bile duct, and the bladder were cannulated (9, 20, 21). To secure a constant production of urine, a solution of 75 mg mannitol/ml saline was infused into the vena jugularis externa cannula at a rate of 2.7 ml/h (20, 21) after an initial bolus of ~0.5 ml. To maintain anesthesia and to prevent thyroidal uptake of radioiodide, 8 mg ketamine and 16 μg KI, respectively, were administered per 100 g body wt/h together with the mannitol infusion. About 1–1.5 h after PTU administration, 12 μCi [^{125}I]T_3 (in 400 μl 10 mM NaOH in saline) was injected in the penile vein (t = 0). Bile and urine were collected over 30-min periods, and four successive blood samples (< 0.8 ml) were taken from the tail vein up to 6 h after tracer injection when the animals were bled by heart puncture. Production rates of bile and urine were 2.05 ± 0.13 and 1.91 ± 0.07 ml/h (mean ± SE, n = 5), respectively, making additional sc injections with saline necessary to prevent dehydration (~2.5 ml every 2 h). Body temperature was maintained using a heated pad and/or an infrared lamp.

Experiment 2

Pentobarbital-anesthetized rats, with or without a bile duct cannula, were injected iv with [^{125}I]T_3 (t =0) about 1 h after a single administration of PTU or vehicle (controls). Plasma and bile were collected as described previously (9, 10).

Experiment 3

Rats were treated for 4 days with twice daily ip injections of 0.25 μg T_3/100 g body wt in combination with PTU. Together with the last two injections animals received ~10 μCi [^{125}I]T_3. Four h after the last injection (*i.e.* 18 h after the first tracer dose) the animals were bled by heart puncture under ether anesthesia. Labeled metabolites in plasma extracts were analyzed by HPLC and compared with determinations of T_3S and iodothyronines by RIA (11).

Analysis of samples

Total radioactivity in plasma, bile, and urine was determined, and samples were stored immediately at −20 C until further HPLC analysis of the radiolabeled compounds. Samples were either fractionated by Sephadex LH-20 chromatography, affording separation between I^-, conjugates, and nonconjugated idothyronine derivatives (8, 10, 13, 19), or processed by C_{18} solid-phase extraction (10, 19). For quantification of the individual metabolites, the appropriate fractions were concentrated as previously described (10, 19) and analyzed by HPLC. Unprocessed bile was diluted in mobile phase and directly injected into the HPLC system (10, 13, 19). Isolated conjugates were incubated with β-glucuronidase type IX or with sulfatase type VIII, and the liberated compounds were identified as previously described (19).

Metabolites were analyzed by HPLC using a Chromspher C_{18} column (Chrompack International BV, Middelburg, The Netherlands) with two different elution systems as previously reported in detail (10, 13, 19). In short, separation of iodothyronines and conjugates was performed by gradient elution with 16–40% acetonitrile in 0.05 M ammonium acetate, pH 4, (system I, see Refs. 10, 13) and for iodothyroacetic acid derivatives with 24–44% acetonitrile in 0.02 M sodium phosphate, pH 2.7 (system II, see Ref. 19). Normally, fractions of 0.3 ml were counted for radioactivity. Columns were calibrated using either commercially available or homemade synthetic or biosynthetic reference compounds (8, 13, 19, 22).

Results

Detection and identification of 3,3′-TA_2S.

Figure 1 shows that PTU pretreatment significantly increased the excretion of biliary radioactivity after [^{125}I]T_3 administration to rats infused with mannitol and ketamine (Exp 1). Total radioactivity eliminated with bile up to 6 h after tracer injection was 2.6 times higher in PTU-treated than in control rats. PTU treatment did not affect plasma radioactivity or plasma T_3 clearance but significantly reduced plasma I^- by an average of 69.5%. HPLC analysis of plasma radioactivity after [^{125}I]T_3 injection (Exp 1) showed that PTU induced marked increases in both plasma T_3S and 3,3′ T_2S compared with control rats (Fig. 2). This was accompanied by increased biliary excretion of these sulfated iodothyronines in PTU-treated rats. The excretion of T_3G, the predominant biliary product in controls, is not signifi-

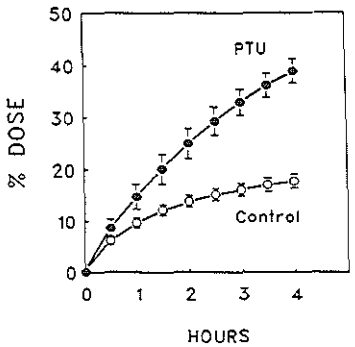

FIG. 1. Effect of PTU on biliary-excreted radioactivity. Normal or PTU-treated rats were iv injected with [^{125}I]T_3, and bile was collected under continuous infusion with mannitol and ketamine (Exp 1). The cumulative excretion of radioactivity is expressed as mean ± SE percent of the injected T_3 dose (n = 3–4).

cantly affected by PTU (Fig. 3).

In PTU-treated rats, but not in control animals, an additional unknown metabolite was detected that eluted just in front of T_3 on HPLC (Figs. 2 and 3). At the later time points, this compound was the major radioactive metabolite in plasma of these animals (Fig. 2). It was identified as 3,3′-TA_2S based on the following findings with the radioactive material isolated from either plasma or bile of PTU-treated rats using HPLC system I where it co-eluted with synthetic 3,3′-TA_2S (at 23 min). 1) The compound was adsorbed quantitatively onto Sephadex LH-20 under acidic conditions while more than 93% was eluted from the columns with water. This behavior is characteristic for conjugated iodothyronine derivatives but differs from the nonconjugated compounds, which require alkaline alcohol for elution. 2) The purified compound co-eluted with synthetic 3,3′-TA_2S (at 10.5 min) when rechromatographed using HPLC system II developed for the analysis of iodothyroacetic acids and their conjugates (19). 3) The compound was resistant to treatment with β-glucuronidase type IX but was completely hydrolyzed by incubation with sulfatase type VIII. The liberated compound was isolated on Sephadex LH-20 in 0.1 M ammonia in ethanol and eluted at the exact position of synthetic 3,3′-TA_2 in both HPLC systems, *i.e.* 31 min with system I and 25 min with system II. 4) No other possible T_3 metabolite tested, including 3′-T_1 and 3′-T_1S, comigrated with this compound before or after hydrolysis in both HPLC systems.

Additional tests were carried out with conjugates isolated on Sephadex LH-20 from plasma or bile of [^{125}I]T_3-injected rats. A representative HPLC chromatogram of the plasma conjugate fraction obtained from rats in Exp 3 is depicted in Fig. 4, showing the presence of 3,3′-TA_2S in addition to 3,3′-T_2S and T_3S. Sulfatase treatment of such conjugate fractions resulted in the liberation of 3,3′-T_2, T_3, and 3,3′-TA_2 in proportions similar to the original sulfates. For example (Fig. 5A), bile obtained 6 h after iv [^{125}I]T_3 administration to PTU-treated rats in Exp 1 contained mainly 3,3′ TA_2S (48 %) besides smaller amounts of 3,3′-T_2S (18%) and T_3S (7%). After incubation with sulfatase type VIII (Fig. 5B), predominantly 3,3′-TA_2 (61%) was identified by HPLC besides 3,3′-T_2 (20%) and T_3 (5%).

Quantification of 3,3′-TA_2S

Plasma of PTU-treated rats infused with mannitol and ketamine (Exp 1; Fig. 2) contained extremely high 3,3′-TA_2S levels, amounting to 0.19% dose/ml 4 h after iv [^{125}I]T_3, which is 20-and 12-fold higher than plasma 3,3′-T_2S and T_3S, respectively. However, also in Exp 2 substantial plasma levels of 3,3′-TA_2S were observed within 2 h after administration of [^{125}I]T_3 to PTU-treated rats. No 3,3′-TA_2S was detected in plasma of control animals (*e.g.* Fig. 2). The plasma levels of the major radiolabeled compounds 4 h after [^{125}I]T_3 injection in

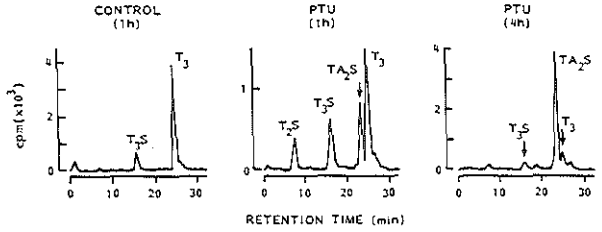

FIG. 2. The effect of PTU on plasma T_3 metabolites. Plasma was collected from control or PTU-treated rats 1 h or 4 h after iv administration of [^{125}I]T_3 (Exp 1). Radiolabeled compounds were determined by HPLC (system I) following C_{18} solid-phase extraction of plasma. Chromatogram A, B, and C represent 40%, 80%, and 83% of total plasma radioactivity, respectively, due to removal of mainly I^- during precleaning of samples. The recovery of radioactivity from HPLC was on average 91%.

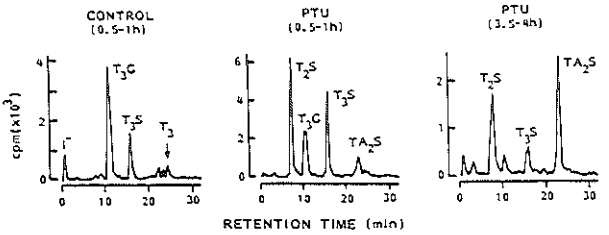

FIG. 3. The effect of PTU on biliary T_3 metabolites. Bile was collected from mannitol- and ketamine-infused rats with or without pretreatment with PTU (Exp 1). Of samples obtained between 0.5–1 h and 3.5–4 h after injection of [^{125}I]T_3, 15–20 μl aliquots were directly analyzed using HPLC system I. The recovery of radioactivity was 92%.

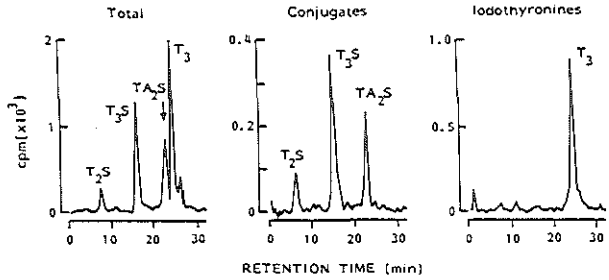

FIG. 4. HPLC analysis of plasma extracts from a rat treated twice daily with 1 mg PTU and 0.25 μg T_3/100 g body wt. [^{125}I]T_3 was coadministered with the last two injections, and plasma was collected 4 h after the final dose (Exp 3). The *left panel* depicts the separation of the noniodide radioactivity by HPLC (system I) after precleaning of plasma on C_{18} solid-phase. Plasma was also fractionated on Sephadex LH-20 for separate HPLC analysis of either conjugated (*middle*) or nonconjugated iodothyronines (*right*). The prepurified fractions contained 73% (*left*), 39% (*middle*) and 33% (*right*) of total plasma radioactivity. The recovery of radioactivity from HPLC was 93–97%.

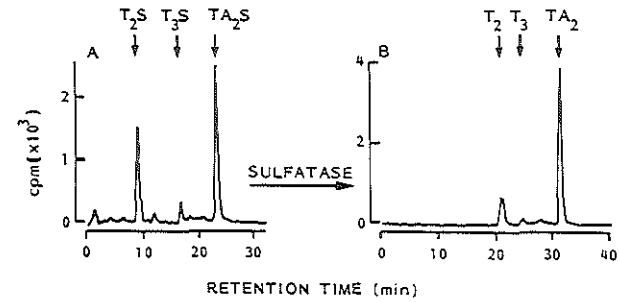

FIG. 5. Hydrolysis of sulfate conjugates in bile from PTU-treated rats injected with [^{125}I]T_3 (Exp 1). Conjugates were isolated from bile (5– 5.5 h after tracer administration) on Sephadex LH-20, lyophilized, and analyzed by HPLC using system I (A). The LH-20 conjugate fraction was also treated with sulfatase type VIII resulting in 74% hydrolysis. Subsequently, the liberated compounds were separated from the remaining conjugates on Sephadex LH-20 and analyzed with the same HPLC system (B). The results are derived from 25 (A) or 200 (B) μl bile samples. *Arrows* indicate the elution position of synthetic reference compounds.

the different experimental groups are summarized in Table 1. In PTU-treated pentobarbital-anesthetized rats (Exp 2) the mean plasma 3,3′-TA_2S level was 0.015% dose/ml, similar to that of 3,3′-T_2S but much lower than the T_3S level (Table 1). The relative proportion of 3,3′-TA_2S in the circulation of these anesthetized animals (Exp 2) was only half of that in the freely moving, PTU-treated rats of Exp 3, *i.e.* 6% *vs.* 13% of total plasma

TABLE 1. Distribution of plasma radioactivity in control and PTU-treated rats 4 h after [^{125}I]T_3 administration

Exp	Treatment	n	Plasma concentration (100 × % dose/ml) [% of plasma radioactivity]				
			I^-	3,3′-T_2S	T_3S	3,3′-TA_2S	T_3
1		4	26.3 ± 3.4 [83.3]	0.1 ± 0.1 [0.4]	0.2 ± 0.1 [0.7]	0 [0.1]	1.1 ± 0.4 [3.7]
1	PTU	3	7.5 ± 3.3 [17.3]	1.0 ± 0.6 [3.2]	1.6 ± 1.1 [5.0]	19.4 ± 7.3 [59.3]	1.6 ± 0.2 [5.1]
2		5	22.2 ± 5.4 [66.7]	0.2 ± 0.1 [0.7]	0.7 ± 0.3 [2.1]	0 [0]	4.0 ± 0.6 [12.2]
2	PTU	6	6.9 ± 0.3 [26.2]	1.6 ± 0.4 [6.1]	5.6 ± 1.8 [21.0]	1.5 ± 0.4 [5.8]	5.4 ± 1.6 [20.4]
3[a]	PTU	5	11.6 ± 7.9 [26.2]	1.7 ± 0.9 [4.5]	8.5 ± 3.8 [20.6]	5.7 ± 3.8 [12.7]	9.1 ± 2.0 [24.0]

The level of 3,3′-TA_2S in rat plasma 4 h after [^{125}I]T_3 injection is compared with the other radiolabeled T_3 metabolites and remaining T_3. Besides plasma I^-, which was determined on Sephadex LH-20, compounds were quantified by HPLC after solid-phase extraction. Data are expressed as mean ± SD percent dose per milliliter plasma, and the mean percent distribution is given within *brackets*. The mean total plasma radioactivity was similar in the different groups (0.26–0.36% dose/ml).

[a] Only the [^{125}I]T_3 dose of the final injection was taken into account.

radioactivity.

In bile of [^{125}I]T_3-injected rats infused with mannitol and ketamine (Exp 1), excretion of 3,3′-TA_2S was significantly increased by PTU, especially at the later time points (Figs. 3 and 5). In Fig. 6 the composition of biliary radioactivity is depicted as a function of time after tracer injection. It is clearly shown that the normal excretion of biliary 3,3′-TA_2S is nearly negligible but is increased 6- to 29-fold between 1.5–4 h in PTU-treated rats, thus becoming the major T_3 metabolite. Biliary excretion of

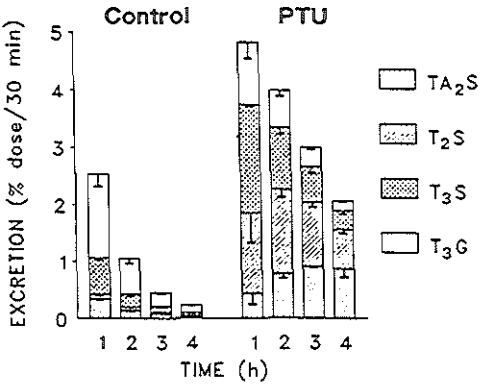

FIG. 6. Biliary excretion of T_3 metabolites as a function of time after administration of [^{125}I]T_3 (t = 0) to normal or PTU-treated rats infused with mannitol and ketamine (Exp 1). Conjugates in bile collected between 0.5–1, 1.5–2, 2.5–3, and 3.5–4 h were determined by HPLC system I. Data are expressed as the mean ± SE percent dose per 30 min collection period (n = 3–4). From 90 min onwards, biliary 3, 3′-TA_2S was significantly increased by PTU ($P < 0.001$).

3,3′TA_2S was negligible in Exp 2, even after PTU treatment.

Relatively little of the administered radioactivity was excreted in urine within 6 h, *i.e.* 4.2% *vs.* 8.2% in PTU-treated *vs.* control animals. The urinary radioactivity of control animals (Exp 1) consisted mainly of radioiodide. Besides I^-, 20–60% of urinary radioactivity in PTU-treated rats was in the form of 3,3′-TA_2S (not shown).

Discussion

Previous studies in our laboratory have demonstrated that although T_3 is conjugated in liver to roughly equal extents with glucuronic acid or sulfate (8), little T_3S is normally observed in plasma and bile of rats (9, 10). This is explained by the rapid degradation of the conjugate by the type I deiodinase of liver—and perhaps other tissues—through inner ring deiodination (IRD) to 3,3′-T_2S and subsequent outer ring deiodination (ORD) as illustrated in Fig. 7 (14–16, 23). Treatment of rats with PTU results in a marked inhibition of iodide release accompanied by the accumulation of T_3S as well as 3,3′-T_2S in the plasma and bile of these animals (9–11). PTU also strongly reduces deiodination of iv injected T_3S in rats; however, besides T_3S no 3,3′-T_2S is excreted in bile (10). Apparently, unlike T_3 itself, T_3S is not a substrate for the PTU-insensitive type III (inner ring) deiodinase; hence, 3,3′-T_2S is chiefly generated via direct IRD of T_3 by this enzyme followed by sulfate conjugation. The type III deiodinase is localized predominantly in extra-hepatic tissues (14, 15) but also exists in liver (8).

Similar to T_3 (8, 16), the side chain analog TA_3 is also metabolized in liver by glucuronidation, sulfation and IRD (19). While TA_3G is a stable conjugate, alternating sulfation and deiodination reactions produce a series of

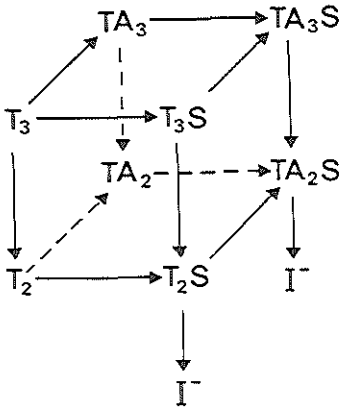

FIG. 7. Pathways for the metabolism of T_3 in rats by sulfation, side-chain modification, and deiodination.

products with 3, 3′-TA_2S as a common intermediate (Fig. 7; Ref. 19). TA_3 and 3,3′-TA_2 are deiodinated more efficiently by the type I deiodinase than the parent compounds T_3 and 3,3′-T_2, which is further enhanced by sulfate conjugation (17). The direct IRD of TA_3 by isolated rat hepatocytes is probably largely catalyzed by the type I deiodinase (17), but *in vivo* TA_3 has also been shown to undergo IRD by the type III deiodinase (19). After iv administration of labeled TA_3 to normal rats, TA_3G is the predominant metabolite excreted in bile, whereas in PTU-treated rats significant amounts of biliary TA_3S also are excreted concomitant with a transient increase of plasma TA_2S (19). Furthermore, in PTU-treated TA_3-injected animals an accumulation of 3,3′-TA_2S is observed in plasma, but little of this conjugate appears in bile.

In humans, production of TA_3 may account for approximately 14% of T_3 turnover (4), but little is known about the quantitative contribution of side-chain modification to the metabolism of T_3 in rats. Conversion of T_3 to TA_3 has been observed *in vitro* in homogenates of rat kidney, liver, muscle, and brain, and the enzyme activity for this reaction has been localized in mitochondria (24, 25). Roche *et al.* (26) identified TA_3 in the kidneys of thyroidectomized rats injected with radioactive T_3 whereas they were also able to detect renal 3,3′-TA_2 after injection of labeled 3,3′-T_2 (27). Flock *et al.* (28) have reported excretion of minor quantities of TA_3G and TA_3S in the bile of rats injected iv with radioiodinated T_3. We have previously noticed a metabolite especially in plasma of rats 2–4 h after iv administration of radioactive T_3, which eluted just in front of T_3 in our reversed-phase HPLC system (10, 11). Although this metabolite has not been fully characterized chemically, all available evidence points to its identity with 3,3′-TA_2S.

Figure 7 shows the multiple metabolic pathways that lead from T_3 to ultimate formation of 3,3′-TA_2S. It is impossible on the basis of our findings to indicate which of these pathways is actually followed, but the involvement of some of these routes may be excluded. The lack of plasma 3,3′-TA_2S production from injected T_3 even in PTU-treated rats (see above), strongly suggests that T_3S is not an intermediate. The accumulation of plasma 3,3′-TA_2S from injected T_3 has been observed not only in PTU-treated rats but also in animals treated with the radiocontrast agent iopanoic acid (IOP) (27; Rutgers, M., S. J. Eelkman Rooda, and T. J. Visser, unpublished work). PTU is a selective, uncompetitive inhibitor of the type I deiodinase, while IOP inhibits other deiodinases as well (14). However, accumulation of 3,3′-T_2S was not observed in the IOP-treated animals injected with T_3 (29), suggesting that $T_3 \rightarrow$ 3,3′-T_2 conversion is completely blocked. Therefore, it is unlikely that 3,3′-T_2 is an intermediate in 3,3′-TA_2S production from T_3. Although these considerations provide insufficient evidence to identify the pathway(s) of 3,3′-TA_2S production from T_3, it is likely that this proceeds through TA_3.

It is remarkable that the amount of 3,3′-TA_2S encountered in plasma is much greater in the rats of Exp 1 than in those studied on Exps 2 and 3. The main difference in experimental conditions between these groups concerns the use of anesthetics, *i.e.* ketamine in Exp 1, pentobarbital in Exp 2, and none in Exp 3. Ketamine was chosen in Exp 1 as an alternative anesthetic because of its lack of effect on the energy status of the liver compared with unanesthetized rats, whereas pentobarbital anesthesia results in decreased hepatic ATP/ADP ratios (30). It is remarkable that the use of ketamine resulted in a bile flow which was two-fold higher[1] than that in animals anesthetized with pentobarbital (Exp 2), irrespective of co-administered mannitol. This phenomenon has previously been reported by others (31, 32) and might be explained by: 1)stimulation of bile flow associated with biliary clearance of ketamine (33); or 2) hemodynamic effects such as cardiac output and (hepatic) arterial pressure (33).

In Exp 1, ketamine was given by iv infusion together with mannitol to stimulate diuresis, a well accepted method in pharmacology to facilitate the study of the urinary clearance of drugs (20). The more pronounced

[1] Bile flow (mean ± SD μl/h·100 g body wt) in bile-diverted rats anesthetized by either ip administration of ketamine or iv infusion of ketamine-plus-mannitol amounted to 1010 ± 112 (n = 4) or 925 ± 101 (n = 7), respectively, but was only 503 ± 73 (n = 32) in pentobarbital anesthetized animals as observed in this and other studies (10, 13, 19, 29).

accumulation of plasma 3,3′-TA_2S induced by PTU in Exp 1 compared with the other experiments cannot be ascribed to the mannitol infusion, because roughly similar plasma 3,3′-TA_2S levels were obtained in IOP-treated rats anesthetized by ip ketamine injections that were not infused with mannitol (not shown). The differences between the plasma 3,3′-TA_2S levels detected in Exps 1 and 2 are, therefore, probably due to the use of different anesthetics. Because of the acute nature of the experiments, differential induction of thyroid hormone-metabolizing enzymes by these anesthetics is unlikely. It is possible that they have different influences on the tissue delivery of iodothyronine derivatives. With pentobarbital this may occur through its effect on tissue ATP (30), resulting in a decreased activity of tissue iodothyronine uptake mechanisms (34), as well as through competitive displacement of iodothyronines from plasma proteins (35). The hemodynamic effect of ketamine (33) may change blood flow in iodothyronine-metabolizing organs, although serum thyroid hormone levels in baboons are not affected by prolonged ketamine anesthesia (36). Therefore, the use of both anesthetics may influence the outcome of these investigations, although the findings obtained in Exp 3 demonstrate that significant 3,3′-TA_2S production occurs under physiological conditions.

In conclusion, we identified 3,3′-TA_2S as a major T_3 metabolite in plasma of PTU-treated rats and in ketamine-plus-mannitol-infused animals also in bile. The physiological relevance of these findings is underscored by the findings in unanesthetized rats. It is not possible to indicate the actual pathway(s) through which 3,3′-TA_2S is generated from T_3 and how much it contributes to the metabolism of T_3. The accumulation of 3,3′-TA_2S in PTU-treated rats is explained by the inhibition of the clearance of this metabolite by the type I deiodinase. The substantial plasma 3,3′-TA_2S levels in PTU-treated rats may reflect a high production of this metabolite and/or slow metabolic clearance rate due to high affinity binding to plasma proteins as we previously demonstrated (19).

References

1. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5:151
2. Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford. p 345
3. Burger AG 1986 Nondeiodinative pathways of thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. p255
4. Gavin LA, Livermore BM, Cavalieri RR, Hammond ME, Castle JN 1980 Serum concentration, metabolic clearance, and production rates of 3, 5, 3′-triiodothyroacetic acid in normal and athyreotic man. J Clin Endocrinol Metab 51:529
5. Nakamura Y, Chopra IJ, Solomon DH 1978 An assessment of the concentration of acetic acid and propionic acid derivatives of 3,5,3′-triiodothyronine in human serum. J Clin Endocrinol Metab 46:91
6. Pittman CS, Buck MV, Chambers JB 1972 Urinary metabolites of ^{14}C-labeled thyroxine in man. J Clin Invest 51:1759
7. Chopra IJ, Boado RJ, Geffner DL, Solomon DH 1988 A radioimmunoassay for measurement of thyronine and its acetic acid analog in urine. J Clin Endocrinol Metab 76:480
8. Eelkman Rooda S-J, Otten MH, van Loon MAC, Kaptein E, Visser TJ 1989 Metabolism of triiodothyronine in rat hepatocytes. Endocrinology 125:2187
9. De Herder WW, Bonthuis F, Rutgers M, Hazenberg MP, Visser TJ 1988 Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Endocrinology 122:153
10. Rutgers M, Bonthuis F, de Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758
11. Eelkman Rooda S-J, Kaptein E, Rutgers M, Visser TJ 1989 Increased plasma 3,5,3′-triiodothyronine sulfate in rats with inhibited type I iodothyronine deiodinase activity, as measured by radioimmunoassay. Endocrinology 124:740
12. De Herder WW, Hazenberg MP, Pennock-Schröder AM, Oosterlaken AC, Rutgers M, Visser TJ 1989 On the enterohepatic cycle of triiodothyronine in rats; importance of the intestinal microflora. Life Sci 45:849
13. Rutgers M, Heusdens FA, Bonthuis F, de Herder WW, Hazenberg MP, Visser TJ 1989 Enterohepatic circulation of triiodothyronine (T_3) in rats. Importance of the microflora for the liberation and reabsorption of T_3 from biliary T_3 conjugates. Endocrinoloby 125: 2822
14. Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 189
15. Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RJB, Van der Molen HJ (eds) Hormones and Their Action, Part I. Elsevier, Amsterdam. p 81
16. Visser TJ, van Buuren JCJ, Rutgers M, Eelkman Rooda S-J, de Herder WW 1990 The role of sulfation in thyroid hormone metabolism. Trends Endocrinol 1:221
17. Rutgers M, Heusdens FA, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. I: Deiodination of TA_3 and TA_3 sulfate by microsomes. Endocrinology 125:424
18. Eelkman Rooda S-J, Kaptein E, Visser TJ 1989 Serum triiodothyronine sulfate in man measured by radioimmunoassay. J Clin Endocrinol Metab 61:552
19. Rutgers M, Heusdens FA, Bonthuis F, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. II: Deiodination and conjugation of TA_3 by rat hepatocytes and in rats *in vivo*. Endocrinology 125:433
20. Mulder GJ, Scholtens E, Meijer DKF 1981 Collections of metabolites in bile and urine from rat. In: Colowick SP, Kaplan NO (eds) Methods in Enzymology. Academic Press, New York, vol 77:21
21. Weitering JG, Krijgsheld KR, Mulder GJ 1979 The availability of inorganic sulphate as a rate limiting factor in the sulphate conjugation or xenobiotics in the rat. Biochem Pharmacol 28:757
22. Mol JA, Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. Endocrinology 117:1
23. Visser TJ, Mol JA, Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112:1547
24. Tomita K, Lardy HA 1960 Enzymic conversion of iodinated thyronines to iodinated thyroacetic acids. J Biol Chem 235:3292
25. Galton VA, Pitt-Rivers R 1959 The identification of the acetic acid analogues of thyroxine and tri-iodothyronine in mammalian tissues. Biochem J 72:319
26. Roche J, Michel R, Jouan P, Wolf W 1956 The recovery of 3:5:3′-triiodothyroacetic acid and 3:3′ diiodothyronine from rat kidney after injection of 3:5:3′-triiodothyronine. Endocrinology 59:425
27. Roche J, Michel R, Nunez J, Jaquemin C 1959 On the metabolism of 3, 3′-diiodothyronine and 3,3′,5′-triiodothyronine. Endocrinology 65:403
28. Flock EV, Bollman JL, Owen CA, Zollman PE 1965 Conjugation of thyroid homrones and analogs by the Gunn rat. Endocrinology 77:303

29. Eelkman Rooda S-J, van Loon MAC, Bonthuis F, Heusdens FA, Kaptein E, Rutgers M 1988 Effects of iopanoic acid (IOP) on the metabolism of T_3 in rats. Ann Endocrinol Paris 49:182 (Abstract)
30. Schwenke W-D, Soboll S, Seitz HJ, Sies H 1981 Mitochondrial and cytosolic ATP/ADP ratios in rat liver *in vivo*. Biochem J 200:405
31. Ireland SJ, Livingston A 1980 Effect of biliary excretion on ketamine anesthesia in the rat. Br J Anaesth 52:23
32. Gourley GR, Mogilevsky W, Arend RA, Siegel FL, Odell GB 1985 Effects of anesthetic agents on bile pigment excretion in the rat. Hepatology 5:610
33. Gelman S 1986 General anesthesia and hepatic circulation. Can J Physiol Pharmacol 65:1762
34. Krenning EP, Docter R 1986 Plasma membrane transport of thyroid hormone. In: Henneman G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 107
35. Langer P, Kokesova H, Gschwendtova K 1976 Acute redistribution of thyroxine after the administration of univalent anions, salicylate, theophilline and barbiturates in rats. Act Endocrinol (Copenh) 81:516
36. Kalk WJ, Fitzpatrick M, van der Walt LA, Smit JA, Myburgh JA 1986 Circulating thyroid hormones in primates with mild or severe hepatitis following liver transplantation. Horm Metab Res 18:323

Chapter VII

EFFECTS OF PROPYLTHIOURACIL ON THE BILIARY CLEARANCE OF THYROXINE (T_4) IN RATS: DECREASED EXCRETION OF 3,5,3'-TRIIODOTHYRONINE GLUCURONIDE AND INCREASED EXCRETION OF 3,3',5'-TRIIODOTHYRONINE GLUCURONIDE AND T_4 SULFATE

Effects of Propylthiouracil on the Biliary Clearance of Thyroxine (T_4) in Rats: Decreased Excretion of 3,5,3′-Triiodothyronine Glucuronide and Increased Excretion of 3,3′,5′-Triiodothyronine Glucuronide and T_4 Sulfate*

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ABSTRACT. The liver metabolizes T_4 by deiodination and conjugation to T_4 glucuronide (T_4G), but little information exists about the formation of T_4 sulfate (T_4S) *in vivo*. We have examined the excretion of T_4G, T_4S, T_3 and rT_3 glucuronide (T_3G and rT_3G) in bile, collected under pentobarbital anesthesia 0–8 h or 17–18 h after iv [^{125}I]T_4 injection to control and 6-propyl-2-thiouracil (PTU)-treated rats. Radioactivity in bile, plasma, feces, and urine was analyzed by Sephadex LH-20 chromatography and HPLC. PTU induced a 2-fold increase in the biliary excretion of total radioactivity (26.6% *vs.* 15.0% dose between 0–8 h; 2.0% *vs.* 1.0% dose between 17–18 h). Biliary metabolites, 17–18 h after T_4 injection, in control *vs.* PTU rats amounted to (percent dose): T_4G, 0.44 *vs.* 0.75; T_3G, 0.19 *vs.* 0.07; rT_3G, 0.02 *vs.* 0.15; and T_4S, 0.06 *vs.* 0.32. Similar results were obtained for control rats when bile was collected between 7–8 h after iv T_4. The excretion rate of T_3G was lower and that of rT_3G higher when bile was continuously collected for 8 h immediately after T_4 administration, probably due to prolonged experimental stress. However, regardless of the period of bile collection, PTU induced a more than 24-fold decrease in the T_3G/rT_3G ratio and a 5-fold increase in T_4S excretion. In the animals killed 18 h after T_4 injection, PTU treatment increased plasma T_4 retention by 50%, reduced urinary I^- excretion by 74%, and increased fecal radioactivity by 47%. No conjugates were detected in feces, and the distribution of fecal T_4:T_3:rT_3 was 70:18:2 in control and 68:7:6 in PTU-treated rats. The results indicate that 1) the glucuronidative clearance of T_4 is not affected by PTU; 2) the T_3G/rT_3G ratio in bile is a sensitive indicator of type I deiodinase inhibition; 3) T_4 undergoes significant sulfation in rats *in vivo*, and 4) biliary excretion of T_4S is enhanced if its type I deiodination is inhibited. (*Endocrinology* 125: 2175–2186, 1989)

IN NORMAL humans and rats approximately 80% of the circulating bioactive thyroid hormone T_3 is produced outside the thyroid gland by outer ring deiodination (ORD) of T_4. An even greater proportion of plasma rT_3 is produced by inner ring deiodination (IRD) of T_4 in peripheral tissues, which is regarded as an inactivation process (1). Deiodination of the different iodothyronines is catalyzed by at least three different deiodinases. Besides the nonselective type I enzyme, which is capable of both ORD and IRD, specific deiodinases for either ORD (type II) or IRD (type III) have been identified. They differ among others in substrate specificity, reaction kinetics, and tissue distribution (2). Both type II and III deiodinases are insensitive to inhibition by the antithyroid drug 6-propyl-2-thiouracil (PTU), in contrast to the type I deiodinase. The latter is predominantly localized in liver and kidney as well as thyroid and is most active in the ORD of rT_3 (2).

In euthyroid rats at least 70% of the peripherally produced T_3 originates from PTU-sensitive conversion of T_4 by the type I deiodinase of liver and other tissues (3). It has also been demonstrated that PTU greatly reduces peripheral T_3 production in euthyroid humans (4). The liver type I enzyme activity probably does not contribute to circulating rT_3, but is largely responsible for plasma rT_3 clearance (5), which is understandable in light of the substrate specificity of this enzyme (6).

The different deiodinative pathways are important for the regulation of thyroid hormone bioactivity, but other metabolic routes, such as conjugation of the phenolic hydroxyl group with sulfate or glucuronic acid, may contribute significantly to the clearance of T_4 and T_3 (1,

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7, 8). Most of the T_4 and T_3 excreted in bile of normal rats is conjugated with glucuronic acid (8–11), and T_4 glucuronide (T_4G) has also been identified in human bile (12). The glucuronides are stable conjugates, although their biliary excretion does not represent the irreversible elimination of thyroid hormone from the body. The intestinal microflora of humans and rats has a high hydrolytic capacity for iodothyronine conjugates (13–15), and a significant fraction of the liberated iodothyronines is reabsorbed (16, 17). However, the extent to which T_4 is engaged in an enterohepatic circulation in humans and rats is not known (7, 12, 18).

In general, sulfation of iodothyronines accelerates the deiodinative degradation of these compounds by the type I deiodinase. Thus, the maximum velocity (V_{max})/K_m ratios of the ORD of 3,3′-diiodothyronine sulfate (3,3′-T_2S) and the IRD of T_3 sulfate (T_3S) and T_4 sulfate (T_4S) are 40–200 times higher than the corresponding values for the nonsulfated substrates, but ORD of T_4S does not occur (2, 19). Because T_3S is rapidly deiodinated it does not accumulate in rat hepatocyte cultures or in bile and plasma of rats after administration of T_3 unless the type I deiodinase is inhibited by PTU (10, 20), butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) (21, 22), or hypothyroidism (23). Preliminary evidence suggested the occurrence of T_4S in plasma and lymph of thyroidectomized dogs (23) as well as in bile of thiouracil-treated rats (21, 22, 24).

In this study we present the first unequivocal evidence for T_4S formation in rats. We have identified and quantified the biliary excretion of this conjugate as well as that of T_4G, T_3G, and rT_3G after iv administration of radioactive T_4 in normal and PTU-treated rats. The excretion of T_4S in bile greatly depends on the rate of its degradation by the type I deiodinase. We also show that the ratio of excreted T_3G/rT_3G is a sensitive parameter of type I deiodinase inhibition by PTU.

Materials and Methods

[3′-^{125}I]T_4 was purchased from Amersham (Amersham, United Kingdom; specific radioactivity, 1500 μCi/μg), purified by Sephadex LH-20 chromatography, and dissolved in 10 mM NaOH in saline supplemented with 1% normal rat serum. At the time of administration the purity of the [^{125}I]T_4 was checked by HPLC. On the average, 94% of the applied radioactivity eluted as T_4, and other compounds were not observed.

Experimental procedures

Male Wistar rats, 200–240 g BW, were used. Conscious animals had free access to food and water. Anesthesia was induced by ip injection of 6 mg pentobarbital sodium/100 g BW. To prevent thyroid uptake of the $^{125}I^-$ released from injected [^{125}I]T_4, 100 μg KI/100 g BW were given ip. Cannulation of the common bile duct was performed as previously described (10). For continuous bile collection anesthesia was maintained by additional ip injections of pentobarbital (2–4 mg) when necessary, and saline (2.5 ml) was administered sc every 2 h to prevent dehydration. Body temperature was maintained at 36–38 C by the use of a heating pad in combination with an infrared lamp.

Exp I. Simultaneously with induction of anesthesia, rats received ip injections of 1 mg PTU plus 100 μg KI in 200 μl alkaline saline (50 mM NaOH)/100 g BW. Control rats received the same volume of vehicle with KI. One hour later 15 μCi [^{125}I]T_4 was administered iv (time zero). The rats were allowed to recover from anesthesia in individual stainless steel metabolic cages to collect urine and feces. The PTU and/or KI injections were repeated at 7 and 15 h, and thereafter, the animals were anesthetized a second time. At 16.5 h the rats were fitted with a bile duct cannula, and bile was collected in preweighed tubes during three successive 30-min periods until 18 h, when the rats were bled by heart puncture.

Exp Ia. Bile collection from two control rats, described in Exp I, was continued under anesthesia for an additional period of 8 h until 26 h after [^{125}I]T_4 injection.

Exp II. Rats were anesthetized and injected with PTU plus KI or KI alone (−1 h), as described for Exp I. Subsequently, the bile duct was cannulated, and 15 μCi [^{125}I]T_4 were administered iv (time zero). Bile was collected continuously under anesthesia in 30-min periods. Repetitive blood samples (0.35 ml) were taken from the tail vein, and the animals were bled by heart puncture at 8 h.

Exp III. Three control rats were injected with pentobarbital and KI; after 15 min they received 15 μCi [^{125}I]T_4, iv (time zero). After regaining consciousness (~1 h) the rats moved freely until 6.5 h, when the bile ducts were cannulated under anesthesia. Bile was collected from 7 until 8 h, when the animals were bled.

Analysis of samples

Production of bile was measured gravimetrically. Fifty or 100 μl bile and serum samples were counted for total radioactivity. The volume and radioactivity of urine were determined after centrifugation to eliminate mainly food particles. Total fecal radioactivity was counted, and about 1 g feces was suspended in 4 or 5 ml ethanol. The suspension was shaken for 1.5 h at 37 C and left for 40–60 h at 4 C. The ethanol extracts were centrifuged, and the clear supernatants were stored at −20 C until further analysis.

Radioactivity in plasma, bile, urine, and extracts of feces was generally analyzed by Sephadex LH-20 chromatography in combination with reverse phase C_{18} HPLC. Small Sephadex LH-20 columns (1.3-ml bed volume) equilibrated in 0.1 M HCl were used for crude fractionation of samples. Aliquots of bile (50–100 μl), urine (250 μl), or fecal extracts were prepared in 1 ml 0.3 M HCl and 10% (vol/vol) ethanol (final concentrations). Serum samples (<100 μl) were prepared in 0.1 M HCl and 25% (vol/vol) ethanol (20). These mixtures were applied to the LH-20 columns, and iodide was eluted with 4 times 1 ml 0.1 M HCl and subsequently conjugates with 11 times 1 ml 20% (vol/vol)

ethanol in water. Columns were rinsed with 0.75 ml 0.1 M ammonia in ethanol, and the eluate was discarded. Finally, nonconjugated iodothyronines were eluted quantitatively with 1.2 ml 0.1 M ammonia in ethanol.

For HPLC analysis of conjugates the relevant 20% ethanol in water fractions were pooled, acidified (final concentration, 0.2 M HCl), and loaded on a second Sephadex LH-20 column (1.1-ml bed volume). After washing the columns with 0.7 ml 0.1 M HCl, 0.7 ml water, and 0.7 ml 0.1 M ammonia in ethanol, 93 ± 3% (mean ± SD; n = 34) of the pooled radioactivity was finally eluted with 1.2 ml 0.1 M ammonia in ethanol.

To identify and quantify iodothyronine glucuronides, 50 μl bile were incubated for 2 h at 37 C with 30 μg β-glucuronidase (type IX, Sigma, St. Louis, MO) in 600 μl 0.05 M phosphate buffer (pH 6.8). After the addition of 300 μl 0.8 M HCl and 100 μl ethanol, the liberated iodothyronines were isolated on Sephadex LH-20 as described above. Bile was also treated for 18–20 h at 37 C with sulfatase (type VIII, Sigma; 250 μg/ml) in 0.05 M sodium acetate (pH 5.0) in the presence of 8 mM D-saccharic acid 1,4-lactone to inhibit possible β-glucuronidase activity. Acid-labile conjugates were identified after treatment of 50 μl bile for 1 h at 80 C in 300 μl 1 M HCl.

Except for T_4S, biliary metabolites were quantified directly by HPLC of untreated bile (see below). Some serum and urine samples were processed by C_{18} solid phase extraction before HPLC analysis, resulting in isolation of conjugates together with nonconjugated iodothyronines in methanol as described previously (20).

Reverse phase HPLC

Chromspher C_{18} analytical columns (10 × 0.3 cm) were used in combination with a 10 × 2.1-mm reverse phase guard column (Chrompack International BV, Middelburg, The Netherlands). For separation of most conjugates a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4) was used (system A), but resolution of nonconjugated iodothyronines, especially T_3 and rT_3, was more accurate if elution was performed with a gradient of methanol in the same buffer (system B). Gradients were programed by an automated gradient controller (model 680, Waters Associates, Milford, MA) and are detailed in Table 1. The HPLC systems described were calibrated using the following reference compounds: synthetic iodothyronines (Henning GmbH, Berlin, West Germany); iodothyroacetic acids (Sigma); ^{125}I-labeled rT_3, T_3, and 3,3′-T_2 (prepared in this laboratory); iodothyronine sulfates [synthetized as previously described (25)]; biosynthetic T_3G and 3,3′-T_2G [isolated from primary cultured rat hepatocytes[1] (13)]; and biosynthetic rT_3G and T_4G (isolated from rat bile; this study). Retention times were estimated by measurement of the radioactivity or absorbance (254 nm) of the eluate (model 440 fixed wavelength detector, Waters Associates).

TABLE 1. Elution schemes of reverse phase C_{18} HPLC using linear gradients of acetonitrile (system A) or methanol (system B) in 0.02 M ammonium acetate (pH 4)

System A		System B	
Min	% Acetonitrile	Min	% Methanol
0*–6	16	0*–10	47
6–18	16–27	10–14	47–51
18–22	27	14–18	51
22–27	27–45	18–25	51–65
27–38	45	25–32	65

* Time of injection; flow, 0.8 ml/min.

Samples were prepared for HPLC by evaporation of the solvents at 45 C under a stream of N_2 and reconstitution of the residues in the appropriate initial mobile phase (Table 1). Unprocessed bile (15–25 μl) was injected after 1:5 dilution with initial mobile phase (system A).

Data analysis

Total radioactivity or radioactivity associated with individual components in the various samples was expressed as a percentage of the injected [^{125}I]T_4 dose. The amount of radioactive T_3G or T_3 was multiplied by 2 to correct for loss of specific radioactivity due to random production of [^{125}I]T_3 and $^{125}I^-$ from injected [^{125}I]T_4. All data are given as the mean ± SD. Statistical analysis was performed by Student's t test for unpaired data or one-way classification [*i.e.* analysis of variance followed by comparison between class means (26)]. $P < 0.05$ was considered significant.

Results

Identification of metabolites

The T_4 metabolites excreted in rat bile were identified by 1) coelution on HPLC with available reference compounds (Fig. 1), and 2) hydrolysis of conjugates with β-glucuronidase or sulfatase or with 1 M HCl at elevated temperatures. The identification of T_3 and 3,3′-T_2 conjugates has been described previously (see footnote 1) (10, 20). T_4G and rT_3G were isolated by HPLC and identified by the liberation of T_4 or rT_3 during β-glucuronidase treatment. Biliary T_4S was identified by coelution with synthetic T_4S. In keeping with previous observations (25), biosynthetic T_4S was rapidly hydrolyzed with acid, but not with sulfatase or β-glucuronidase. The presence of 15–25 μl bile in HPLC samples accelerated the elution of T_4S by at least 1 min without affecting the retention times of other known compounds. rT_3S, which coelutes with T_4G, was not observed after treatment of bile with β-glucuronidase.

Recovery of radioactivity applied to HPLC amounted to 94.1 ± 5.5% (n = 73) for unprocessed bile, 97.5 ± 7.5% (n = 45) for biliary conjugates isolated on Sephadex LH-20, and 90.0 ± 8.1% (n = 53) for iodothyronines extracted from feces, serum, or hydrolyzed bile. Recovery of radioactivity fractionated on Sephadex LH-20 was always complete. When T_4S was added to bile, 74.2 ± 4.4% (n = 9) was recovered in the 20% ethanol in water fraction after Sephadex LH-20.

[1] Eelkman Rooda, S. J., M. H. Otten, M. A. C. van Loon, E. Kaptein, and T. J. Visser, submitted for publication.

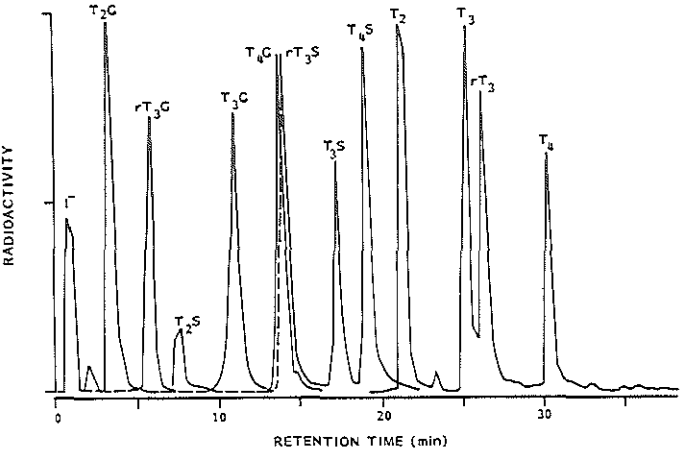

FIG. 1. Elution profile of ^{125}I-labeled iodothyronines and their sulfate (S) and glucuronide (G) conjugates after reverse phase HPLC. The C_{18} column was eluted with a two-step gradient of 16–45% acetonitrile in 0.02 M ammonium acetate (pH 4) at a flow of 0.8 ml/min (system A, Table 1), and fractions of 0.3 min were collected and counted for radioactivity. The chromatogram was constructed from four successive runs with different sets of reference compounds in the absence of bile as described in *Materials and Methods*. The *dotted curve* represents rT_3S coeluting with T_4G. The retention times (minutes) of other analogs are: 3′-monoiodothyronine, 10.2; 3′,5′-T_2, 20.0. 3,3′-diiodothyroacetic acid (3,3′-TA_2), 32.7; 3,5,3′-triiodothyroacetic acid (TA_3), 33.7; and tetraiodothyroacetic acid (TA_4), 37.0.

Exp I

Table 2 shows that within 15 h after injection of [^{125}I]T_4 total radioactivity excreted in urine was decreased to one third by PTU treatment, while fecal excretion was increased, although not significantly. In urine of control and PTU-treated rats, 98.8 ± 0.4% and 93.0 ± 1.0%, respectively, of radioactivity consisted of iodide. Thus, excretion of iodide in urine was decreased by 74% during PTU treatment ($P < 0.001$). On Sephadex LH-20, 1.0 ± 0.3% (control) or 6.6 ± 1.3% (PTU) of urinary radioactivity eluted in the conjugate fraction, but this was not further identified.

Distribution of radioactivity after Sephadex LH-20 chromatography of 50–100 μl rat serum spiked with purified [^{125}I]T_4 was 2.9 ± 1.3%, 2.6 ± 1.3%, and 92.1 ± 2.8% for the fractions eluted with 0.1 M HCl, 20% ethanol in water, and 0.1 M ammonia in ethanol, respectively (n = 15). Eighteen hours after iv injection of [^{125}I]T_4 to control or PTU-treated rats, 10.5 ± 1.7% or 4.1 ± 0.1% ($P < 0.001$) of plasma radioactivity eluted in the I^- fractions, 2.2 ± 0.6% or 4.2 ± 1.5% ($P < 0.05$) eluted in the conjugate fractions, and 85.3 ± 3.0% or 89.6 ± 2.7% (P = NS) eluted in the iodothyronine fractions. Radioactivity in the latter fraction eluted as authentic T_4 on HPLC; labeled T_3 and rT_3 were undetectable. Similar results were obtained by HPLC analysis after solid phase extraction of serum (not shown). In neither control nor PTU-treated rats was T_4S detected in plasma. PTU increased plasma T_4 retention (percent dose per ml) 1.5-fold ($P < 0.005$; Table 2).

Total radioactivity excreted in bile 17–18 h after iv [^{125}I]T_4 was doubled in rats treated with PTU, but bile flow was not affected (Table 2). HPLC analysis showed that the distribution of major metabolites in bile of control rats was, on the average, 43% T_4G, 9.3% T_3G (without correction for difference in specific radioactivity), less than 2% rT_3G, 6.2% T_4S, and 10.8% I^-. The T_4S could only be quantified properly by HPLC after isolation of conjugates on Sephadex LH-20 to eliminate

TABLE 2. Radioactivity (RA) of bile, urine, feces, and plasma collected from rats injected with [^{125}I]T_4

	Bile (17–18 h)		Urine (0–15 h): RA (% dose)	Feces (0–15 h): RA (% dose)	Plasma (18 h)	
	RA (% dose)	Vol (μl/100 g)			Total RA (% dose/ml)	T_4 (% dose/ml)
Control	1.03 ± 0.16	517 ± 55	20.3 ± 4.7	13.3 ± 4.5	0.80 ± 0.11	0.68 ± 0.11
PTU	1.97 ± 0.40[a]	568 ± 61	5.8 ± 0.7[b]	19.6 ± 6.0	1.14 ± 0.07[b]	1.02 ± 0.08[a]

Control and PTU-treated rats (n = 5 each) were anesthetized with pentobarbital, and bile ducts were cannulated about 16.5 h after iv [^{125}I]T_4 injection (time zero). Bile was collected between 17–18 h, and the animals were bled at 18 h (Exp I). The statistical significance (by Student's t test) of the effect of PTU is indicated.

[a] $P < 0.005$.

[b] $P < 0.001$.

interfering biliary components. This is illustrated by representative HPLC profiles in Fig. 2. In bile of PTU-treated rats the mean distribution of radioactivity was 38% T_4G, 1.9% T_3G, 7.4% rT_3G, 16.6% T_4S, and 3.6% I^-. A small amount of 3,3′-T_2S, eluting 2 min after rT_3G, was observed in PTU-treated, but not in control, rats.

Excretion of biliary metabolites 17–18 h after [^{125}I]T_4 injection, expressed as a percentage of the administered dose, is given in Table 3. No significant differences were observed in excretion of biliary iodide (0.112 ± 0.041% *vs.* 0.072 ± 0.033% dose) or nonconjugated T_4 (0.073 ± 0.026% *vs.* 0.128 ± 0.057% dose) between normal *vs.* PTU-treated rats. The opposite effects of PTU on T_3G and rT_3G excretion resulted in a 24-fold decrease in the biliary T_3G/rT_3G ratio. The deiodinase inhibitor caused a 5-fold increase in biliary T_4S. The excretion of T_4G, the major metabolite in bile of all rats, was also higher after PTU treatment, apparently in parallel with plasma [^{125}I]T_4.

HPLC analysis of iodothyronines liberated by β-glucuronidase treatment of bile is illustrated in Fig. 3 (A and C), and corresponding data are presented in Table 4. The T_3/T_4 and rT_3/T_4 ratios obtained (Table 4) were in agreement with the direct estimation of the glucuronides mentioned above (Table 3), and a 15-fold reduction in the T_3/rT_3 ratio by PTU was observed.

No conjugates were observed in the ethanol extracts of feces, and HPLC anlaysis of fecal iodothyronines is illustrated in Fig. 3 (B and D). Similar results were obtained by HPLC of fecal extracts with or without prepurification of iodothyronines on Sephadex LH-20. For both control and PTU-treated rats ratios between iodothyronines in feces (Table 5) were slightly different from the distribution of corresponding conjugates in bile (Table 3). However, the effect of PTU was similar in so far as it decreased the proportion of radioactivity excreted as T_3 while it increased that excreted as rT_3, resulting in an 8-fold decrease in the fecal T_3/rT_3 ratio.

When collection of bile was prolonged for an additional 8 h in two control rats (Exp Ia), bile flow gradually decreased from 492 μl/100 g BW at 17–18 h to 325 μl/100 g BW at 25–26 h. Plasma radioactivity was 0.36% dose/ml at 26 h (consisting of 54% T_4 and 43% I^-) compared with 0.96% dose/ml at 18 h. Total radioactivity excreted in bile was reduced from 1.00% at 17–18 h to 0.33% dose at 25–26 h. The proportion of radioactivity excreted as T_3G was greatly diminished (T_3G/T_4G, 0.18), while that excreted as rT_3G was highly increased (rT_3G/T_4G, 0.29), so that the T_3G/rT_3G ratio in bile collected between 25–26 h was 20-fold lower than that in bile obtained 8 h earlier (0.7 *vs.* 13.2 in Exp I). This was confirmed by determination of iodothyronines liberated by β-glucuronidase treatment.

Exp II

Biliary clearance was analyzed until 8 h after iv [^{125}I]T_4 administration to pentobarbital-anesthetized rats by continuous bile collection. Cumulative excretion of total radioactivity amounted to 15.0 ± 2.9% dose in

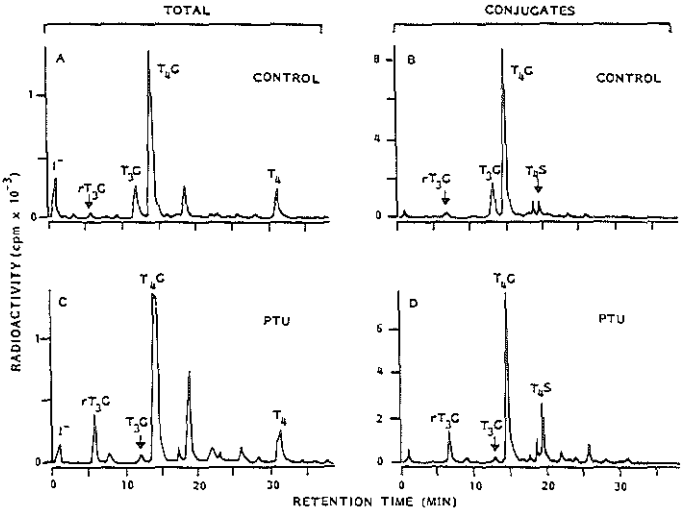

FIG. 2. HPLC analysis of bile collected from control (*upper panels*) and PTU-treated (*lower panels*) rats 17–18 h after injection of [^{125}I]T_4. A and C, Distribution of biliary metabolites was analyzed directly after diluting 25 μl bile with 100 μl mobile phase. B and D, Conjugates were isolated on Sephadex LH-20, concentrated (see *Materials and Methods*), and dissolved in mobile phase. The equivalents of 225 (B) and 150 (D) μl bile were injected into the HPLC system. Elution was performed using system A (Table 1, see also Fig. 1). The results are representative for the distribution of biliary metabolites in each group.

TABLE 3. Effect of PTU on the biliary excretion of T_4 metabolites

Metabolite	Biliary excretion ($10^3 \times$ % dose/h)				
	Exp I (n = 5), 17–18 h		Exp II (n = 3), 7.5–8 h		Exp III (n = 3), 7–8 h, control
	Control	PTU	Control	PTU	
T_4G	441 ± 78	750 ± 112[a]	327 ± 50	609 ± 209	656 ± 85
T_3G	187 ± 36	72 ± 9[a]	47 ± 16	18 ± 32	210 ± 46
rT_3G	17 ± 5	150 ± 58[a]	39 ± 10	652 ± 446	25 ± 5
T_4S	62 ± 10	322 ± 50[a]	46 ± 15	353 ± 133[b]	93 ± 30
T_3G/T_4G	0.43 ± 0.11	0.10 ± 0.02[a]	0.14 ± 0.03[c]	0.02 ± 0.04[b]	0.32 ± 0.05[d]
rT_3G/T_4G	0.04 ± 0.01	0.20 ± 0.05[c]	0.12 ± 0.02[c]	1.10 ± 0.64	0.04 ± 0.01[d]

Experimental protocols have been described in detail in *Materials and Methods* with the period of bile collection indicated. Note that in Exp I and III rats were anesthetized and cannulated less than 0.5 h before collection of bile, whereas the above data for Exp II were derived from samples completing more than 8 h continuous bile collection. Biliary conjugates were quantified by HPLC using system A (Table 1, see also Figs. 1 and 2). T_4S excretion was corrected for the 74% recovery of its isolation on Sephadex LH-20. The significance of the differences (by Student's t test) between control and PTU-treated rats is indicated.

[a] $P < 0.001$.

[b] $P < 0.025$.

[c] Different from control rats in Exp I ($P < 0.005$).

[d] Different from control rats in Exp II ($P < 0.001$), but not from controls in Exp I.

controls (n = 3) and was increased 1.8-fold to 26.6 ± 3.9% by PTU treatment (n = 3; $P < 0.001$). Figure 4 shows the disappearance of plasma radioactivity and the excretion rate of biliary radioactivity. Plasma iodide was lower (*i.e.* 0.09% *vs.* 0.19% dose/ml at 8 h) and conjugates were higher (*i.e.* 0.07% *vs.* 0.03% dose/ml) in PTU-treated *vs.* control rats, but these differences were not significant. Semilogarithmic plots of plasma T_4 *vs.* time yielded estimates of t_{ω} that were not significantly different (7.0 ± 1.4 *vs.* 9.3 ± 1.5 h, control *vs.* PTU).

HPLC analysis of biliary conjugates in relation to the time after [^{125}I]T_4 injection is depicted in Fig. 5. T_4G was the major metabolite observed in bile of all animals, and even in control rats little T_3G was detected (Table 3). In PTU-treated rats rT_3G markedly increased and even exceeded T_4G excretion in the latest bile sample. Especially in the PTU-treated animals relatively large amounts of T_4S were excreted throughout the period of bile collection. Figure 5 also shows the total excretion of conjugates in bile pools of normal and PTU-treated rats. PTU increased biliary T_4G, rT_3G, and T_4S by 1.4-, 13.4-, and 4.6-fold, respectively. Already low in control rats, PTU further decreased T_3G 2.5 times. Again, a dramatic reduction of 28-fold was observed in the biliary T_3G/rT_3G ratio after PTU treatment. No significant differences were observed in the biliary excretion of iodide (1.9 ± 0.4% *vs.* 1.5 ± 0.2% dose) or nonconjugated T_4 (1.7 ± 0.4% *vs.* 1.9 ± 0.3%, dose, controls *vs.* PTU). The latter was excreted in bile mainly during the first hour after T_4 injection. Table 4 includes data on the HPLC determination of iodothyronines liberated by treatment of bile pools with β-glucuronidase. Ratios between T_4, T_3, and rT_3 were very similar to those between the corresponding glucuronides (Fig. 5).

Exp III

This was a modification of Exp II carried out in control rats with the intention to expose the animals to less experimental stress. Part of the results obtained are included in Figs. 4 and 5 in comparison with those from rats subjected to prolonged bile diversion and anesthesia. In Exp III, rats were anesthetized and cannulated immediately before bile collection 7–8 h after [^{125}I]T_4 administration. Biliary excretion of radioactivity amounted to 1.54 ± 0.17 % dose (n = 3), *i.e.* 81% higher than in the corresponding time period in Exp II (Fig. 4), and also the T_3G/T_4G ratio was 2.3-fold higher in Exp III (Table 3). The HPLC distribution of biliary metabolites was very similar to that observed after 18 h in Exp I (Table 3). Again, β-glucuronidase treatment of bile provided consistent results (Table 4). The proportion of radioactivity excreted as nonconjugated T_4 was higher after 8 h than after 18 h (13.6 ± 2.4% *vs.* 7.0 ± 1.6%; $P < 0.005$).

Biliary clearance of plasma T_4

Biliary clearance of injected T_4 was estimated from the total radioactivity excreted in bile and the plasma T_4 level determined simultaneously and corrected for body weight (Table 6). In Exp I, biliary clearance 18 h after T_4 was increased, but not significantly, by PTU. Based on the estimation of T_4G in bile, PTU was shown to have no effect on the glucuronidative clearance of T_4 (Table 6). In Exp II, the biliary T_4 clearance varied between 3.3–4.1 ml/h·kg in controls, but gradually increased from 3.9 (t = 0.5 h) to 9.9 ml/h·kg (t = 8 h) in PTU-treated rats. The biliary clearance by disposition of T_4G determined during the entire 8-h observation

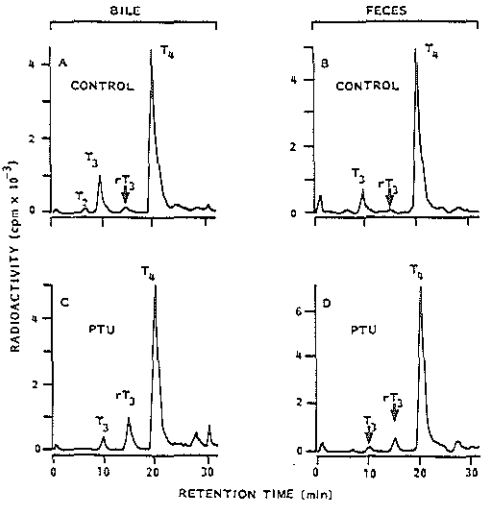

FIG. 3. HPLC analysis of iodothyronines in bile treated with β-glucuronidase (*left*) and in fecal extracts (*right*). [^{125}I]T_4 was injected iv in control (A and B) and PTU-treated (C and D) rats. A and C. Bile collected between 17–18 h was incubated with β-glucuronidase. The hydrolyzed iodothyronines were isolated on Sephadex LH-20 (see *Materials and Methods*) and analyzed by reverse phase C_{18} HPLC using a two-step gradient of 47–65% methanol in 0.02 M ammonium acetate (pH 4) at a flow of 0.8 ml/h (system B, Table 1). Fractions of 0.4 ml were collected and counted for radioactivity. The equivalents of 165 (A) and 150 (C) μl bile were applied to the column. B and D, Fecal radioactivity excreted within 15 h after [^{125}I]T_4 injection was extracted in ethanol (see *Materials and Methods*), with a recovery of 43 ± 9% (n = 16). About 160 μl of this extract were dried, reconstituted in mobile phase, and analyzed using the same HPLC system. The chromatograms shown are representative for the distribution of metabolites in each group. In this system 3′-monoiodothyronine, 3′,5′-T_2, 3,3′-TA_2, TA_3, and TA_4 eluted at 2.6, 5.3, 22.8, 26.2, and 30.8 min, respectively. Iodothyronine conjugates, if present, eluted within 4 min.

period was, on the average, 1.55 ± 0.20 (control) and 2.17 ± 0.41 ml/h kg (PTU).

Discussion

Biliary excretion of thyroid hormone has been poorly documented, although this is a significant metabolic pathway in rats and probably also in humans (7). Neither the identity nor the quantity of the T_4 metabolites in human or rat bile have been established directly with current chromatographic techniques. Many endogenous compounds are converted by hepatic glucuronidation and sulfation into more hydrophilic derivatives to facilitate their biliary and urinary excretion (27). In principle, these are reversible reactions, since hydrolysis of the conjugates is catalyzed by ubiquitous β-glucuronidases and sulfatases. However, sulfation of T_4 and T_3 leads to the irreversible inactivation of these compounds, since T_3S and T_4S are rapidly converted by the type I deiodinase of liver in contrast to the glucuronides (see footnote 1) (2). After biliary excretion, iodothyronine glucuronides are hydrolyzed in the intestine, especially by bacterial glucuronidases, which enables reabsorption of the free hormones (14, 16, 17).

We have studied the biliary clearance of T_4 in rats in two different experimental situations. Initially, this was done by bile duct cannulation before iv [^{126}I]T_4 administration and prolonged bile collection under continuous pentobarbital anesthesia, as described in Exp II. The biliary excretion of total radioactivity after [^{125}I]T_4 injection to normal rats in this type of experiment, *i.e.* 14% in 8 h, is very similar to that reported by other researchers, *i.e.* 6–10% in 3–6 h (11, 24, 28–32). Also, the 2-fold increase in biliary radioactivity induced by PTU is in accordance with the report by Lang and Premachandra (32), although more variable results were obtained by Flock and Bollman (24) after long term treatment of rats with thiouracil.

For the determination of biliary T_4 metabolites we used a HPLC system that resulted in separation of the sulfate and glucuronide conjugates of different iodothyronines. In addition, T_4 excretory products were identified after enzymatic or acid hydrolysis of conjugates using another HPLC system for separation of native iodothyronines. In agreement with previous publications (8, 11, 21, 22, 24), conjugates in bile of normal rats represents mainly T_4G and smaller amounts of T_3G and rT_3G. In addition, we present unequivocal evidence for the excretion of T_4S in rat bile. This is reminiscent of the finding by Flock and Bollman (24) of an acid-labile T_4 conjugate in the sulfate region of their chromatographic system (termed T_4X) which was resistant to arylsulfatase (Mylase P). The biliary metabolite eluting at the T_4S position of our HPLC system is also hydrolyzed to T_4 in 1 M HCl at 80 C, but is resistant to Sigma sulfatase type VIII, consistent with authentic T_4S (25). It has also been reported by others that bile of [^{125}I]T_4-injected rats did not liberate substantial amounts of T_4 after digestion with Sigma sulfatase type III (33) or type IV (11), leading to the suggestion that T_4S is not an important constituent of rat bile. After injection of radioactive T_4 to thyroidectomized dogs, Roche and Michel (23) noted the presence of a metabolite in plasma and lymph that was hydrolyzed by treatment with acid or Taka-Diastase with liberation of T_4. Sulfatases produced by certain obligately anaerobic bacterial strains from human and rat intestinal microflora also effectively hydrolyze T_4S (15), which further underscores the variable susceptibility of T_4S to different sulfatase preparations.

The T_3G/T_4G ratio in bile of the control rats in Exp

TABLE 4. Effect of PTU on T_4 metabolites in β-glucuronidase-treated bile

Metabolite	% Distribution of iodothyronines				
	Exp I (n = 5), 17–18 h		Exp II (n = 3), 0–8 h		Exp III (n = 3), 7–8 h, control
	Control	PTU	Control	PTU	
T_3[a]	14.9 ± 3.1	3.6 ± 0.8[b]	5.0 ± 1.9	1.1 ± 0.2[c]	10.5 ± 2.2
rT_3	3.1 ± 0.6	11.6 ± 2.7[b]	3.7 ± 0.9	20.1 ± 9.0[d]	2.4 ± 0.3
T_4	64.0 ± 2.0	63.8 ± 2.4	75.4 ± 3.0	58.8 ± 5.5[c]	71.3 ± 0.7
$2 \times T_3/T_4$	0.47 ± 0.11	0.11 ± 0.02[a]	0.13 ± 0.05	0.04 ± 0.006[d]	0.30 ± 0.06
rT_3/T_4	0.05 ± 0.01	0.18 ± 0.05[b]	0.05 ± 0.01	0.36 ± 0.19[d]	0.03 ± 0.004

Detailed experimental protocols are given in *Materials and Methods*, with the period of bile collection indicated. The experiments are the same as those shown in Table 3. Biliary glucuronides were hydrolyzed with β-glucuronidase, and the liberated iodothyronines were isolated on Sephadex LH-20 before HPLC analysis (Fig. 3). The significance of the differences (by Student's t test) between control and PTU-treated rats is indicated.

[a] Not corrected for loss of specific radioactivity due to random production of $[^{125}I]T_3$ and $^{125}I^-$ from injected $[^{125}I]T_4$.

[b] $P < 0.001$.

[c] $P < 0.025$.

[d] $P < 0.05$.

TABLE 5. Effect of PTU on fecal excretion products of T_4

Metabolite	% Distribution of fecal radioactivity	
	Controls	PTU-Treated
T_3[a]	9.0 ± 2.6	3.7 ± 1.4[b]
rT_3	2.2 ± 0.7	6.2 ± 0.6[c]
T_4	70.4 ± 4.5	68.1 ± 6.9
$2 \times T_3/T_4$	0.26 ± 0.09	0.11 ± 0.04[d]
rT_3/T_4	0.03 ± 0.01	0.09 ± 0.01[c]

Feces were collected during 15 h after $[^{125}I]T_4$ injection (Exp I) and extracted with ethanol. Analysis of fecal radioactivity by HPLC was performed using system B (Table 1, see also Fig. 3) either directly or after isolation of iodothyronines on Sephadex LH-20. Differences between control and PTU rats (n = 5 each) were statistically significant (by Student's t test) as indicated.

[a] Not corrected for loss of specific radioactivity due to random production of $[^{125}I]T_3$ and $^{125}I^-$ from injected $[^{125}I]T_4$.

[b] $P < 0.005$.

[c] $P < 0.001$.

[d] $P < 0.01$.

II remains relatively constant (0.11–0.14) during the 8 h of collection after $[^{125}I]T_4$ injection, whereas the rT_3G/T_4G ratio steadily increases from 0.01 (0.5–1 h) to 0.12 (7.5–8 h). By HPLC of β-glucuronidase-digested bile, Takai *et al.* (11) estimated similar T_3G/T_4G[2] (0.09) and rT_3G/T_4G (0.04) ratios for bile collected 2–2.5 h after $[^{125}I]T_4$ injection. However, higher T_3G/T_4G (see footnote 2) (0.14) and rT_3G/T_4G (0.09) ratios were found by Flock *et al.* (22, 34) in bile collected up to 6 h after $[^{125}I]T_4$ administration. This was determined using a cumbersome technique with poor resolution of the different metabolites. In our Exp II, T_4S is a relative constant fraction of total biliary radioactivity independent of time after $[^{125}I]T_4$ administration; its proportion (5–8%) is in agreement with that reported for T_4X by Flock *et al.* (24, 34).

We also investigated the biliary clearance of T_4 using the experimental protocol previously used by Bastomsky and Papapetrou (35), where iv injected $[^{125}I]T_4$ is allowed to exchange with the different tissue pools before collection of bile 17–18 h after tracer administration (Exp I). Bastomsky *et al.* (30, 35) were unable to identify biliary constituents other than T_4, T_4G, and I^-, which were reported to be excreted in a ratio of 8:55:11. The mean biliary T_3G/T_4G ratio of 0.43 in the control animals in our Exp I is 3-fold higher, while the relative proportions of rT_3G (0.04) and T_4S (0.14) are similar or even lower compared with the control values of Exp II. By RIA of endogenous iodothyronines in bile from normal rats digested with β-glucuronidase and sulfatase, Földes *et al.* (36) determined a T_3/T_4 ratio of 0.24 and a rT_3/T_4 ratio of 0.06. Possible explanations for this discrepancy in biliary $T_3(G)/T_4(G)$ ratio are 1) the difference in biliary clearance of endogenous T_4 and that determined at a single time point after distribution of exogenous $[^{125}I]T_4$, and 2) the use of older rats by Földes *et al.* (36), *i.e.* about 350 g BW as opposed to about 220 g in our experiment.

In normal rats the low T_3G/T_4G ratio determined even in the last bile sample (7.5–8 h) of Exp II compared with that estimated in the bile sample (17–18 h) collected in Exp I could be due to differences in 1) the time of equilibration of injected $[^{125}I]T_4$ with T_3-producing tissues, or 2) the duration of anesthesia and bile diversion. Additional experiments were conducted to discriminate between these possibilities. The results of Exp Ia demonstrate that prolonged anesthesia and bile collection after complete distribution of $[^{125}I]T_4$ (see also Ref. 33) produce a dramatic decrease in the T_3G/T_4G ratio and an even greater increase in the rT_3G/T_4G ratio. The findings obtained in Exp III suggest that a near-maximum T_3G/T_4G ratio is already achieved 8 h after T_4

[2] In contrast to our study, these previous reports (11, 22, 34) do not mention the correction of T_3G excretion for the loss of specific radioactivity due to random deiodination of injected $[3',5'\text{-}^{125}I]T_4$.

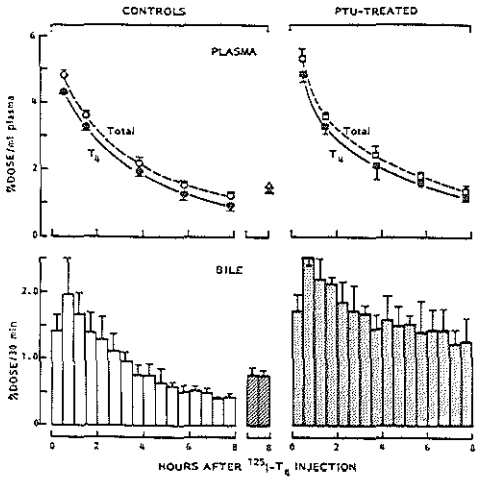

FIG. 4. Plasma and biliary radioactivity after administration of [^{125}I]T_4 (time zero) to control and PTU-treated rats. Radioactivity in plasma (*upper panels*) and bile (*lower panels*) collected up to 8 h after iv [^{125}I]T_4 injection in normal rats is depicted by *circles* and *open bars*, and that in PTU-treated rats by *squares* and *dotted bars*. Rats were anesthetized, and bile was collected during the entire experiment (II). Results are compared with those obtained in Exp III (*triangles and hatched bars*) in which control rats were anesthetized and cannulated just before the collection of bile from 7–8 h after T_4 injection. All groups consisted of three rats. The *open symbols* show total plasma radioactivity, and the *solid symbols* plasma [^{125}I]T_4 isolated on Sephadex LH-20. Data are expressed as the mean ± SD percent dose per ml (unless SD smaller than symbol). Plasma T_4 retention was significantly increased by PTU ($P < 0.005$), as estimated by one-way classification. Radioactivity in bile (mean ± SD percent dose) was determined in successive 30-min periods. PTU significantly increased biliary radioactivity after T_4 injection ($P < 0.001$, one-way classification). Bile flow in Exp II decreased gradually from 563 ± 35 in the first hour to 367 ± 17 μl/h·100 g BW after 8 h (n = 6) independent of PTU. Bile flow in Exp III amounted to 494 ± 45 μl/h·100 g BW (n = 3).

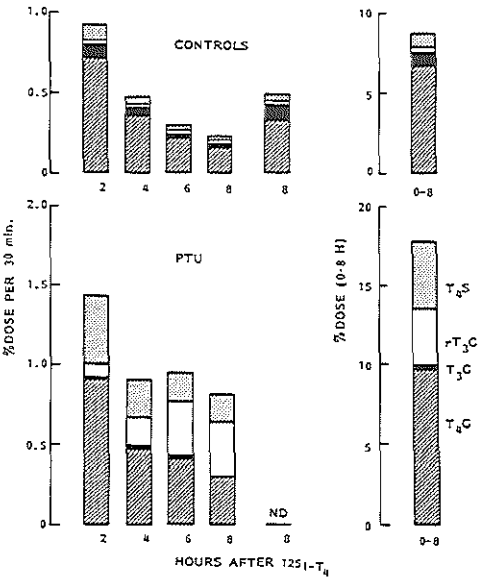

FIG. 5. Effect of PTU on biliary excretion of T_4 metabolites. Anesthetized bile-diverted control (*upper panel*) and PTU-treated (*lower panel*) rats were injected iv with [^{125}I]T_4 (time zero). Iodothyronine conjugates in bile collected at 1.5–2, 3.5–4, 5.5–6, and 7.5–8 h were quantified by HPLC using gradient system A (Table 1 and Figs. 1 and 2). The results are shown in the panels on the *left* (Exp II; n = 3). Distribution of biliary T_4 metabolites in control rats that were anesthetized just before cannulation and bile collection 7–8 h after T_4 injection (Exp III; n = 3) is shown as the *extra bar* in the *upper panel*. Data are expressed as percent dose excreted in 30 min, and PTU significantly decreased excretion of T_3G as well as increased biliary T_4G, rT_3G, and T_4S ($P < 0.005$, one-way classification). The panels on the *right* represent total excretion of biliary T_4 metabolites over 8 h, determined by HPLC of bile pools, and are expressed as percent dose (n = 3). *Bar sections* represent T_4G (*hatched*), T_3G (*black*), rT_3G (*white*), and T_4S (*dotted*). ND, Not determined.

injection. Therefore, prolonged experimental stress is the main cause of the low T_3G excretion in control animals of Exp II, in agreement with the stress-induced reduction in peripheral T_3 production recently reported by Bianco *et al.* (37). This conclusion indicates that data on biliary T_4 clearance obtained by prolonged bile collection under continuous anesthesia after [^{125}I]T_4 administration should be interpreted with caution. It is not entirely certain if the results obtained in the present study using pentobarbital anesthesia even in case of Exp I truly reflect the physiological situation. In contrast to its analog, phenobarbital, relatively little is known about the possible effects of pentobarbital on rat thyroid parameters (38). Prolonged administration of phenobarbital to rats does not affect binding of T_4 to plasma proteins, but it induces microsomal enzymes with a consequent increase in the glucuronidation and deiodination of iodothyronines (38). Acute administration of pentobarbital as in Exp I does not have such effects, although bile flow may change a little (39).

In both Exp I and II, PTU treatment of rats produces an almost 2-fold increase in T_4G excretion, a 2.6-fold decrease in biliary T_3G, a 9- to 17-fold increase in rT_3G, and a 5.8-fold increase in T_4S excretion. After prolonged administration of thiouracil, Flock and Bollman (24) noted similar changes in biliary T_3G/T_4G and rT_3G/T_4G ratios, but the relative amount of T_4X (T_4S; see above) was only increased 50% by thiouracil. These workers also showed that acute treatment with BHDB, another type

TABLE 6. The effect of PTU on the biliary clearance of T_4

	Biliary clearance of plasma T_4 (ml/h·kg BW)				
	Exp I (n = 5), 18 h		Exp II (n = 3), 8 h[a]		Exp III (n = 3), 8 h, control
	Control	PTU	Control	PTU	
Total	6.5 ± 1.7	9.1 ± 2.2	4.1 ± 1.2[b]	9.9 ± 3.2[b]	5.6 ± 0.6
Via T_4G	2.9 ± 0.8	3.0 ± 1.2	1.5 ± 0.5	2.4 ± 0.9	2.4 ± 0.3

Experimental details of anesthesia, bile duct cannulation, and injection of T_4 are described in *Materials and Methods*. Either total radioactivity or that associated with the T_4G in bile (percent dose per h) was divided by plasma T_4 (percent dose per ml) and expressed per kg BW (mean ± SD). Differences between corresponding control and PTU groups were not significant except as indicated.

[a] Data derived from samples more than 8 h after induction of anesthesia and bile diversion in contrast to less than 1.5 h in Exp I and III.

[b] $P < 0.05$ (by Student's t test).

I deiodinase inhibitor (40), induces a 2-fold decrease in biliary T_3G/T_4G, a 3-fold increase in rT_3G/T_4G, and a 3-fold increase in the proportion of T_4X (21, 22). Taken together, these findings support the view that the PTU (and BHDB)-sensitive type I deiodinase is an important site for the peripheral production of T_3 and degradation of rT_3 and T_4S. The magnitude of the PTU-induced decrease in biliary T_3G is consistent with the findings of Silva *et al.* (3) that in euthyroid rats at least 70% of peripheral T_3 generation is derived by type I deiodination of T_4. The PTU-induced increase in biliary T_4G excretion apparently results from the increased plasma T_4 retention.

Although the recovery of radioactivity in feces and urine within 15 h of $[^{125}I]T_4$ administration in Exp I is far from complete, the ratio of fecal:urinary radioactivity in control rats, *i.e.* 13%:20%, is similar to that found by complete collection of excreted radioactivity in rats given a single $[^{125}I]T_4$ injection or a constant $[^{125}I]T_4$ infusion (41–45). In these studies the exact composition of fecal T_4 metabolites has not been established, and our findings may be influenced by the incomplete ethanol extraction of fecal radioactivity. There is general agreement that iodothyronines are not normally eliminated as conjugates in the feces, and that free T_4 is the main excretory product. Boonnamsiri *et al.* (43) found in their ^{126}I-equilibrated rats a T_3/T_4 ratio of 0.26, while DiStefano and Sapin (42) reported a value of 0.18 after a bolus $[^{125}I]T_4$ injection. Our T_3/T_4 ratio of 0.26 for control rats is consistent with the above data.

The PTU-induced shift in the clearance of T_4 from urine to feces has been documented in numerous studies (41, 46, 47). The decrease in urinary radioactivity is due to a diminished iodide production accompanied by a slight increase in the excretion of unidentified conjugates. The increased fecal excretion of T_4 metabolites correlates well with the PTU-induced alterations in biliary T_4 clearance. However, in both control and PTU-treated rats the ratios between the different iodothyronines in feces are not identical to those between the corresponding conjugates in bile. Possible explanations for this difference are 1) the time lag of the appearance of radioactive products in feces compared with bile (intestinal transit), 2) different rates of hydrolysis of the conjugates and subsequent reabsorption of the free iodothyronines (enterohepatic circulation), and 3) secretion of iodothyronines from blood to intestine (mesenteric arterial flux) (48).

In the control animals of Exp I and III, 18 and 8 h after $[^{125}I]T_4$ injection, respectively, at least 85% of plasma radioactivity consists of T_4, and the remainder represents mainly iodide (33, 49). The proportion of plasma iodide is increased during prolonged bile collection in Exp Ia and II. We were unable to determine the concentrations of labeled T_3 and rT_3 in plasma by HPLC. This is understandable in view of the low levels of endogenous T_3 and rT_3 in plasma relative to those of T_4.[3] PTU induces an increase in plasma conjugates, but no significant accumulation of T_4S is observed. This is in contrast to previous observations of the appearance of T_3S in plasma after $[^{125}I]T_3$ injection, which is greatly stimulated by PTU treatment (20). The difference is probably explained by the slower metabolic clearance of T_4 (48) and the smaller proportion of this compound undergoing sulfate conjugation.

We calculated the biliary clearance of T_4, *i.e.* that part of the plasma T_4 clearance that is accounted for by the biliary excretion of T_4 products (total biliary clearance) or specifically by the excretion of T_4G (glucuronidative clearance). Values for total biliary T_4 clearance are in general agreement with the literature. Using data obtained 3–4 h after labeled T_4 injection, a biliary clearance of 3–4 ml/h·kg BW has been reported (29, 30, 32), which is similar to our observations in Exp II. In studies analogous to our Exp I, Bastomsky and Papapetrou (28, 35) estimated a biliary T_4 clearance in normal rats of 3.3–4 ml/h·kg, which is lower than the 6.5 ml/h·kg that we

[3] In our laboratory, plasma iodothyronine levels in normal rats amounted to 51 ± 10 nM T_4 (n = 66), 1.4 ± 0.2 nM T_3 (n = 62), and 0.03 ± 0.01 nM rT_3 (n = 35).

have determined. In a similar study, Oppenheimer *et al.* (49) found a biliary clearance of 2–2.5 ml/h·kg 24 h after tracer T_4 administration. Using rats equilibrated with radioiodide, Galton and Nisula (50) showed that the biliary T_4 clearance amounts to roughly 3 ml/h·kg. The results of the latter two studies, however, must be interpreted with caution since they were carried out in ether-anesthetized rats. Ether has been shown to greatly affect hepatic glucuronidation by extensive depletion of UDP-glucuronic acid levels (51).

The glucuronidative clearance of T_4 (GC_4) is approximately 40% of total biliary clearance in untreated rats, which is in agreement with the report of Bastomsky and Papapetrou (35). We find that GC_4 is not affected by PTU. From previous data on the biliary excretion of T_3G up to 4 h after injection of T_3 (10, 20), a mean glucuronidative clearance for T_3 (GC_3) of 46 ml/~~min~~·kg (n = 8) was calculated. The T_3G/T_4G ratio in bile of control rats in Exp I is, on the average, 0.43. If this value is divided by the GC_3/GC_4 ratio (15.8), an approximate figure for the plasma T_3/T_4 ratio 18 h after [^{125}I]T_4 injection of 0.027 is obtained. Although this is identical to the ratio of endogenous T_3 and T_4 levels in plasma (see footnote 3), it should be emphasized that 1) GC_3 was determined under nonsteady state conditions; 2) the thus-calculated plasma T_3/T_4 ratio neglects the contribution of thyroidal T_3 secretion; and 3) the excretion of T_3G determined after a bolus injection of tracer T_4 may not reflect production of T_3G from endogenous T_4.

The biliary T_3G/rT_3G ratio appears to be a sensitive indicator of type I deiodinase activity, showing a 24-fold decrease after PTU treatment. Takai *et al.* (11) report on a decreased biliary T_3G/rT_3G ratio in 72-h fasted rats which was not observed if the fasting-induced hypothyroid state in these animals (52) was prevented with replacement doses of T_4. The decreased T_3G/rT_3G ratio in the nonsubstituted fasted rats is consistent with a decrease in type I deiodinase activity in hypothyroid animals (52). Direct effects of food deprivation on peripheral thyroid hormone metabolism independent of thyroid function may, therefore, not involve a decrease in type I deiodinase activity. Evidence has been presented that fasting-induced changes in thyroid hormone metabolism are mainly due to a diminution in the tissue uptake of iodothyronines (53, 54).

In conclusion, we have performed two types of experiments to determine the biliary clearance of T_4 in rats. The results demonstrate that it is necessary 1) to allow sufficient time for complete exchange of injected tracer T_4 (>8 h) with the different tissues, and 2) to keep stress associated with anesthesia and bile collection to a minimum. Our findings indicate that sulfation is a significant pathway of T_4 metabolism in rats, although its precise contribution remains to be assessed. The effects of PTU on the excretion of T_4S indicate that the conjugate is cleared mainly by type I deiodination. The T_3G/rT_3G ratio in bile reflects the activity of the type I deiodinase and can be a useful parameter in further studies of the metabolism of thyroid hormone in different pathophysiological conditions.

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References

1. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5:151
2. Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 189
3. Silva JE, Gordon MB, Crantz FR, Leonard JL, Larsen PR 1984 Qualitative and quantitative differences in the pathways of extrathyroidal triiodothyronine generation between euthyroid and hypothyroid rats. J Clin Invest 73:898
4. Cooper DS 1984 Antithyroid drugs. N Engl J Med 311:1353
5. Bauer AGC, Wilson JHP, Lamberts SWJ, Docter R, Hennemann G, Visser TJ 1987 Handling of iodothyronines by the liver and kidney in patients with chronic liver disease. Acta Endocrinol (Copenh) 116:339
6. Visser TJ, Kaptein E, Terpstra OT, Krenning EP 1988 Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 67:17
7. Burger A 1986 Nondeiodinative pathways of thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 276
8. Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford, p 345
9. Taurog A 1955 Conjugation and excretion of the hormone. Brookhaven Symp Biol 7:111
10. De Herder WW, Bonthuis F, Rutgers M, Hazenberg MH, Visser TJ 1988 Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Endocrinology 112:153
11. Takai NA, Rapoport B, Yamamoto M 1980 Biliary excretion of iodothyronines in rats as determined by high pressure liquid chromatography: effect of starvation. Endocrinology 107:176
12. Myant NB 1956 Biliary excretion of thyroxine in humans. Clin Sci 15:227
13. De Herder WW, Hazenberg MP, Pennock-Schröder AM, Hennemann G, Visser TJ 1986 Hydrolysis of iodothyronine glucuronides by obligately anaerobic bacteria isolated from human fecal flora. FEMS (Fed Eur Microbiol Soc) Microbiol Lett 35:249
14. De Herder WW, Hazenberg MP, Pennock-Schroder AM, Hennemann G, Visser TJ 1986 Rapid and bacteria-dependent *in vitro* hydrolysis of iodothyronine conjugates by intestinal contents of humans and rats. Med Biol 64:31
15. Hazenberg MP, de Herder WW, Visser TJ 1988 Hydrolysis of iodothyronine conjugates by intestinal bacteria. FEMS (Fed Eur Microbiol Soc) Microbiol Rev 54:9
16. De Herder WW, Bonthuis F, Hazenberg MP, Otten MH, Visser TJ 1986 Biliary excretion and reabsorption of T_3 and its metabolites. Ann Endocrinol (Paris) 47:23 (Abstract)
17. Rutgers M, Hazenberg MP, Heusdens FA, Bonthuis F, Visser TJ 1987 Further evidence for the enterohepatic circulation (EHC) of T_3 in rats. Ann Endocrinol (Paris) 48:134 (Abstract)
18. Miller LJ, Gorman CA, Go VW 1978 Gut-thyroid interrelationships. Gastroenterology 75:901
19. Mol JA, Visser TJ 1985 Rapid and selective inner ring deiodination of T_4 sulfate by rat liver deiodinase. Endocrinology 117:8

20. Rutgers M, Bonthuis F, de Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758
21. Flock EV, Bollman JL 1964 Effect of butyl-4-hydroxy-3,5-diiodobenzoate on the metabolism of L-thyroxine and related compounds. Endocrinology 75:721
22. Flock EV, Bollman JL, Owen CA, Zollman PE 1965 Conjugation of thyroid hormones and analogs by the Gunn rat. Endocrinology 77:303
23. Roche J, Michel R 1960 On the peripheral metabolism of thyroid hormones. Ann NY Acad Sci 86:454
24. Flock EV, Bollman JL 1962 The effect of thiouracil on the metabolism of L-thyroxine. Biochem J 84:621
25. Mol JA, Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. Endocrinology 117:1
26. Snedecor GW, Cochran WG 1967 One-way classifications. Analysis of variance. In: Statistical Methods. Iowa State University Press, Ames, p 258
27. Klaassen CD, Watkins JB 1984 Mechanisms of bile formation, hepatic uptake, and biliary excretion. Pharmacol Rev 36:1
28. Bastomsky CH 1973 The biliary excretion of thyroxine and its glucuronic acid conjugate in normal and Gunn rats. Endocrinology 92:35
29. Bastomsky CH 1974 Effects of a polychlorinated biphenyl mixture (Aroclor 1254) and DDT on biliary excretion in rats. Endocrinology 95:1150
30. Bastomsky CH, Wyse JM 1975 Enhanced thyroxine metabolism following cutaneous application of microscopic immersion oil. Res Commun Chem Pathol Pharmacol 10:725
31. Goldstein JA, Taurog A 1968 Enhanced biliary excretion of thyroxine glucuronide in rats pretreated with benzpyrene. Biochem Pharmacol 17:1049
32. Lang S, Premachandra BN 1963 Propylthiouracil and hepatic clearance of thyroid hormones. Am J Physiol 204:133
33. Pittman CS, Shimizu C 1966 Metabolism of ^{131}I- and ^{14}C-labeled thyroxines. Endocrinology 79:1109
34. Flock EV, David C, Hallenbeck GA, Owen CA 1963 Metabolism of D-thyroxine. Endocrinology 73:764
35. Bastomsky CH, Papapetrou PD 1973 The effect of methylcholanthrene on biliary thyroxine excretion in normal and Gunn rats. J Endocrinol 56:267
36. Földes O, Langer P, Straussova K, Brozmanova H, Gachwendtová K 1982 Direct quantitative estimation of several iodothyronines in rat bile by radioimmunoassay and basal data on their biliary excretion. Biochim Biophys Acta 716:383
37. Bianco AC, Nunes MT, Hell NS, Maciel RMB 1987 The role of glucocorticoids in the stress-induced reduction of extrathyroidal 3,5,3′-triiodothyronine generation in rats. Endocrinology 120:1033
38. Cavalieri RC, Pitt-Rivers R 1981 The effects of drugs on the distribution and metabolism of thyroid hormones. Pharmacol Rev 33:55
39. Kuipers F, Dijkstra T, Havinga R, Van Asselt W, Vonk RJ 1985 Acute effects of pentobarbital-anesthesia on bile secretion. Biochem Pharmacol 34:1731
40. Hesch RD, Koehrle J 1986 Intracellular pathways of iodothyronine metabolism. In: Ingbar SH, Braverman LE (eds) The Thyroid. Lippincott, Philadelphia, p 154
41. Morreale de Escobar G, Escobar del Rey F 1967 Extrathyroid effects of some antithyroid drugs and their metabolic consequences. Recent Prog Horm Res 23:87
42. DiStefano JJ, Sapin V 1987 Fecal and urinary excretion of six iodothyronines in the rat. Endocrinology 121:1742
43. Boonnamsiri V, Kermode JC, Thompson BD 1979 Prolonged intravenous infusion of labelled iodocompounds in the rat: [^{125}I]thyroxine and [^{125}I]triiodothyronine metabolism and extrathyroidal conversion of thyroxine to triiodothyronine. J Endocrinol 82:235
44. Van Doorn J, van der Heide D, Roelfsema F 1983 Sources and quantity of 3,5,3′-triiodothyronine in several tissues of the rat. J Clin Invest 72:1778
45. Newman WC, Moon RC 1967 Altered thyroxine metabolism resulting from the chemical carcinogen 3-methylcholanthrene. Endocrinology 80:896
46. Jones SL, Van Middlesworth L 1960 Normal I^{131} L-thyroxine metabolism in the presence of potassium perchlorate and interrupted by propylthiouracil. Endocrinology 67:855
47. Van Doorn J, Roelfsema F, van der Heide D 1983 The effect of propylthiouracil and methimazole on the peripheral conversion of thyroxine to 3,5,3′-triiodothyronine in athyreotic thyroxine maintained rats. Acta Endocrinol (Copenh) 103:509
48. DiStefano III JJ 1986 Modeling approaches and models of the distribution and disposal of thyroid hormones. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 39
49. Oppenheimer JH, Bernstein G, Surks MI 1968 Increased thyroxine turnover and thyroidal function after stimulation of hepatocellular binding of thyroxine by phenobarbital. J Clin Invest 47:1399
50. Galton VA, Nisula BC 1972 The enterohepatic circulation of thyroxine. J Endocrinol 54:187
51. Watkins JB, Klaassen CD 1988 Chemically-induced alteration of UDP-glucuronic acid concentration in rat liver. Drug Metab Dispos 11:37
52. Kaplan MM 1986 Regulatory influences on iodothyronine deiodination in animal tissues. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 231
53. Van der Heyden JTM, Docter R, van Toor H, Wilson JHP, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251:E156
54. De Jong M, van der Hoek H, Docter R, Krenning EP, Hennemann G 1987 Decreased thyroid hormone metabolism in the intact fasted rat liver is not due to inhibition of deiodination but caused by diminished transport into the cells. Ann Endocrinol (Paris) 48:133 (Abstract)

Chapter VIII

ENTEROHEPATIC CIRCULATION OF TRIIODOTHYRONINE (T_3) IN RATS: IMPORTANCE OF THE MICROFLORA FOR THE LIBERATION AND REABSORPTION OF T_3 FROM BILIARY T_3 CONJUGATES

Enterohepatic Circulation of Triiodothyronine (T_3) in Rats: Importance of the Microflora for the Liberation and Reabsorption of T_3 from Biliary T_3 Conjugates*

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ABSTRACT. In normal rats, T_3 glucuronide (T_3G) is the major biliary T_3 metabolite, but excretion of T_3 sulfate (T_3S) is greatly increased after inhibition of type I deiodinase, *e.g.* with 6-propyl-2-thiouracil (PTU). In this study, the fate of the T_3 conjugates excreted with bile was studied to assess the significance of a putative enterohepatic circulation of T_3 in rats. Conventional (CV) or intestine-decontaminated (ID) rats received iv [^{125}I]T_3G or [^{125}I]T_3S, the latter usually after pretreatment with PTU (1 mg/100 g BW). Radioactivity in plasma and bile or feces was analyzed by Sephadex LH-20 chromatography and HPLC. Within 1 h, 88% of injected T_3G was excreted in bile of CV or ID rats, independent of PTU. About 75% of the injected T_3S was excreted within 4 h in PTU-treated rats, in contrast to only 20% in controls. Up to 13 h after iv administration of T_3G or T_3S (+PTU) to intact ID and CV rats, fecal radioactivity consisted of more than 90% T_3 in all CV rats, 95% of T_3S in T_3S-injected ID rats, and 30% T_3 and 67% T_3G in T_3G-injected ID rats. In overnight-fasted CV rats injected with T_3G, total plasma radioactivity rapidly declined until a nadir of 0.10% dose/ml at about 2.5 h, but radioactivity reappeared with a broad maximum of 0.12% dose/ml between 5.5–10 h. In the latter phase, plasma radioactivity consisted of predominantly I^- and T_3 in a ratio of 2:1. Reabsorption was diminished in fed CV rats and prevented in ID rats. Plasma T_3 4–10 h after iv T_3G injection to overnight-fasted CV rats was 12, 2, and 3 times higher than that in bile-diverted rats, fed CV rats, and ID rats, respectively, and similar to that 4 h after the injection of T_3 itself. Total plasma radioactivity as well as plasma T_3 6–13 h after iv administration T_3S in PTU-treated rats were significantly increased in CV *vs.* ID rats, *e.g.* T_3 0.016% *vs.* 0.005% dose/ml. These results demonstrate a significant enterohepatic circulation of T_3 in rats in which bacterial hydrolysis of T_3 conjugates excreted with bile plays an important role. (*Endocrinology* 125: 2822–2830, 1989)

THE THYROID gland produces mainly the prohormone T_4, which is activated in peripheral tissues by outer ring deiodination (ORD) to T_3. Inner ring deiodination (IRD) of T_4 and T_3 results in the formation of iodothyronines with little or no thyromimetic activity, *i.e.* rT_3 and 3,3′-diiodothyronine (3,3′-T_2), respectively (1, 2). Thus, IRD and ORD are important metabolic routes in the regulation of the peripheral effect of thyroid hormone. In humans, about 80% of T_4 produced daily is degraded by deiodination, but for the metabolic clearance of T_3 deiodinative and nondeiodinative pathways are equally important (1, 3).

Many drugs, food additives, pollutants, as well as endogenous compounds are conjugated in the liver and other tissues with glucuronic acid (4) or sulfate (5), which increases their water solubility and, hence, facilitates their biliary and renal clearance (4–6). However, compounds that are excreted as conjugates with bile are not necessarily eliminated from the body, but may undergo enterohepatic circulation (EHC) as reported for bile acids, bilirubin, steroids, and vitamins (6–8). Intestinal hydrolysis of the conjugates, predominantly by bacterial enzymes, and subsequent reabsorption of the parent compound in the portal system are essential events in such an EHC. A proportion of reabsorbed material that escapes extraction by the liver reappears in the systemic circulation. It has been proposed that T_4 is engaged in an EHC in humans and rats (9, 10), but conflicting data have been reported (11, 12).

It has been shown that thyroid hormones are excreted predominantly as glucuronides in bile of normal humans and rats (13–16). Sulfated iodothyronines undergo enhanced deiodinative clearance (16–18) because they are better substrates than the parent hormones for the type

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I deiodinase in human and rat liver and perhaps other tissues (2, 19, 20). However, under conditions in which hepatic deiodinase activity is impaired, iodothyronine sulfates accumulate in bile and plasma (16–18).

In general, iodothyronines are excreted in the feces in the nonconjugated form (21, 22). Several strains of obligately anaerobic bacteria have been isolated from human and rat intestinal microflora which effectively hydrolyze iodothyronine glucuronides and sulfates *in vitro* (23–25). The role of the microflora *in vivo* was recently illustrated by showing that fecal excretion of T_3 conjugates was higher in intestine-decontaminated (24) than in conventional animals after iv injection of [^{125}I]T_3 (21). Furthermore, after oral administration of [^{125}I]T_3 conjugates, plasma [^{125}I]T_3 was significantly higher in conventional than in decontaminated rats (21).

We have further investigated in rats the fate of T_3 conjugates that, in contrast to the above-mentioned studies, enter the intestine with the bile. Preliminary experiments showed that iv injected T_3G and T_3S are rapidly excreted in bile as intact conjugates, provided that deiodination of the latter is prevented by treatment with 6-propyl-2-thiouracil (PTU). Our results provide additional evidence for the existence of an EHC of T_3, since reabsorption of T_3 in the circulation was significantly increased in normal *vs.* decontaminated animals after iv injection of [^{125}I]T_3 conjugates.

Materials and Methods

Chemicals

[3′-^{125}I]T_3 (~3300 μCi/μg) was synthesized by radioiodination of 3,5-diiodothyronine (Henning GmbH, Berlin, West Germany), using the chloramine-T method and purified by Sephadex LH-20 chromatography. PTU was purchased from Sigma (St. Louis, MO), salicylamide from Riedel-de Haën AG (Hannover, West Germany), ampicillin from Beecham (Heppignies, Belgium), neomycin from Pharmachemie (Haarlem, The Netherlands), and polymyxin-B from Pfizer (Brussels, Belgium).

Preparation of [^{125}I]T_3 conjugates for iv injection

T_3 sulfate (T_3S) synthesis. T_3S was prepared by reaction of solid [^{125}I]T_3 for 75 min at 37 C with 200 μl $ClSO_3H$ (15 M) in anhydrous dimethylformamide (1:4, vol/vol) (26) and isolated on Sephadex LH-20 with about 90% yield. For iv injection, [^{125}I]T_3S was made up in 5 mM NaOH and 0.9% (wt/vol) NaCl. The purity of the injected tracer was confirmed by HPLC, as illustrated in Fig. 1.

T_3 glucuronide (T_3G) biosynthesis. Sulfate-deplete cultures of 10^6 rat hepatocytes in 10-cm^2 wells, prepared as previously described (20, 27), were incubated for 3 h at 37 C with less than 25 μCi [^{125}I]T_3 in 2 ml protein-free Dulbecco's medium or Hanks' Balanced Salt Solution with 25 mM HEPES (pH 7.4). Iodothyronine glucuronides were isolated from medium and cell extracts in the 0.1-M sodium acetate (pH 4) fractions of a stepwise Sephadex LH-20 chromatography procedure. Fractions consisting of more than 85% T_3G (HPLC) were pooled and adsorbed onto a second LH-20 column (17) from which radioactivity was quantitatively eluted in about 1 ml 0.1 M ammonia in ethanol. The solvent was evaporated at 45 C under a stream of nitrogen, and the residue was dissolved in 5 mM NaOH in saline for iv injection. The purity of the prepared [^{125}I]T_3G batches was analyzed by HPLC (Fig. 1). The final yield of [^{125}I]T_3G amounted to 16 ± 2% (mean ± SE; n = 12) of the added [^{125}I]T_3. A small amount of 3,3′-T_2G (4.2 ± 0.3%) was detected in the T_3G preparation by HPLC (Fig. 1).

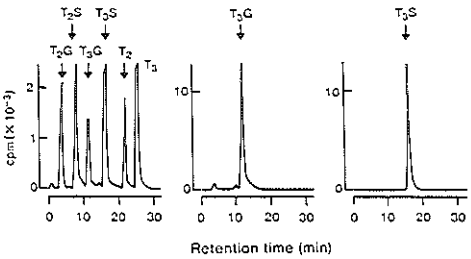

FIG. 1. Analysis of [^{125}I]T_3 conjugates by reverse phase HPLC. Representative batches of synthetic [^{125}I]T_3S (*right*) or biosynthetic [^{125}I]T_3G (*middle*) prepared for injection were analyzed on a C_{18} column. Elution was performed with a gradient of 16–40% acetonitrile in 0.02 M ammonium acetate (pH 4) at a flow of 0.8 ml/min (see *Materials and Methods*). Of the applied radioactivity 92 ± 2.5% (n = 4) eluted as authentic T_3S, and 86 ± 0.9% (n = 11) eluted as T_3G, with a mean recovery of 97% for total radioactivity. Separation of T_3 and available metabolites is depicted in the *left panel*.

Experimental procedures

Male Wistar rats, 200–360 g BW, were fed *ad libitum*, except on the night before the experiment. Intestinal decontamination was performed by adding ampicillin, neomycin, and polymyxin-B (all 1 g/liter) to the drinking water 12–14 days before and during the experiment. The drinking water of all rats, including controls, was supplemented with sucrose (10 g/liter). Two to 3 days before the experiment, decontaminated rats were screened by microscopical analysis of Gram-stained fecal smears to examine the effectiveness of the treatment. Cultures of fecal dilutions (28) showed that the antibiotics had reduced the number of obligately anaerobic bacteria per g feces from, on the average, 1.5×10^{10} (control) to less than 10^3–10^5 (decontaminated), resulting in a significant loss of hydrolytic activity toward T_3 conjugates (24).

Exp I. Conventional and decontaminated rats were anesthetized by ip injection with pentobarbital sodium (5 mg/100 g BW), and the common bile duct was cannulated (16). When appropriate, rats were given a single iv injection of 1 mg PTU/100 g BW, while controls received vehicle. After about 40 min, 7 μCi [^{125}I]T_3G or 12 μCi [^{125}I]T_3S were injected iv (at time zero). Rats were kept anesthetized with additional ip injections of pentobarbital when necessary, and dehydration was pre-

vented with sc injections of saline. Body temperature was maintained by the use of a heating pad in combination with an infrared lamp. Bile was continuously collected, first in 5- to 15-min periods followed by intervals of 15 or 30 min, and the volume was determined gravimetrically. Repetitive blood samples (0.4–0.8 ml) were taken from the tail vein, and the animals were bled finally by heart puncture after 3 or 4 h. Serum and bile were counted for total radioactivity and kept at −20 C until further analysis.

Exp II. Conventional and decontaminated rats, either fed or fasted overnight, received 7 μCi [^{125}I]T_3G, iv, under light ether anesthesia (at time zero). They were placed in individual stainless steel metabolic cages for the collection of feces. Small blood samples (~0.4 ml) were taken at 1- to 1.5-h intervals from the tail vein under light ether anesthesia. After bleeding the rats by heart puncture at 11.5 or 13 h the content of the cecum was collected. Part of the feces collected between 5–13 h, and cecal material was extracted with 4 vol ethanol and centrifuged. The clear supernatants and serum samples were stored at −20 C until further analysis.

Exp III. Conventional and decontaminated rats received a single iv injection of 1 mg PTU/100 g BW. About 40 min later, 7 μCi [^{125}I]T_3S was administered iv (time zero), and regular blood samples were obtained until the animals were bled by heart puncture at 13 h. Feces and cecum were obtained as described for Exp II.

Analysis of samples

The radioactivity in bile was analyzed directly by HPLC (see below). Plasma radioactivity was fractionated on Sephadex LH-20 as previously reported (17). In short, serum samples (<100 μl) were prepared in 0.1 M HCl and 25% (vol/vol) ethanol and applied to small LH-20 columns. The columns were eluted successively with 0.1 M HCl (five times; 1 ml), water (nine times; 1 ml), and 0.1 M ammonia in ethanol (three times; 1 ml) for the isolation of I^-, conjugates, and nonconjugated iodothyronines, respectively. Recovery of radioactive compounds added to rat serum (<250 μl) amounted to 97.7 ± 1.8 for I^-, 96.0 ± 2.7% for T_3G, 91.9 ± 4.3% for T_3S, or 95.5 ± 1.8% for T_3 (mean ± SD; n = 7–14) in the corresponding fractions. Radioactive metabolites in feces and cecum were separated similarly by chromatography of mixtures of 100 μl ethanolic extract with 900 μl 0.3 M HCl on Sephadex LH-20. For HPLC analysis, mixtures of conjugates and iodothyronines were isolated together on Sephadex LH-20 by omission of the water fractions and, instead, direct elution with 0.1 M ammonia in ethanol.

Serum obtained by heart puncture as well as pools of preceding collections were processed by C_{18} solid phase extraction. Conjugated and nonconjugated iodothyronines were collectively isolated in methanol, as previously described (17), and submitted to HPLC analysis.

HPLC was performed with a 10 × 0.3-cm Chromspher C_{18} analytical column protected by a 10 × 2.1-mm reverse phase guard column, both supplied by Chrompack International BV (Middelburg, The Netherlands). Elution was performed with a 25-min nonlinear gradient of 16–40% acetonitrile in 0.02 M ammonium acetate (pH 4) (17), using program 7 of the model 680 automatic gradient controller (Waters Associates, Milford, MA). For analysis of samples the alcoholic solvents were evaporated at 45 C under a stream of nitrogen, and the residues were dissolved in initial mobile phase. Bile (<25 μl) was diluted five times in mobile phase and applied directly to the C_{18} column.

Data analysis

Data are given as the mean ± SE unless indicated otherwise. Statistical analysis was done by one-way classification (*i.e.* by analysis of variance followed by comparison between class means) (29). $P < 0.05$ was considered significant.

Results

Biliary excretion

Figure 2 shows the biliary excretion of radioactivity after iv injection of [^{125}I]T_3G or [^{125}I]T_3S to bile duct-cannulated rats under pentobarbital anesthesia (Exp I), and Fig. 3 shows the disappearance of radioactivity in plasma of these animals. T_3G was eliminated very rapidly from the circulation, reflecting effective biliary clearance.

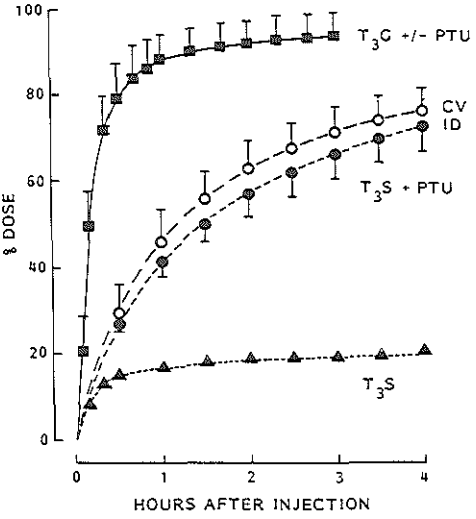

FIG. 2. Biliary excretion of [^{125}I]T_3 conjugates. Conventional and decontaminated rats were fitted under pentobarbital anesthesia with a bile duct cannula and injected iv with [^{125}I]T_3G, with or without PTU pretreatment (■; n = 12). Similarly, [^{125}I]T_3S was given to both PTU-treated conventional (○; n = 5) and decontaminated (●; n = 2) rats as well as to untreated controls (▲; n = 2). Cumulative excretion of radioactivity in bile is expressed as the mean ± SD percentage of the dose. In PTU-treated rats, T_3S excretion was significantly lower in decontaminated than in normal rats ($P < 0.025$).

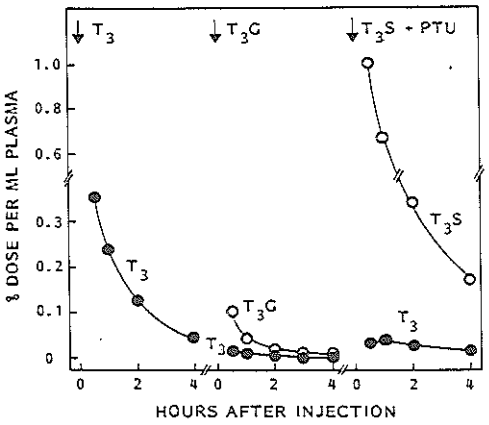

FIG. 3. Plasma radioactivity in bile-diverted rats as a function of time after injection of [^{125}I]T_3 or [^{125}I]T_3 conjugates. Normal rats were fitted under pentobarbital anesthesia with a bile duct cannula and injected iv (time zero) with [^{125}I]T_3 (*left*), [^{125}I]T_3G (*middle*), or, after PTU treatment, [^{125}I]T_3S (*right*). Plasma T_3 conjugates (○) and T_3 (●) were isolated on Sephadex LH-20 (see *Materials and Methods*). Data are expressed as percent dose per ml plasma and represent mean results of four (T_3), eight (T_3G), or five (T_3S) rats.

On the average, 88% of the injected [^{125}I]T_3G was excreted within 1 h, independent of treatment with PTU or antibiotics. As reported previously (17), T_3S is rapidly eliminated from the circulation in normal rats due to effective deiodination of the conjugate by type I deiodinase in liver and perhaps other tissues. Plasma T_3S in PTU-treated rats decreased much slower in keeping with the less rapid biliary clearance in comparison with T_3G, amounting to about 75% of the dose after 4 h. Biliary excretion of T_3S was slightly but significantly increased in conventional rats *vs.* decontaminated rats ($P < 0.025$). With both conjugates, no difference was observed in biliary radioactivity between overnight-fasted and normally fed rats. All radioactivity in bile consisted of the injected conjugate, as demonstrated by HPLC analysis (not shown).

For comparison, data from similar experiments with iv [^{125}I]T_3-injected rats are included in Fig. 3, showing that plasma T_3 is eliminated at an intermediate rate compared with T_3G and, in PTU-treated rats, T_3S. Some radioactivity in plasma of rats injected with either conjugate coeluted with T_3 from Sephadex LH-20. This was negligible compared with that in rats injected with T_3 alone (Fig. 3) and was probably due to slight contamination of the T_3 fraction with conjugates. Chromatography of rat serum spiked with [^{125}I]T_3S or [^{125}I]T_3G showed that 2.0 ± 0.4% (n = 10) of the applied radioactivity eluted in the iodothyronine fraction from the Sephadex LH-20 column, although [^{125}I]T_3 was not detectable by HPLC (<1%; Fig. 1).

Bile flow amounted to 486 ± 18 μl/h·100 g BW (n = 17) in overnight-fasted or fed conventional rats. However, production of bile was significantly lower in decontaminated rats, *i.e.* 356 ± 20 μl/h·100 g BW (n = 4) *vs.* 482 ± 24 μl/h·100 g BW in five conventional rats tested simultaneously ($P < 0.01$).

Fecal excretion

Within 13 h after the administration of either T_3 conjugate, conventional rats excreted, on the average, 24% and decontaminated rats 13% of the dose in the feces. Fecal excretion of radioactivity in the limited period of collection was highly variable, *i.e.* ranging from 5–35% of the dose, and no clear differences were observed between normal and decontaminated animals or between T_3S- and T_3G-injected rats. The cecum of conventional and decontaminated rats contained up to 14% of the dose.

Fractionation on Sephadex LH-20 of ethanol-extracted radioactivity from feces and cecum is summarized in Table 1. These data were confirmed by quantitation of individual components by HPLC, as illustrated in Fig. 4. After iv injection of T_3S or T_3G to conventional rats more than 91% of fecal radioactivity consisted of nonconjugated T_3, indicating effective intestinal hydrolysis of conjugates excreted with bile. The role of the microflora in this deconjugation is illustrated by the findings that in feces of decontaminated rats virtually all radio-

TABLE 1. Distribution of radioactivity extracted from feces and cecum of rats injected with [^{125}I]T_3G or [^{125}I]T_3S

Injected conjugate	Rats[a]	Extract	n	Conjugates (%)	Iodothyronines (%)
T_3G	CV	Feces	12	3.0 ± 0.5	91.9 ± 2.0
		Cecum	8	6.2 ± 2.1	86.3 ± 6.7
T_3G	ID	Feces	8	67.1 ± 13.5	29.6 ± 13.4
		Cecum	4	88.2 ± 9.6	9.6 ± 4.2
T_3S (+PTU)	CV	Feces	4	5.2 ± 1.2	90.8 ± 1.2
		Cecum	4	9.9 ± 4.3	85.0 ± 3.3
T_3S (+PTU)	ID	Feces	4	95.1 ± 2.4	3.3 ± 2.2
		Cecum	4	96.5 ± 0.9	2.1 ± 0.6

Conventional (CV) or decontaminated (ID) rats were injected iv (time zero) with [^{125}I]T_3G alone or [^{125}I]T_3S after PTU treatment. Feces collected between 5–13 h, and material isolated from the cecum at 13 h were extracted with ethanol and fractionated on Sephadex LH-20 as described in *Materials and Methods*. The data are the mean ± SD percentages of total radioactivity eluting in the conjugate or iodothyronine fraction.

[a] Rats were fasted overnight or normally fed before T_3G injection, or fasted overnight before T_3S administration.

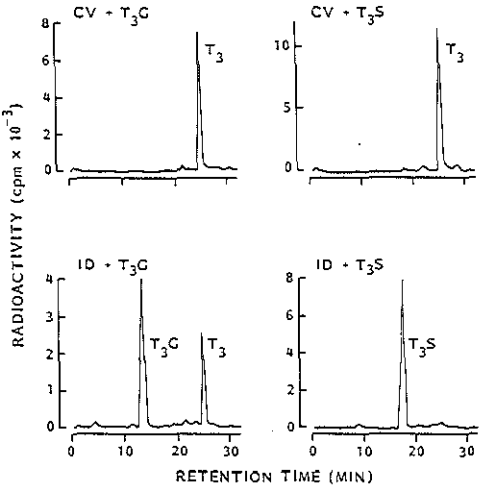

FIG. 4. HPLC analysis of fecal radioactivity. Intact conventional (*upper panels*) or decontaminated rats (*bottom panels*) were injected iv (time zero) with [^{125}I]T_3G (*left*) or after PTU treatment with [^{125}I]T_3S (*right*). Feces collected between 5–13 h were extracted with ethanol and analyzed by HPLC with or without precleaning on Sephadex LH-20. The figure shows a set of representative profiles, and the recovery of radioactivity applied to HPLC was 84.5 ± 1.3% (n = 27).

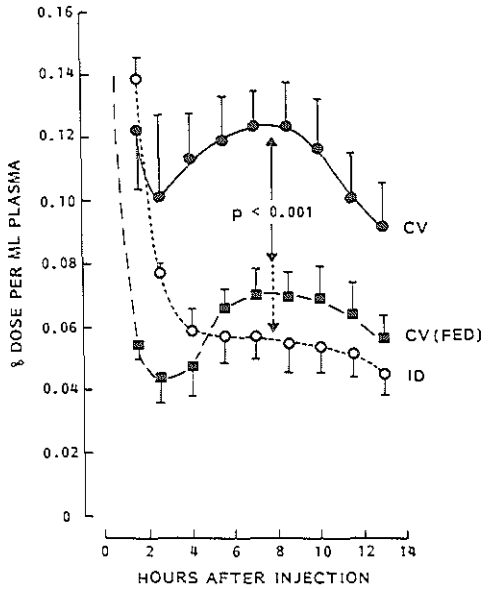

FIG. 5. Total plasma radioactivity in intact mobile rats as a function of time after iv injection of [^{125}I]T_3G (time zero). Blood was collected at regular intervals under light ether anesthesia from overnight-fasted conventional (●) or decontaminated rats (○) and from normally fed conventional rats (■) until 13 h. Radioactivity is expressed as the mean ± SE percent dose per ml plasma (n = 4). The significance of the differences *vs.* overnight-fasted conventional rats is indicated.

activity is still in the form of injected T_3S, while only part of the injected T_3G is excreted as free T_3 (Table 1 and Fig. 4). Incomplete prevention of T_3G hydrolysis in decontaminated rats is probably due to β-glucuronidase activity in intestinal mucosa cells.

Plasma radioactivity after [^{125}I]T_3G injection

Figure 5 depicts the total radioactivity in plasma of rats as a function of time after iv administration of [^{125}I]T_3G (Exp II). In conventional rats plasma radioactivity rapidly declined until a nadir at about 2.5 h of 0.104 ± 0.023% dose/ml in overnight-fasted or 0.043 ± 0.007% dose/ml in fed animals. Instead of a further decline, plasma radioactivity reappeared after 4 h in the conventional rats, showing a broad maximum between 5.5–10 h of 0.121 ± 0.014% *vs.* 0.069 ± 0.008% dose/ml in overnight-fasted *vs.* fed rats ($P < 0.001$). After tracer administration to overnight-fasted decontaminated rats, total plasma radioactivity initially decreased at a similar rate, but, unlike conventional rats, this continued to decrease until a plateau between 5.5–10 h of 0.056 ± 0.008% dose/ml (Fig. 5). This was the same as in normally fed decontaminated rats, *i.e.* 0.0054 ± 0.005% dose/ml (n = 4; not shown). In all groups, plasma radioactivity at the later time points consisted of primarily I^- and T_3 and very little conjugate. The percent distribution of I^-:T_3:T_3G in plasma, as determined with Sephadex LH-20, amounted to, on the average, 57:27:11 from 4 h after T_3G injection onward.

Figure 6 shows the Sephadex LH-20 fractionation of plasma radioactivity from intact overnight-fasted conventional rats (Exp II) compared with that in bile-diverted rats (Exp I) injected with T_3G. Table 2 summarizes the plasma T_3 levels at 4–10 h in intact decontaminated and conventional rats and at 4 h in bile duct-cannulated rats. In overnight-fasted conventional rats with normal bile flow, plasma T_3 at 4–10 h amounted to 0.038 ± 0.006% dose/ml, *i.e.* 12 times higher than in bile-diverted rats at 4 h and similar to that 4 h after iv injection of [^{126}I]T_3 itself (0.050 ± 0.004% dose/ml; n = 12; see also Fig. 3). Compared with overnight-fasted conventional rats, plasma T_3 was 61% lower in overnight-fasted or fed decontaminated rats and 53% lower in normally fed conventional rats. Similar data were obtained by HPLC analysis of solid phase extracts of pooled sera. The observed decrease in plasma T_3 due to bile

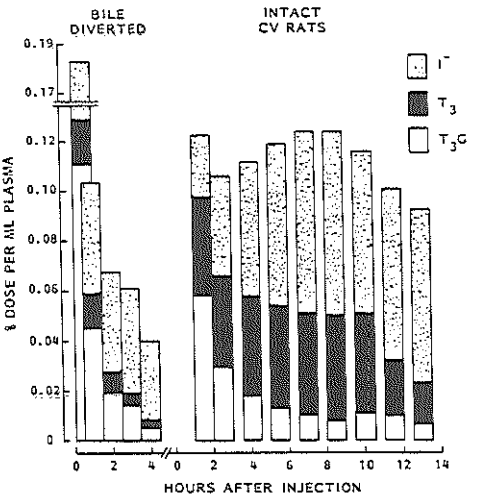

FIG. 6. Distribution of plasma radioactivity in conventional rats *vs.* time after [^{125}I]T_3G injection. [^{125}I]T_3G was administered iv (time zero) to either bile duct-cannulated pentobarbital-anesthetized rats (*left*; fed or overnight-fasted) or intact mobile rats (*right*; overnight-fasted). The radioactivity in plasma samples obtained at the indicated times was separated on Sephadex LH-20 in I^- (□), conjugates (□), and iodothyronines (■). The latter two fractions consisted of T_3G and T_3, respectively, as confirmed by HPLC analysis. Data are expressed as the mean percent dose per ml plasma (n = 4).

TABLE 2. Plasma T_3 after iv administration of [^{125}I]T_3G

Rats	n	Time (h)	Plasma T_3 (% dose/ml × 100)
Bile diverted CV	4	4	0.32 ± 0.005[a]
Intact CV, fasted	4	4–10	3.75 ± 0.59
Intact CV, fed	8	4–10	1.78 ± 0.34[a]
Intact ID, fasted or fed	8	4–10	1.46 ± 0.26[a,b]

Intact mobile conventional (CV) or decontaminated (ID) rats or bile-diverted anesthetized rats received iv [^{125}I]T_3G (time zero). For each animal, plasma [^{125}I]T_3 was determined on Sephadex LH-20 (see *Materials and Methods*) at regular intervals after tracer injection, and the mean value was calculated over the indicated period.

[a] $P < 0.001$ *vs.* intact fasted CV rats.

[b] $P < 0.025$ *vs.* intact fed CV rats.

diversion or intestinal decontamination compared with that in the animals with normal gastrointestinal physiology was highly significant (Table 2). Thus, substantial amounts of T_3 are normally reabsorbed from biliary T_3G after bacterial hydrolysis, processes that apparently are affected by the presence of food in the intestine.

Plasma radioactivity after [^{125}I]T_3S injection

Figure 7 shows that in Exp III, total plasma radioactivity leveled off from 6 h onward after iv administration

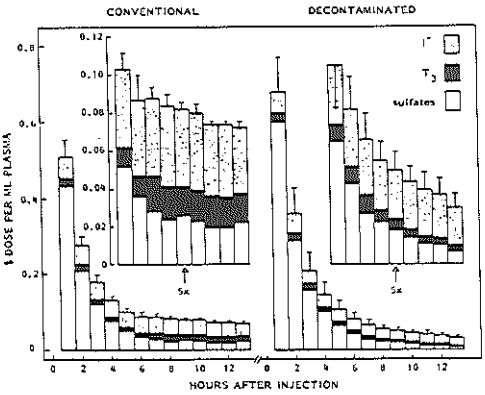

FIG. 7. Distribution of plasma radioactivity in relation to time after administration of [^{125}I]T_3S to overnight-fasted conventional (*left*) or decontaminated (*right*) rats. Intact mobile rats were treated with PTU and subsequently injected iv with [^{125}I]T_3S (time zero). Repetitive blood samples were collected up to 13 h. Plasma was fractionated on Sephadex LH-20, resulting in the separation of I^- (□), sulfate conjugates (□), and T_3 (■), as confirmed by HPLC. Data are expressed as the mean ± SE percent dose per ml plasma (n = 4). *Insets* show a 5-fold magnification of the distribution of plasma radioactivity from 5 h onward. Total plasma radioactivity in conventional rats was significantly increased between 8–13 h compared with that in decontaminated rats ($P < 0.001$).

TABLE 3. Plasma T_3 after iv administration of [^{125}I]T_3S

Rats	n	Time (h)	Plasma T_3 (% dose/ml × 100)
Bile diverted CV	3	4	1.79 ± 0.26
Intact CV	4	8–13	1.61 ± 0.15
Intact ID	4	8–13	0.45 ± 0.09[a]

Overnight-fasted intact mobile conventional (CV) or decontaminated (ID) rats or bile-diverted anesthetized rats received iv [^{125}I]T_3S (time zero) after pretreatment with PTU. Plasma T_3 was determined in the same way as in the T_3G-injected rats described in Table 2.

[a] $P < 0.001$ *vs.* intact CV rats.

of [^{125}I]T_3S to PTU-treated conventional rats, amounting to 0.078 ± 0.004% dose/ml between 8–13 h. However, plasma radioactivity in similarly treated decontaminated rats steadily declined to 0.043 ± 0.009% dose/ml between 8–13 h, significantly lower than that in conventional rats ($P < 0.001$). The mean percent distribution of plasma I^-:T_3:T_3S at 8–13 h amounted to 50:21:28 in conventional rats and 58:11:30 in decontaminated rats. Table 3 gives the mean plasma T_3 levels in the different groups of T_3S-injected rats (Exp I and III). On the average, plasma of conventional rats contained 4 times more T_3 than plasma of decontaminated rats 8–13 after T_3S administration. Again, these data were confirmed by HPLC analysis of solid phase extracts of serum pools.

Stability and protein binding of T_3 conjugates in plasma

As demonstrated by HPLC, [^{126}I]T_3 conjugates were fully stable during 18-h incubation at 37 C in rat and human serum or whole blood (not shown).

The nonprotein-bound fraction of T_3G in normal rat serum, as determined by equilibrium dialysis (n = 4–6), was 0.92 ± 0.02%, which is higher than that of T_3S (0.20 ± 0.02%) or T_3 (0.35 ± 0.01%) (17). In pooled human serum similar differences between the free fractions of tracers were observed (n = 4), *i.e.* 0.69 ± 0.01% for T_3G, 0.11 ± 0.01% for T_3S, and 0.20 ± 0.01% for T_3. The presence of 1 mM PTU did not affect the free fractions of the different compounds.

Discussion

In rats, circulating T_3 is taken up by the liver and conjugated with glucuronic acid and sulfate. T_3G is not further metabolized, but is rapidly excreted with bile (15, 16, 20). Normally, little T_3S appears in bile because this conjugate is deiodinated very rapidly, via successive IRD and ORD, by type I deiodinase of liver (2, 16, 20), and the iodide produced is released into the circulation (17). However, T_3S accumulates in the bile and plasma of rats treated with inhibitors of the type I deiodinase such as PTU (16, 17) and butyl-4-hydroxy-3,5-diiodobenzoate (30, 31). Thyroidectomized rats and dogs have also been shown to excrete significant amounts of T_3S (and 3,3′-T_2S) in bile and urine, which is explained by the impaired hepatic type I deiodinase activity in hypothyroid animals (32).

Comparison of plasma iodothyronine kinetics and tissue distributions in humans and rats suggests similar pathways of thyroid hormone metabolism and excretion in these species (33, 34), making the rat a suitable model for the study of a putative EHC of iodothyronines. We here demonstrate that iv injected T_3G and T_3S are rapidly excreted in rat bile as intact conjugates, provided that deiodination of T_3S is inhibited. Plasma T_3G is much more rapidly disposed into the bile than plasma T_3S (in PTU-treated rats), which may be explained at least in part by the 5-fold higher plasma protein binding of T_3S compared with T_3G. These biliary conjugates are normally completely hydrolyzed during intestinal transport, as demonstrated by the lack of conjugates in cecum and feces of conventional rats injected with T_3G or T_3S. It is not known how much T_3 in the bowel is derived from direct secretion from the mesenteric venous system, as was recently suggested (35). Apparently, biliary excretion of plasma T_3 conjugates was not affected by intestinal decontamination, despite the markedly reduced bile flow in the animals treated with antibiotics. This diminished bile production is probably due to the interrupted EHC of bile acids (6, 36). The capacity to hydrolyze the T_3 conjugates excreted with bile is greatly reduced in decontaminated *vs.* normal animals. This points to the importance of intestinal bacteria for the hydrolysis of thyroid hormone conjugates. In cecal extracts of rats little T_3S and T_3G are detected, which is explained, at least for T_3G, by the high bacterial β-glucuronidase activity at this site of the intestine (7). The possible role of β-glucuronidase activity in intestinal mucosal cells is indicated by partial hydrolysis of T_3G in decontaminated rats.

Early findings obtained in subjects infused with bile from [^{131}I]T_4-injected donors suggested that between 25–68% of the biliary radioactivity is reabsorbed in rats (10, 37) and about 30–50% in humans (3, 9).

Previous studies in our laboratory (21) have shown that after oral administration of [^{125}I]T_3S or [^{125}I]T_3G, resorption of T_3 is 3–5 times higher in conventional than in decontaminated rats. Direct experimental data concerning the possible EHC of T_3 is lacking, which prompted us to carry out the present investigations in intact mobile animals in which T_3 conjugates enter the intestine via the normal route, *i.e.* by excretion with the bile. The putative EHC of T_3 was interrupted by diversion of the bile (Exp I) or by intestinal decontamination with antibiotics to reduce bacterial deconjugation (Exp II and III).

Our results show that in normal T_3G-injected rats significant amounts of T_3 are reabsorbed and appear in the systemic circulation after excretion of T_3G with the bile. The resulting plasma T_3 levels are 2.6-fold higher than those in decontaminated rats, which indicates an essential role of bacterial hydrolases (24, 25) for T_3 reabsorption. The amounts of circulating T_3 cannot be explained by contamination of the injected glucuronide with T_3 as determined by chromatography of plasma from bile-diverted rats after iv injection of either [^{125}I]T_3 or [^{126}I]T_3G. Taking into account the clearance of T_3 itself, a considerable proportion of iv administered [^{126}I]T_3G reappeared as T_3 in the circulation of conventional rats, resulting in plasma T_3 levels similar to those observed 4 h after iv T_3 injection. Since T_3G is not deiodinated in rat liver (Exp I in this study) (20), most of the iodide observed in plasma of T_3G-injected rats must have originated by metabolism of reabsorbed T_3 (17).

Fecal excretion of thyroid hormone metabolites is influenced by the diet (7, 12). After iv T_3G administration, the level of radioactivity reappearing in plasma (6–10 h) was significantly lower in normally fed conventional rats than in the overnight-fasted conventional animals. This might be due to a reduced availability of conjugates for the bacterial hydrolases and/or to binding of the liberated iodothyronines to food constituents with a consequent decrease in their reabsorption (7). An additional factor may be a prolonged intestinal transit time in

overnight-fasted rats (7), which may increase T_3 resorption. In humans, oral T_4 is resorbed more effectively if taken on an empty stomach (38).

Normally, T_3G is the most important biliary T_3 metabolite, but inhibition of the type I deiodinase greatly enhances the excretion of biliary sulfates in rats, resulting in a 50% increase in biliary T_3 clearance (16). To maximize the biliary excretion of iv administered T_3S it is necessary to prevent its deiodination by the type I enzyme, which is achieved with a single dose of PTU (16, 17). These studies with PTU-treated T_3S-injected rats are complicated because of the slower biliary clearance of T_3S compared with T_3G, which may be the main reason for the lack of a distinct reabsorption phase in these animals. It is noted that considerable levels of plasma sulfates are present 8–13 h after T_3S injection, which are probably largely due to secondary sulfation of reabsorbed T_3. The newly formed T_3S accumulates because the type I deiodination is still diminished due to PTU treatment. This also explains the plateau level of plasma T_3S in conventional rats in contrast to the steady decline of plasma T_3S in decontaminated rats, although initially levels were higher in the latter animals. Our data suggest that the T_3S eliminated with bile undergoes effective bacterial hydrolysis in conventional animals, and this is followed by reabsorption of the liberated T_3. However, absorption of T_3 from biliary T_3S appears less than that from biliary T_3G, in agreement with previous observations after oral administration of these conjugates (21).

In humans, iodothyronine glucuronides are excreted with bile (9, 39) and are hydrolyzed by major residents of the intestinal microflora (23, 25). Recent studies show that the human liver contains phenol sulfotransferases which are active in the sulfation of T_3 (40) and a type I deiodinase which deiodinates T_3S much more rapidly than T_3 itself (19). It is likely that in euthyroid humans also T_3S is deiodinated before biliary excretion, as is the case in normal rats (16). This latter assumption is underscored by recent findings of elevated plasma T_3S levels in human volunteers with inhibited deiodinase activity due to IOP treatment (41). If in humans, as in rats, biliary excretion of T_3S is increased when type I deiodinase is diminished (16), reabsorption of T_3 is possible since the human intestinal microflora is capable of hydrolyzing iodothyronine sulfates (24, 25).

Fecal clearance of thyroid hormone is much less in humans than in rats (34, 42). This difference may be due to a lesser excretion rate of metabolites with bile (14, 39, 42) and/or a more effective EHC in humans. Further studies are needed to explore the EHC of iodothyronines in humans as well as to determine the amount of thyroid hormones circulating in the enterohepatic subsystems of humans and rats. We conclude that a significant EHC of T_3 exists in rats in which bacterial hydrolysis of T_3 conjugates excreted with bile plays an important role, being partly influenced by the presence of food in the intestine.

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References

1. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5:151
2. Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RJB, Van der Molen HJ (eds) Hormones and Their Action, part I. Elsevier, Amsterdam, p 81
3. Burger A 1986 Nondeiodinative pathways of thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 255
4. Dutton GJ 1980 Glucuronidation of Drugs and Other Compounds. CRC Press, Boca Raton
5. Mulder GJ 1981 Sulfation of Drugs and Related Compounds. CRC Press, Boca Raton
6. Klaassen CD, Watkins JB 1984 Mechanisms of bile formation, hepatic uptake, and biliary excretion. Pharmacol Rev 36:1
7. Gregus Z, Klaassen CD 1986 Enterohepatic circulation of toxicants. In: Rozman R, Hänninen O (eds) Gastrointestinal Toxicology. Elsevier, Amsterdam, p 57
8. Renwick AG 1986 Gut bacteria and the enterohepatic circulation of foreign compounds. In: Hill MJ (ed) Microbial Metabolism in the Digestive Tract. CRC Press, Boca Raton, p 135
9. Myant NB 1956 Enterohepatic circulation of thyroxine in humans. Clin Sci 15:551
10. Briggs FN, Taurog A, Chaikoff IL 1953 The enterohepatic circulation of thyroxine in the rat. Endocrinology 52:559
11. Galton VA, Nisula BC 1972 The enterohepatic circulation of thyroxine. J Endocrinol 54:187
12. Cottle WH 1964 Biliary and fecal clearance of endogenous thyroid hormone in cold-acclimated rats. Am J Physiol 207:1063
13. Taurog A 1955 Conjugation and excretion of the hormone. Brookhaven Symp Biol 7:111
14. Myant NB 1956 Biliary excretion of thyroxine in humans. Clin Sci 15:227
15. Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford, p 345
16. De Herder WW, Bonthuis F, Rutgers M, Hazenberg MH, Visser TJ 1988 Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Endocrinology 122:153
17. Rutgers M, Bonthuis F, de Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758
18. Rutgers M, Pigmans IGAJ, Bonthuis F, Visser TJ 1988 Effects of PTU on the biliary clearance of T_4 in rats. Ann Endocrinol (Paris) 49:181 (Abstract)
19. Visser TJ, Kaptein E, Terpstra OT, Krenning EP 1988 Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 67:17
20. Eelkman Rooda SJ, Otten MH, van Loon MAC, Kaptein E, Visser TJ, Metabolism of triiodothyronine in rat hepatocytes. Endocrinology, in press
21. De Herder WW, Hazenberg MP, Pennock-Schroder AM, Oosterlaken AC, Rutgers M, Visser TJ 1989 On the enterohepatic cycle of triiodothyronine in rats: importance of the intestinal microflora. Life Sci 45:849

22. DiStefano JJ, Sapin V 1987 Fecal and urinary excretion of six iodothyronines in the rat. Endocrinology 121:1742
23. De Herder WW, Hazenberg MP, Pennock-Schroder AM, Hennemann G, Visser TJ 1986 Hydrolysis of iodothyronine glucuronides by obligately anaerobic bacteria isolated from human fecal flora. Fed Eur Microbiol Soc Microbiol Lett 35:249
24. De Herder WW, Hazenberg MP, Pennock-Schroder AM, Hennemann G, Visser TJ 1986 Rapid and bacteria-dependent in vitro hydrolysis of iodothyronine conjugates by intestinal contents of humans and rats. Med Biol 64:31
25. Hazenberg MP, de Herder WW, Visser TJ 1988 Hydrolysis of iodothyronine conjugates by intestinal bacteria. Fed Eur Microbiol Soc Microbiol Rev 54:9
26. Mol JA, Visser TJ 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. Endocrinology 117:1
27. Eelkman Rooda SJ, van Loon MAC, Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. J Clin Invest 79:1740
28. Hazenberg MP, Bakker M, Verschoor-Burggraaf A 1981 Effects of the human intestinal flora on germ-free mice. J Appl Bacteriol 50:95
29. Snedecor GW, Cochran WG 1967 Statistical Methods. Iowa State University Press, Ames
30. Flock EV, Bollman JL, Owen CA, Zollman PE 1965 Conjugation of thyroid hormones and analogs by the Gunn rat. Endocrinology 77:303
31. Hesch RD, Koehrle J 1986 Intracellular pathways of iodothyronine metabolism. In: Ingbar SH, Braverman LE (eds) The Thyroid. Lippincott, Philadelphia, p 154
32. Kaplan MM 1986 Regulatory influences on iodothyronine deiodination in animal tissues. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 231
33. DiStefano JJ, Feng D 1988 Comparative aspects of the distribution, metabolism and excretion of 6 iodothyronines in the rat. Endocrinology 123:2514
34. DiStefano JJ 1986 Modeling approaches and models of the distribution and disposal of thyroid hormones. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 39
35. DiStefano JJ, Sternlicht M, Harris DR 1988 Rat enterohepatic circulation and intestinal distribution of enterally-infused thyroid hormones. Endocrinology 123:2526
36. Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ 1985 Enterohepatic circulation in the rat. Gastroenterology 88:403
37. Myant NB 1957 Faecal clearance rate of endogenous thyroid hormone in rats. J Physiol 136:198
38. Wenzel KW, Kirschsieper HE 1977 Aspects of the absorption of oral L-thyroxine in normal man. Metabolism 26:1
39. Langer P, Moravec R, Ohradka B, Foldes O 1988 Iodothyronines in human bile. Endocrinol Exp 22:35
40. Young WF, Gorman CA, Weinshilboum RM 1988 Triiodothyronine: a substrate for the thermostable and thermolabile forms of human phenol sulfotransferase. Endocrinology 122:1816
41. Eelkman Rooda SJ, Kaptein E, Visser TJ 1989 Serum triiodothyronine sulfate in man measured by radioimmunoassay. J Clin Endocrinol Metab 69:552
42. Larsen PR, Atkinson AJ, Wellman HN, Goldsmith RE 1970 The effect of diphenylhydantoin on thyroxine metabolism in man. J Clin Invest 49:1266

Chapter IX

INTERACTIONS BETWEEN IODOTHYRONINE METABOLIC PATHWAYS

1 TYPE I DEIODINATION OF IODOTHYRONINE DERIVATIVES BY RAT LIVER MICROSOMES

Table 1 lists the V_{max} and apparent K_m values for the type I deiodination of iodothyronines and derivatives. These were determined under initial reaction rate conditions using microsomal protein mixtures, and data were analyzed using Lineweaver-Burk plots. The chemical structures of the various T_3 derivatives are given in Figure 1. In view of the ping-pong kinetic mechanism of the type I deiodination, the ratio of V_{max} over K_m is independent of the level of cofactor and provides a measure of the catalytic efficiency of the different reactions (Table 1). The turnover number of rat liver type I deiodinase even with the preferred substrate rT_3 is rather low, i.e. 222 min^{-1} (196).

In microsomal incubations, the actual free concentration of the different substrates in the deiodinase assay may vary due to differences in sequestration by the microsomal membranes. However, the kinetic parameters have usually been determined under conditions where reaction rates are linear with the microsmal protein concentration, suggesting that most added substrate is available for reaction with enzyme (see also discussion in Chapter II and IV). This does not exclude, however, that the access to the enzyme active site in the microsomal membranes differs between the various substrate. Such interaction with the lipid environment of the enzyme will have a greater impact on the estimation of K_m than on V_{max} values. To determine true K_m values, therefore, highly purified enzyme preparations have to be used.

1.1 4'-Derivatives

Glucuronides

Table 1 lacks glucuronides of iodothyronines and analogs, because they are not deiodinated but excreted as such with the bile. This is supported by the following experimental findings. 1) Unlike T_3S, T_3G is not converted by the type I enzyme, because it is fully stable in incubations with liver microsomes or hepatocytes (56). Moreover, bile duct-cannulated rats excrete approximately 80 % of iv injected T_4G or T_3G unaltered in bile in 60 min (6,8,88), which is not enhanced by PTU-treatment (Chapter VIII). 2) Glucuronides of TA_3 and 3,3'-TA_2 are also resistant to the type I deiodinase, because they are stable in incubations with rat liver microsomes (Chapter V).

Sulfates

In the last decade, Visser and coworkers have established that sulfation of the 4'-OH group of iodothyronines facilitates their type I deiodination (for reviews, see Refs. 197,198). The V_{max}/K_m ratios in Table 1 show that sulfation of iodothyronines markedly increases deiodination efficiencies 40- to 200-fold, with exception of the ORD of the

preferred substrate rT_3, which is not further stimulated by sulfation. Sulfation has the most dramatic effect on the substrate behavior of T_4, i.e. the ORD of T_4S is completely blocked, whereas the IRD to rT_3S is enhanced 200-fold (123,198). With regard to deiodination kinetics, sulfation of iodothyronines may either increase the V_{max} value or decrease the apparent K_m value. The former change dominates for the IRDs of T_4S and T_3S, and the latter for the ORD of 3,3'-T_2S.

Thus, iodothyronine sulfates in general are better substrates for the rat liver type I deiodinase than the corresponding nonconjugated iodothyronines. Similar findings have been documented for T_3S using human liver microsomes (194,197). The involvement of the type I enzyme in the deiodination of iodothyronine sulfates by liver microsomes, is indicated by the 1) specific nature of the DTT dependence, 2) susceptibility to PTU inhibition, and 3) competitive inhibition by other iodothyronines such as rT_3 (194,198).

1.2 Side chain derivatives

In comparison with iodothyronine sulfates, which have a negative charge at the 4'-position and a zwitterionic alanine side chain, we have studied the substrate behavior of iodothyroacetic acid (TA_i), iodothyronine sulfamate (T_iNS) and N-acetyl-iodothyronine (AcT_i) derivatives for the type I deiodinase of rat liver (Fig. 1). The side chains of these modified iodothyronines have either a single (TA_i, AcT_i) or a double (T_iNS) net negative charge.

These investigations revealed that the introduction of a negative charge in the iodothyronine side chain, i.e. opposite of the 4'-sulfate group, stimulates the deiodination of such derivatives 2-17 times with exception of rT_3 (Table 1). The improved deiodination efficiencies of these side chain analogs compared with underivatized iodothyronines are largely accounted for by a general 3- to 33-fold reduction in the apparent K_m value, while no consistent changes in V_{max} were observed. Furthermore, Körhle *et al.* (104) determined apparent K_m values of 0.2 μM for TA_4 as well as AcT_4, consistent with an IC_{50} of 0.22 μM reported by Shulkin (174) for TA_4 inhibition of T_4 ORD. Together, these findings indicate that N-acetylated and acetic acid derivatives bind also more avidly to the type I enzyme than native iodothyronines.

The apparent inhibitor constant (K_i value) of one iodothyronine analog, acting as a competitive inhibitor of the deiodination of another iodothyronine, is identical to its K_m value as substrate (196), provided identical free hormone levels in the microsomal incubation mixtures. For example, TA_3 and 3,3'-TA_2 were found to be much more potent inhibitors of rT_3 ORD than expected from their K_m values (Chapter IV). This is likely due to the 20-fold lower microsomal protein levels in the rT_3 deiodinase assay compared with the conditions under which TA_3 and 3,3'-TA_2 were tested as substrates. Therefore, K_m values for deiodination of, in particular, iodothyroacetic acids may be overestimated

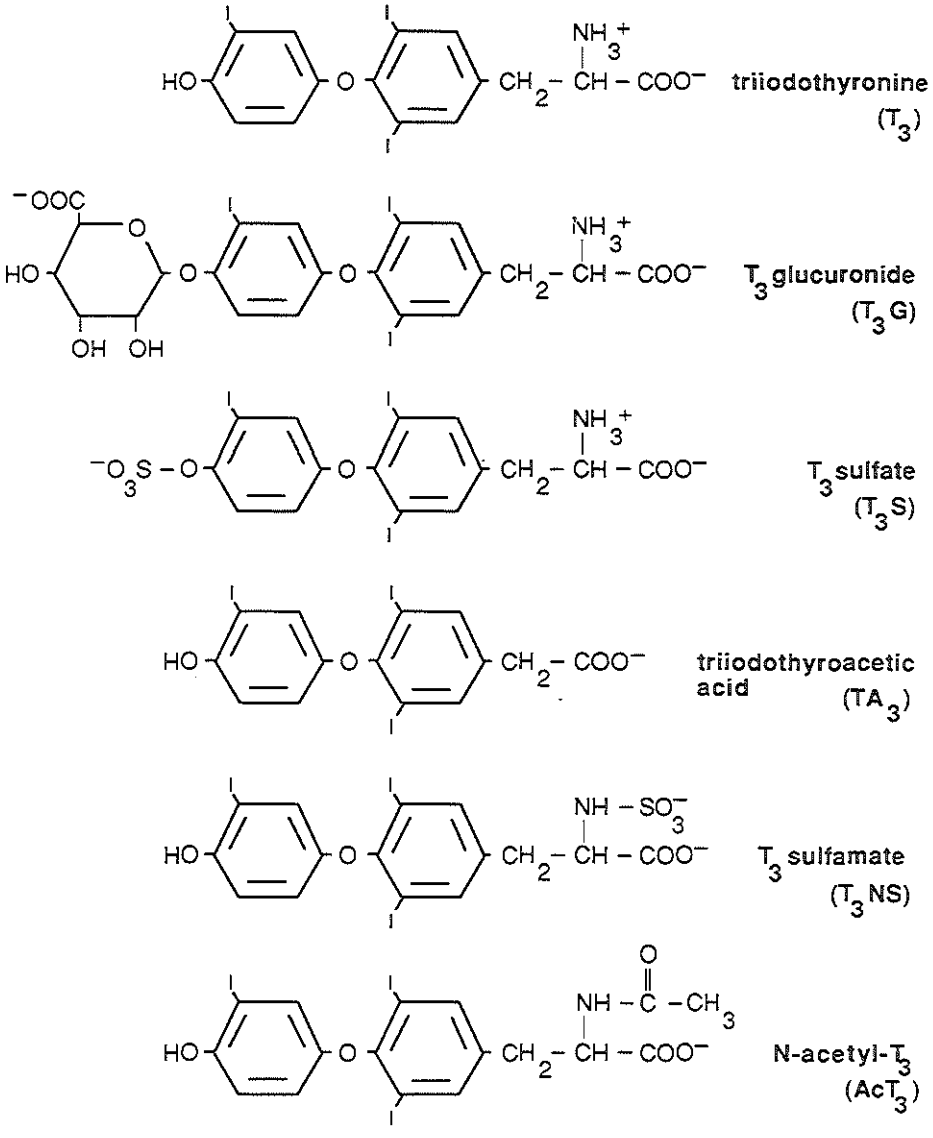


Figure 1. Structures of T_3 and derivatives

Table 1. **Kinetic parameters for the deiodination of native iodothyronines and derivatives by rat liver microsomes**

Substrate	Reaction	K_m [a]	V_{max} [b]	V_{max}/K_m	Reference
T_4	ORD	2.3	30	13	189
T_4S	ORD	undetectable		123	
T_4NS	ORD	0.067	14	204	168[c]
T_4	IRD	1.9	18	9	189
T_4S	IRD	0.29	527	1817	123
T_4NS	IRD	0.060	9	155	168[c]
rT_3	ORD	0.064	559	8730	189
rT_3S	ORD	0.060	516	8600	123
rT_3NS	ORD	0.061	631	10344	168[c]
T_3	IRD	6.2	36	6	189
T_3S	IRD	4.6	1050	230	190
T_3NS	IRD	1.4	79	57	168[c]
AcT_3	IRD	1.06	12	11	168[c]
TA_3	IRD	1.79	174	97	164[d]
TA_3S	IRD	0.004	21	5568	164[d]
3,3'-T_2	ORD	8.9	188	21	139
3,3'-T_2S	ORD	0.34	353	1040	139
3,3'-T_2NS	ORD	4.8	415	87	168[c]
3,3'-AcT_2	ORD	0.76	60	79	168[c]
3,3'-TA_2	ORD	0.75	49	65	164[d]
3,3'-TA_2S	ORD	0.023	64	2783	164[d]
3'-T_1	ORD	undetectable			198
3'-T_1S	ORD	5.9	1900	322	198

Studies were carried out at 37 °C in 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA and 3-5 mM DTT.

[a]) μM

[b]) pmol/min/mg protein

[c]) Chapter II of this thesis

[d]) Chapter IV of this thesis

as they are probably influenced by their relatively high lipid solubility and, hence, sequestration in membranes.

1.3 4'-Sulfated iodothyroacetic acid derivatives

The facilitatory effect of 4'-sulfation on the type I deiodination is not restricted to native iodothyronines, but is also observed for iodothyroacetic acids. Due to improved enzyme affinities, the deiodination efficiencies of TA_3S and 3,3'-TA_2S are 57 and 43 times higher than nonconjugated TA_3 and 3,3'-TA_2, respectively (Table 1). TA_3S is deiodinated by rat liver microsomes only in the inner ring, followed by rapid ORD of the transient intermediate 3,3'-TA_2S, resembling the reaction sequence of T_3S (190; Chapter IV). Remarkably, the stimulation of the deiodination by sulfation is more pronounced for TA_3 than for T_3 (Table 1). Deamination in combination with sulfation of T_3 culminates in nearly 10^3 times more efficient IRD of TA_3S. This is especially due to the dramatic reduction in apparent K_m value to 4 nM, which makes TA_3S the best known substrate for type I IRD, even despite its relative low V_{max} value (Table 1). Furthermore, TA_3S inhibits rT_3 ORD with a K_i value of 4 nM, confirming its extremely high affinity for the type I enzyme (Chapter IV).

1.4 Structure-activity relationship

The structural requirements for optimal binding of substrates and inhibitors to the deiodinase ligand binding site has been studied extensively by Cody, Köhrle and coworkers (4,30,31,104,105). They have used advanced computer graphic modeling techniques to analyze the interaction of iodothyronine analogs with TBPA. TPBA is the best characterized iodothyronine-binding protein, i.e. its crystal structure has been fully elucidated, and its ligand-binding requirements are similar to those of the type I deiodinase. With regard to my own study, a few aspects of the structure-activity relationship will be highlighted.

A negatively charged side chain and a negatively charged 4'-substituent both strongly favor the interaction of the substrate with the ligand-binding site of the type I deiodinase (105). This view is supported by our findings that compounds with a 4'-sulfate group or with an N-acetylated or N-sulfonated alanine or an acetic acid side chain are all better substrates than the corresponding iodothyronines (Table 1). This is probably not due to the alterations of the electron density of the aromatic rings, since modifications at these opposite ends of the molecule may influence deiodination of either ring. Substrates with both modifications, such as TA_3S, show very high affinity for the deiodinase. It has been suggested that these negatively charged groups provide better interaction with positively charged residues in the deiodinase, known to be a basic protein (Chapter I, section 2.2).

However, conjugation of the 4'-hydroxyl group with the bulky, negatively charged glucuronic acid group completely blocks type I deiodination of iodothyronines.

The kinetic parameters listed in Table 1 show that the stimulatory effect of 4'-sulfation on the type I deiodination of iodothyronines and iodothyroacetic acids is not correlated with a consistent change in V_{max} or K_m. The type I deiodinase is capable of both inner ring and outer ring deiodination, even with the same substrate. This suggests distinct modes of binding of substrate to the enzyme, such that either the inner ring or outer ring iodines are in close proximity to the catalytic center, i.e. the selenolate group (Chapter I, section 2.2). The quality of the substrate-enzyme interaction in both modes will be determined by the structures of the 4'-substituent and the derivatized side chain as well as by the iodine substitution pattern. The exact influence of these parameters on the three-dimensional structure of the iodothyronine, e.g. spatial orientation of the rings around the ether bridge, is unknown. Therefore, their impact on the efficiency and specificity of the deiodination process cannot be predicted. However, it is clear that modifications of the 4'-OH group and the alanine side chain may change the preference of the enzyme for the position of the iodine to be removed. This was noted for the "symmetric" substrates T_4 and 3,3'-T_2 (with the same number of iodines in both rings), but not for T_3 and rT_3 (with different numbers of iodines in outer and inner ring). The extent to which 3,3'-T_2 analogs are also deiodinated in the inner ring depends upon the type of modification (Chapters II and IV), and increases from 3,3'-T_2 (a pure ORD substrate) via 3,3'-T_2S, 3,3'-AcT_2, 3,3'-TA_2S and 3,3'-T_2NS to 3,3'-TA_2, which is deiodinated to similar extents in the inner and outer ring. Notably, T_4 and T_4NS undergo both IRD and ORD with the latter being somewhat more efficient, whereas T_4S is only deiodinated in the inner ring, and much more efficiently so than T_4 itself (Table 1).

2 METABOLISM OF IODOTHYRONINE DERIVATIVES BY RAT HEPATOCYTES *IN VITRO*

Freshly isolated rat liver cells in monolayer cultures metabolize iodothyronines by glucuronidation, sulfation and deiodination. Hepatocyte monolayers are, therefore, suitable to study the interaction between these pathways in the metabolism of thyroid hormones (52,56,140,171,197) as well as various naturally occurring derivatives (Chapter V).

Knowing that the microsomal deiodination of 3,3'-T_2 is rather slow compared with rT_3 (Table 1), the unexpected rapid conversion of 3,3'-T_2 by rat hepatocytes resembling that of rT_3 itself, lead in 1983 to the discovery that sulfation markedly accelerates the hepatic deiodination of 3,3'-T_2 (139,140). Accumulation of 3,3'-T_2 from added rT_3 was not observed unless the sulfation capacity of the rat hepatocytes was impaired, thus preventing sulfation and subsequent ORD of generated 3,3'-T_2S (52).

It is nowadays clear, that sulfate conjugation is not only required for the rapid deiodination of 3,3'-T_2 (and 3'-T_1), but it also accelerates deiodination of T_3 (198). The metabolic clearance of these iodothyronines is greatly impeded in the presence of a competitive substrate (salicylamide) or inhibitor of phenol sulfotransferase (DCNP) as well as after sulfate depletion of the hepatocytes (52,56,139,198). Furthermore, in sulfate-containing hepatocyte cultures, the deiodinase inhibitor PTU does not affect the metabolic clearance of 3'-T_1, 3,3'-T_2 or T_3. It induces the accumulation of equivalent amounts of sulfated iodothyronines instead of iodide, which clearly proves that sulfation precedes type I deiodination of these iodothyronines (197,198).

2.1 T_3

Figure 2 depicts the major routes of the hepatic metabolism of T_3. Roughly similar proportions are metabolized by 1) glucuronidation, 2) successive sulfation and type I deiodination (via the intermediate 3,3'-T_2S), and 3) direct IRD without prior sulfate conjugation. This latter route is primarily mediated by a low-K_m, PTU-insensitive, type III-like deiodinase (56,197,198).

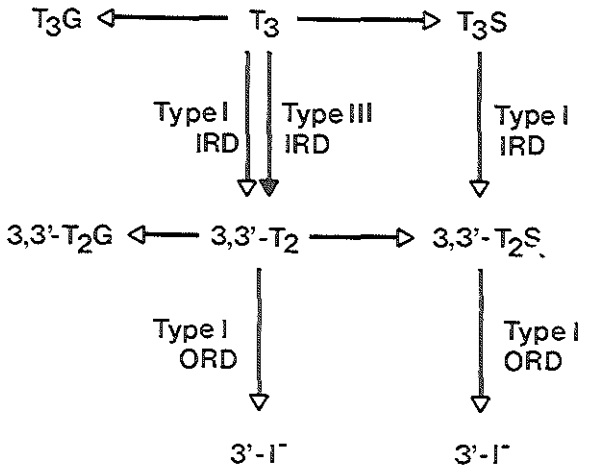


Figure 2. **Metabolic pathways of T_3 in rat hepatocytes**

Only the radioactive products generated from [3'-^{125}I]T_3 are shown (copied from Ref. 56).

* Normally, the ultimate products of T_3 are T_3G and 3'-derived iodide (3'-I^-).
* If type I deiodination is blocked by PTU, no 3'-I^- is released but T_3S and 3,3'-T_2S accumulate in roughly similar amounts. The clearance of T_3 and production of T_3G are not affected.
* A diminished sulfation capacity also decreases the amount of 3'-I^-, accompanied by the accumulation of 3,3'-T_2 (and 3,3'-T_2G). Under such conditions T_3G is slightly elevated and T_3 clearance significantly reduced.

2.2 TA_3

The hepatic metabolism of TA_3 (Fig. 3) roughly resembles that of T_3, although the various routes contribute differently to the overall clearance as broadly outlined below.

An important intermediate in TA_3 metabolism is 3,3'-TA_2, which is predominantly degraded by successive sulfation and rapid deiodination (Chapter V), which is in agreement with 3,3'-TA_2S being a very good ORD substrate (Table 1). However, if the sulfation capacity of the cells is impaired, glucuronidation and direct type I deiodination are efficient alternative pathways. Because 3,3'-TA_2 and, to a lesser extent, 3,3'-TA_2S are converted by both ORD and IRD (section 1.4), various TA_1 derivatives do also arise from TA_3 (Fig. 3). For reasons of clarity these mono-iodinated metabolites are not taken into consideration here, but have been described elsewhere (Chapter V).

In incubations with monolayers of freshly isolated rat hepatocytes, TA_3 is cleared as least as rapidly as T_3, despite its higher protein-bound fraction. This is largely explained by a more extensive glucuronidation of TA_3 relative to T_3.

Some TA_3 is metabolized by succesive sulfation and IRD as co-incubation with PTU results in a moderate accumulation of TA_3S. Similar to T_3, the overall clearance of TA_3 is not affected by PTU. However, for TA_3 this is due to a considerable increase of TA_3G, which even exceeds the TA_3S accumulation. These results indicate that sulfation contribu-

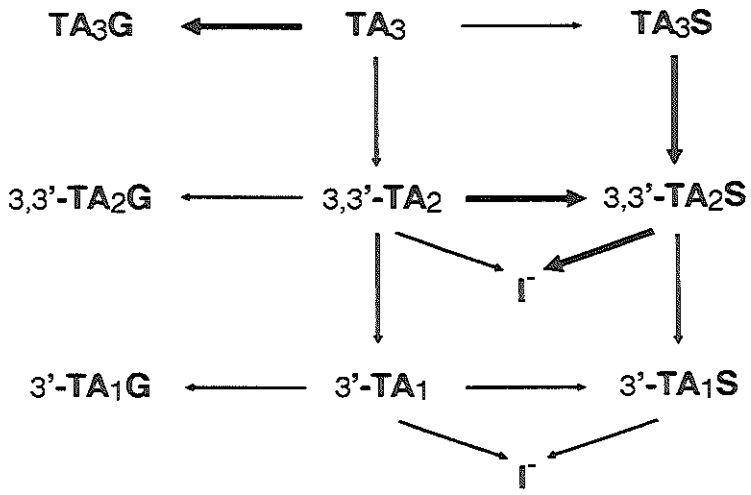


Figure 3. **Metabolic pathways of TA_3 in rat hepatocytes**

Only the radioactive products generated from [3'-^{125}I]TA_3 are shown.

tes less to the hepatic metabolism of TA_3 than T_3. This is rather surprising, because it has been shown that TA_3 is a better substrate for hepatic phenol sulfotransferase (173; Chapter V).

The importance of direct IRD of TA_3 is demonstrated in sulfate-depleted hepatocytes by the production of 3,3'-TA_2, which is then no longer sulfated (and subsequently deiodinated) but glucuronidated or converted by direct ORD. In contrast to T_3, this direct deiodination of TA_3 is largely inhibited by PTU and, therefore, mediated by the type I enzyme.

In comparison with T_3 (56,197), hepatic metabolism of TA_3 occurs more through glucuronidation to TA_3G and direct type I deiodination to 3,3'-TA_2 (with subsequent sulfation) and less through sulfation (to TA_3S) and subsequent deiodination (Chapter V).

2.3 T_4

This section describes the routes of hepatic T_4 metabolism demonstrated *in vitro* (Fig. 4), while an overview of T_4 metabolism in rats is presented in section 3 of this Chapter.

In 2-day old primary cultures of rat hepatocytes, both inner and outer ring ^{125}I-labeled T_4 are metabolized to mainly radioiodide (Fig. 1 in Chapter I). However, if T_4 is incubated at increasing substrate concentrations, both T_4 ORD and IRD become progressively saturated, resulting in accumulation of T_4G and T_4S (171,198). Sulfation is involved in the normal cascade of hepatic deiodination of T_4. Therefore, inhibition of deiodination enables the detection of the "labile" sulfated intermediates in the T_4 metabolism, because they are otherwise rapidly cleared by type I deiodination (Table 1). This was recently illustrated by incubating T_4 with freshly isolated rat hepatocytes of which the conjugation capacity was greatly dimished by coincubation with salicylamide (197). These conditions increased the amount of T_3 by roughly 30 % and induced the appearance of roughly equal amounts of 3,3'-T_2 which does not accumulate in control cultures. Clearly, the T_3 levels in hepatocyte cultures strongly depends on both its production rate from T_4 as well as on its clearance rate via conjugation.

Clearance of T_4, incubated with rat hepatocytes in the presence of PTU, is dramatically reduced, although some rT_3 accumulates, which suggests the involvement of PTU-insensitive, type III-like deiodinase.

3 METABOLISM OF IODOTHYRONINE DERIVATIVES IN RATS *IN VIVO*

Recent studies in rats support the *in vitro* findings with regard to successive sulfation and deiodination of thyroid hormones. Since sulfate conjugates of T_4, T_3, 3,3'-T_2 and TA_3 undergo efficient type I deiodination also *in vivo*, little sulfates are normally detected in

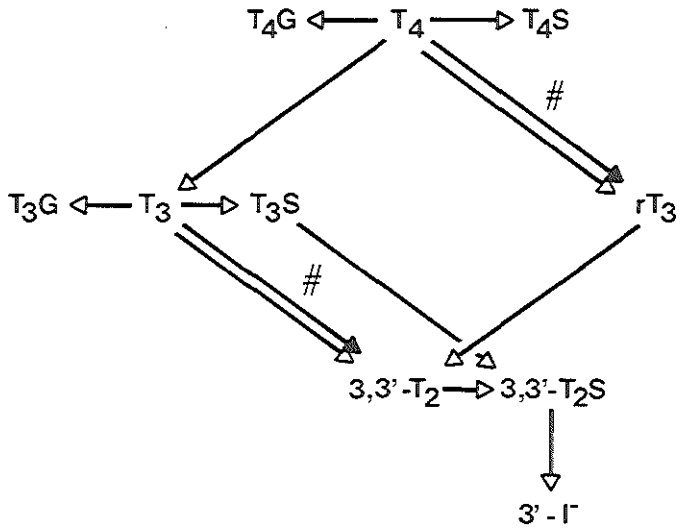


Figure 4. **Major pathways of T_4 metabolism in rat hepatocytes**

#) Besides type I deiodination, T_4 and T_3 are also converted by a type III-like deiodinase

bile, plasma or urine (197,198). The identification of these sulfated intermediates is facilitated when the experimental animals are treated with type I deiodinase inhibitors such as PTU (192,198), thiouracil (65), IOP (53), or butyl 4-hydroxy-3,5-diiodobenzoate (BHDB; Refs. 16,67,68,86). Other experimental conditions associated with diminished type I deiodinase activity are hypothyroidism due to thyroidectomy (29,33,64,155,157) or fasting (177). In fasting, type I deiodinase activity is reduced due to ensuing hypothyroidism and is restored by thyroid hormone replacement (99,197).

Although accumulation of iodothyronine sulfates under the above-mentioned conditions has already been reported many years ago (155,157), we can only explain these findings now the role of the type I deiodinase in the clearance of these conjugates has been recognized (198). It further explains the PTU-induced shift in excretion of radioactivity from urine to feces of rats equilibrated with radioiodinated thyroid hormones (126,185). Experiments with bile duct-cannulated rats and isolated rat liver perfusions indicate that this involves an increased biliary excretion of iodothyronine sulfates (16,40,53,65,110; Chapter VII).

This section highlights that sulfate conjugates are important intermediates in the nor-

Table 2. **Biliary excretion of major thyroid hormone metabolites in control and PTU-treated rats**

Hormone	PTU	Time (h)[a]	Dose (%)	Distribution of major metabolites (% of biliary radioactivity)							Reference or chapter
				T_4G	T_4S	rT_3G	T_3G	T_3S	T_2S[b]		
T_4	—	17-18[c]	1.0	43	6.2	1.6	18.4[d]				VII
T_4	+	17-18	2.0	38	16.6	7.4	3.8[d]				VII
T_3	—	0-4	22				74	8	≤1		40
T_3	+	0-4	36				44	24	14		40
							TA_3G	TA_3S	TA_2S[e]	XS[f]	
TA_3	—	0-4	55				70	8	0	13	V
TA_3	+	0-4	85				57	22	2	12	V

[a]) after iv administration of outer ring ^{125}I-labeled hormone

[b]) 3,3'-T_2S

[c]) similar data when bile was collected between 7-8 h

[d]) corrected for difference in specific radioactivity

[e]) 3,3'-TA_2S

[f]) unidentified sulfate conjugate

mal tissue conversion of thyroid hormones. Tables 2 and 3 summarize the distribution of metabolites in bile and plasma, respectively, of control and PTU-treated rats after injection of outer ring ^{125}I-labeled T_4, T_3 or TA_3 as presented in Chapters III, V-VII. Our data are in good agreement with previous reports, reviewed in references 16, 22, 36 and 197. Before the introduction of a scheme of the various pathways involved with the handling of T_4 (Fig. 6), the relevant routes in the metabolism of T_3 and TA_3 will be discussed.

3.1 T_3

Radioiodide and T_3G are the major metabolites in plasma and bile, respectively, of control [^{125}I]T_3-injected rats (Tables 2 and 3). Deiodination is drastically diminished after PTU treatment, with plasma radioiodide levels being 60 % lower than in control animals. Such acute PTU treatment induces a $\geq$5-fold increase in the biliary excretion of T_3S (40), accompanied by at least 4-fold increment of plasma T_3S (Tables 2 and 3). Furthermore, RIA measurements of plasma T_3S in T_4- or T_3-substituted rats undergoing chronic PTU treatment also revealed 4 times higher levels than in the methimazole-treated controls (54,198). Most important, plasma T_3 clearance is not affected by PTU in rats, indicating that direct type I deiodination is a minor pathway in the *in vivo* metabolism of T_3 (175,198; Chapter III). Clearance of T_3S, however, depends primarily on type I deiodination, as we demonstrated using rats injected with [^{125}I]T_3S (Chapter III). Disappearance of plasma T_3S as well as production of plasma radioiodide were strongly diminished by PTU, and nearly all injected T_3S was recovered in the bile.

Moreover, the appearance of 3,3'-T_2S in bile of PTU-treated, T_3-injected rats is even more pronounced than that of T_3S (Table 2). The 3,3'-T_2S production is sensitive to IOP treatment (53), which inhibits both type I and type III deiodinases, indicating that this metabolite most likely arises by extrahepatic type III IRD of T_3 and subsequent sulfation of 3,3'-T_2. Within 2 h after T_3 administration to PTU-treated rats, the biliary excretion of sulfate conjugates even exceeds that of the glucuronides, due to this marked stimulation of 3,3'-T_2S (40).

It is furthermore noteworthy that 3,3'-TA_2S is a persistent metabolite of T_3 in PTU-treated rats, and is probably generated via TA_3 (Chapter VI). This sulfate conjugate clearly appears in plasma (Table 3) but scarcely in bile, and is also observed in IOP-treated animals (Chapter VI). Studies with rat hepatocyte monolayers have shown that 3,3'-TA_2 is very effectively cleared via successive sulfation and deiodination (Chapter V). The striking accumulation of 3,3'-TA_2S in T_3-injected animals with impaired type I activity is fully explained by its diminished degradation, but its origin remains unclear. In rats, 3,3'-TA_2S appears to be a physiological significant intermediate in the T_3 metabolism, because plasma 3,3'-TA_2S levels are at least as high as plasma 3,3'-T_2S

Table 3. **Iodothyronines and metabolites in plasma of control and PTU-treated rats**

Hormone	PTU	Time (h)[a]	% Dose per ml	Distribution of major metabolites (% of plasma radioactivity)										Chapter
				T_4	T_3	T_3S	T_2S[b]	TA_3	TA_3S	TA_2S[c]	I^-	X[e]	XS[e]	
T_4	—	18	0.8[d]	85							11			VII
T_4	+	18	1.1[d]	90							4			VII
T_3	—	4	0.33		10	2	1			0	84			III,VI
T_3	+	4	0.26		20	21	6			6	37			III,VI
TA_3	—	4	0.29					14	1	1	74	3	2	V
TA_3	+	4	0.18					18	9	31	19	4	6	V

[a]) after iv injection of outer ring ^{125}I-labeled hormone. For data on earlier time points, see chapters listed.
[b]) 3,3'-T_2S
[c]) 3,3'-TA_2S
[d]) rT_3 and T_3 undetectable by HPLC; total conjugates ≈2% (Ctrl) or ≈4 % (+PTU) by Sephadex LH-20
[e]) unidentified metabolite (X) and its sulfate conjugate (XS)

(Table 3). Moreover, even much higher 3,3'-TA_2S levels were detected under the least invasive experimental conditions (Chapter VI). The relatively low biliary 3,3'-TA_2S disposal in PTU-treated rats may be explained by its extremely strong binding to plasma proteins (Chapter VI).

The biological relevance of the pathway of successive sulfation and deiodination of T_3 has been established not only in rats, but also in humans (198). As measured by RIA, T_3S is undetectable in plasma of healthy humans but is observed in hypothyroid patients (28) and in PTU- or IOP-treated volunteers (55). However, in these latter persons, the plasma T_3S/T_3 ratio is much lower than in PTU-treated rats (54), suggesting that sulfation of T_3 is less important in humans than in rats (198).

3.2 TA_3

The metabolism of TA_3 in rats follows the same pathways as T_3, although the biliary clearance rate of TA_3 is at least 2-fold higher than that of T_3 due to extensive glucuronidation (Table 2). This also explains the more rapid plasma disappearance of injected TA_3, despite its higher binding to serum proteins compared to that of T_3 (Chapter V).

Analogous to T_3, PTU treatment does not affect plasma TA_3 clearance but stimulates total biliary TA_3 clearance 1.5-fold (Table 2). Besides a 4-fold increment in biliary TA_3S, the elimination of TA_3G was increased 30 %, in contrast to the biliary T_3G excretion which is not affected by PTU. As TA_3 is a better substrate than T_3 for the type I deiodinase (Table 1), inhibition of the enzyme by PTU may increase the hepatic availability of TA_3 for glucuronidation.

Figure 5 shows that the effect of PTU on plasma TA_3 metabolites mimics that of T_3. Normally, generated TA_3S and 3,3'-TA_2S undergo very rapid deiodination (Table 1), but when this is blocked by PTU, the plasma level of sulfated intermediates increase 3-fold with a concomittant 85 % decrease in plasma iodide averaged over the first 4 h (see also Table 3). This is in keeping with a 60 % reduction of urinary radioactivity in [^{125}I]TA_3-injected animals (67,68). Irrespective of PTU treatment, a significant proportion of TA_3 metabolites in bile and plasma consists of an unidentified sulfate conjugate (Chapter V).

The accumulation of sulfated derivatives in bile after [^{125}I]TA_3 injection in PTU-treated rats was already noticed by Roche *et al.* in 1958 (154). Moreover, other conditions that affect type I deiodinase activity, such as BDBH treatment (67) and hypothyroidism, have previously been shown to increase TA_3S in experimental animals, analogous to the accumulation of iodothyronine sulfates (16,154,157; Chapter V). In conclusion, the majority of TA_3 is metabolized by effective glucuronidation and subsequent rapid biliary excretion, while some TA_3 is also degraded via successive sulfation and type I deiodination as well as by direct deiodination.

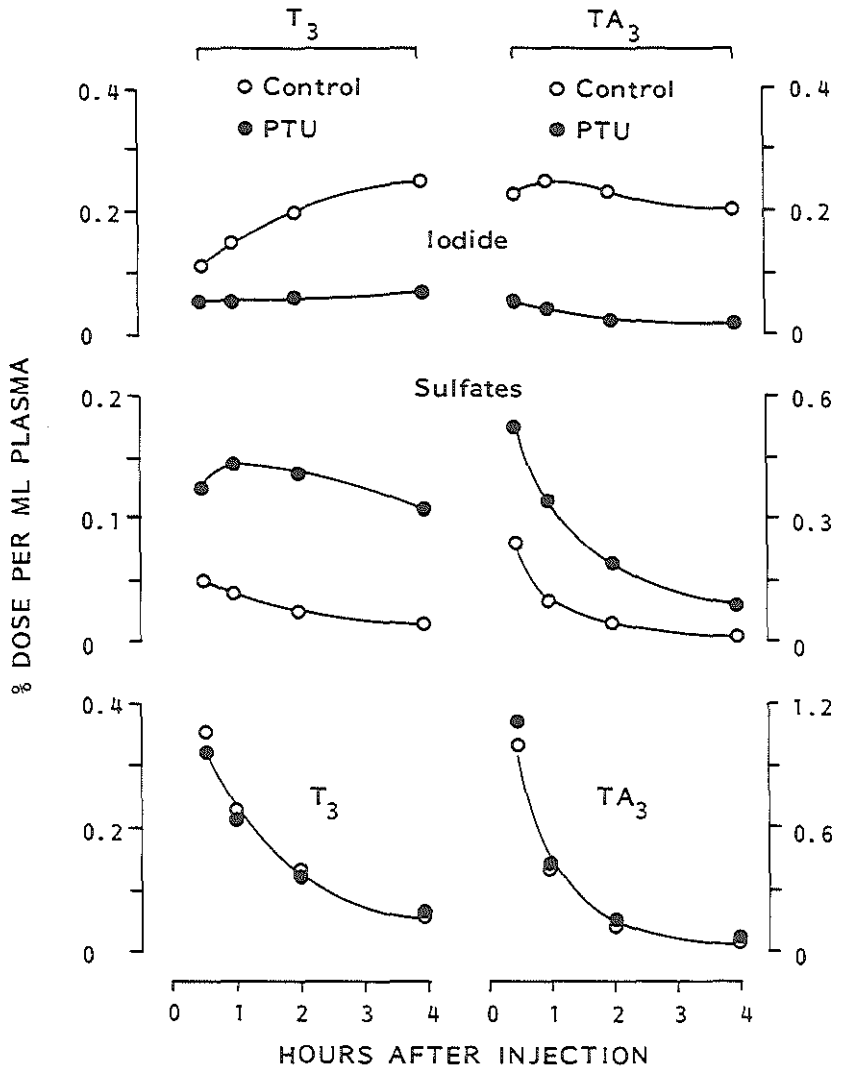


Figure 5. **Plasma metabolites in control (open symbols) and PTU-treated (closed symbols) rats after iv administration of [^{125}I]T_3 (left) or [^{125}I]TA_3 (right)**

Note the difference in scales of the left and right panels.
Sulfated metabolites consist of mainly T_3S in T_3-injected rats.
In TA_3-injected rats, sulfates represent mainly TA_3S at the early time points, but 3,3'-TA_2S dominates from 2 h onwards.
Data are taken from Chapters III and V.

3.3 Multiple pathways of T_4 metabolism

When allowing complete tissue distribution after [3',5'-^{125}I]T_4 administration to normal rats, >85 % of plasma radioactivity consists of T_4 and the remainder of radioiodide (Table 3). Radiolabeled T_3 and rT_3 cannot be detected using HPLC, which is understandable from steady-state plasma iodothyronine concentrations, i.e. 51 nM T_4, 1.4 nM T_3 and 0.03 nM rT_3 (54,198). Urinary excretion of radioactivity exceeds that in feces (95,126,185; Chapter VII) with radioiodide being the sole radiolabeled urinary product, while fecal radioactivity consists of 70 % T_4, 18 % T_3 and 2 % rT_3 (45; Chapter VII). Absence of iodothyronine glucuronides or sulfates in feces is in keeping with effective intestinal hydrolysis of the conjugates excreted with bile (Chapter I, section 2.3). This biliary excretion accounts for a large fraction of total T_4 clearance. Normally, about 70 % of the biliary radioactivity consists of T_4G, T_3G, rT_3G and T_4S in a ratio of 1 : 0.42 : 0.04 : 0.14 (Table 2).

The importance of the type I deiodinase in the activation and stepwise degradation of T_4 has been assessed by selective blocking of this enzyme with PTU. Our studies have focused on the shifts in excretion of T_4 metabolites, especially in relation to sulfate conjugation (Chapter VII). By the identification of T_4S especially in bile of PTU-treated rats we have clearly proven that, like other iodothyronines, part of T_4 is sulfated prior to type I deiodination. Injection of PTU-pretreated rats with [^{125}I]T_4 induces the following changes (Chapter VII):

1) Plasma and urinary radioiodide are decreased to <50 % (Table 3). This reduced deiodinative clearance is compensated by a 1.5-fold stimulation of fecal radioactivity (95,126,185) due to increased biliary clearance of [^{125}I]T_4.
2) The total radioactivity excreted with bile is doubled (Table 2). Biliary T_4G is increased two-fold in parallel with prolonged plasma T_4 retention (54) and, hence, without a change in the glucuronidative clearance of T_4 (197; Chapter VII). Excretion of biliary rT_3G increases 9-fold, whereas that of T_3G drops 2.6-fold, resulting in a 23-fold lower biliary T_3G/rT_3G ratio.
3) Excretion of T_4S in bile is enhanced at least 5-fold (Table 2), but T_4S is not observed in plasma (Table 3). In early studies of Flock *et al.* (65), an acid-hydrolyzable T_4 conjugate was detected in bile of thiouracil-treated rats, which was not identified, however, as T_4S at that time.

Because the type I deiodinase is responsible for both peripheral T_3 production and rT_3 clearance, a decreased biliary T_3G/rT_3G ratio reflects diminished enzyme activity. Other conditions associated with decreased type I deiodinase activity, that result in a significant reduction of the biliary T_3G/rT_3G ratio, are treatment with BHDB (67,68) or fasting-induced hypothyroidism (177) (Chapter VII). Even in "control" animals this ratio can decrease 20-fold due to experimental stress, which rapidly diminishes biliary T_3G

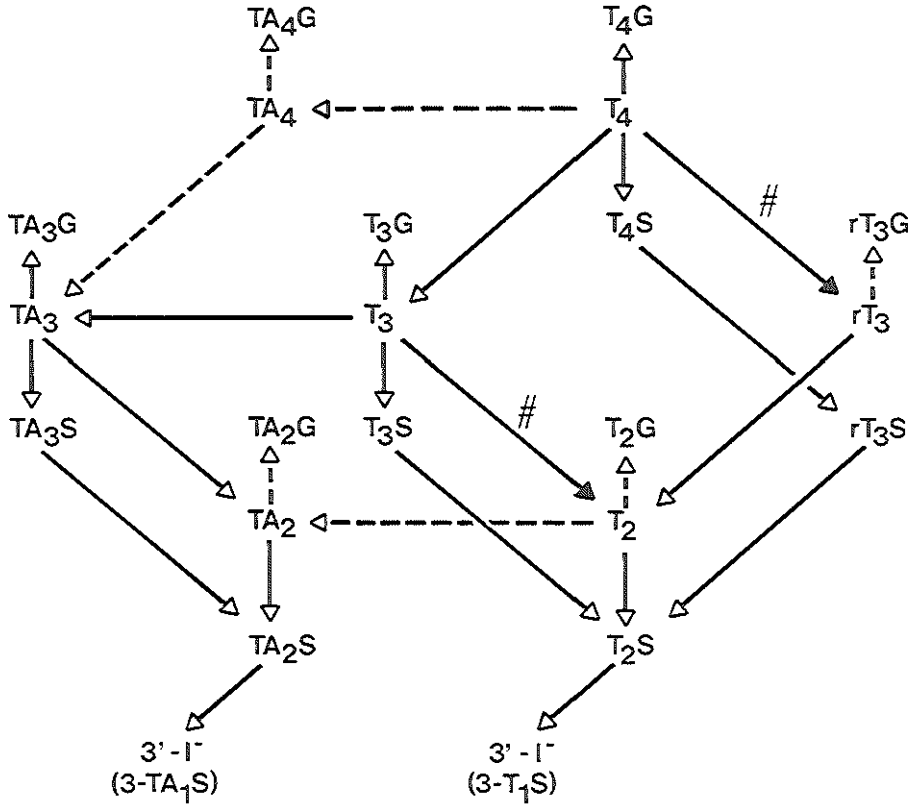


Figure 6. **Model of the multiple pathways of T_4 metabolism**

All T_2 derivatives are 3,3'-diiodinated

(Oxidative deamination and decarboxylation ←, Glucuronidation ↑; Sulfation ↓; type I ORD ↙ ; type I IRD ↘ , except for # which represent predominantly type III IRD)

excretion as discussed in Chapter VII. The effect of stress may be mediated by plasma glucocorticoids which may reduce hepatic and renal T_3 production (14,23). This clearly limits the use of the biliary T_3G/rT_3G ratio as a sensitive index of type I deiodinase activity.

Without indicating the different tissues involved or the role of enterohepatic recycling, the biochemical pathways of T_4 metabolism in rats are depicted in Figure 6 and summarized below. The biliary clearance of endogenous T_4 via the various metabolites is assessed on the basis of a daily T_4 production of approximately 1 nmol/100 g rat (197) and the biliary excretion data of [^{125}I]T_4 under steady state conditions (Chapter VII). The latter data are compatible with previous reports (16,36,69,177).

1) If the extent of side chain modification is similar with that in humans, production of TA_4 is a negligible pathway of T_4 metabolism in rats.
2) Approximately 60 % of T_4 is directly deiodinated by ORD to T_3 or by IRD to rT_3 (44[a]), catalyzed by different deiodinases in different tissues. Glucuronidation of T_4 and biliary excretion of T_4G accounts for about 36 % of T_4 disposal. A significant proportion of T_4 is sulfated and, although most T_4S is then rapidly deiodinated, about 5% of total T_4 is excreted as T_4S with bile. Moreover, if type I deiodination capacity is impaired, at least 15 % of T_4 is cleared via biliary T_4S.
3) The metabolism of rT_3 depends primarily on rapid type I ORD to 3,3'-T_2. Biliary excretion of rT_3G contributes to less than 2 % of T_4 turnover in rats, indicating that conjugation of rT_3 is a minor pathway. However, if type I deiodinase activity is impaired, biliary excretion of rT_3G becomes a significant metabolic pathway for T_4 disposal.
4) T_3 secreted by the thyroid or produced peripherally from T_4 is partly metabolized by glucuronidation, which accounts for roughly 15 % of total T_4 clearance. The remainder undergoes successive sulfation and type I deiodination via 3,3'-T_2S, although some T_3 is directly deiodinated to 3,3'-T_2 by the type III deiodinase (54; Chapter III). In addition, conversion to TA_3 may significantly contribute to the disposal of T_3. This pathway may lead to the appearance of 3,3'-TA_2S in PTU-treated, T_3-injected rats (Chapter VI).
5) 3,3'-T_2 is preferentially cleared by sulfation prior to rapid type I deiodination.

In rats, the importance of the sulfation pathway to the overall metabolism of the various iodothyronine derivatives varies greatly (36,197; this thesis). In summary, sulfation contributes very much to the clearance of 3,3'-T_2, is of equal importance as glucuronidation for T_3, but seems of less importance in the normal metabolism of T_4 and TA_3, because the majority of these latter compounds is cleared via direct deiodination and glucuronidation. Inactivation of iodothyronines by sulfation rapidly liberates iodide, becoming available for thyroid hormones (re)synthesis.

The role of sulfation in the metabolism of T_3 and T_4 in humans remains to be fully explored. Iodothyronine conjugation in humans is further discussed in section 3.4 below.

3.4 Enterohepatic cycling of iodothyronines

Some patients who suffer from severe diarrhea or subjects consuming a soy flour diet have abnormal fecal loss of thyroid hormone (143), which can cause thyroid dysfunction unless compensated by increased thyroid hormone production. These findings are understandable if thyroid hormone undergoes substantial enterohepatic circulation (EHC). Despite various animal studies (1,20,112,120,131,197) and mathematical analysis (46-48), no conclusive experimental evidence is available for the existence of an EHC of iodothyronines. We could show that T_3 is indeed involved in an EHC in rats, involving intestinal hydrolysis of T_3G and subsequent reabsorption of the active hormone (41; Chapter VIII). The main events involved in this T_3 recycling are depicted in Figure 7, which illustrates the fate of T_3 in control (A), PTU-treated (B), and intestine-decontaminated rats (C). Thus, after glucuronidation of endogenous or iv injected T_3, biliary excretion of T_3G does not necessarily represent the elimination of T_3 from the body.

The efficacy of the deconjugation by intestinal bacteria was previously shown by De Herder *et al.* (41), who administered [^{125}I]T_3G to rats by stomach tube. After such treatment, plasma T_3 absorption in normal animals was 5 times higher than that in intestine-decontaminated rats, and was similar compared with oral administration of [^{125}I]T_3 itself, which points to quantitative hydrolysis. However, the natural route for iodothyronine conjugates to enter the gut is via biliary excretion, and the rate of T_3G excretion in bile is greatly increased when T_3G, instead of T_3, is injected iv as reported in Chapter VIII. Within 1 h, about 90 % of T_3G is excreted in bile, irrespective of PTU treatment or intestinal decontamination, and plasma radioactivity rapidly declines in these [^{125}I]T_3G-injected rats. The existence of an EHC of T_3 becomes evident within a few hours, as plasma radioactivity reappeares only in the control animals, consisting of mainly T_3 and iodide, but not T_3G. In intestine-decontaminated animals, however, plasma T_3

Legend to Figure 7.
In the liver, T_3 is largely metabolized by 1) glucuronidation, and 2) successive sulfation and type I deiodination, with T_3G and I^- as the major products. Normally, all T_3G is excreted with bile (A). T_3S appears in significant amounts in bile and plasma only if its rapid deiodination is prevented, for instance due to PTU treatment (B). Biliary-excreted conjugates are hydrolysed by the intestinal microflora, allowing the liberated T_3 to be reabsorbed in the portal blood, while the remaining T_3 is discarded in feces (A,B). Intestine-decontaminated animals are largely incapable of deconjugation as they lack the necessary bacteria and, therefore, loose the biliary conjugates with feces (C).

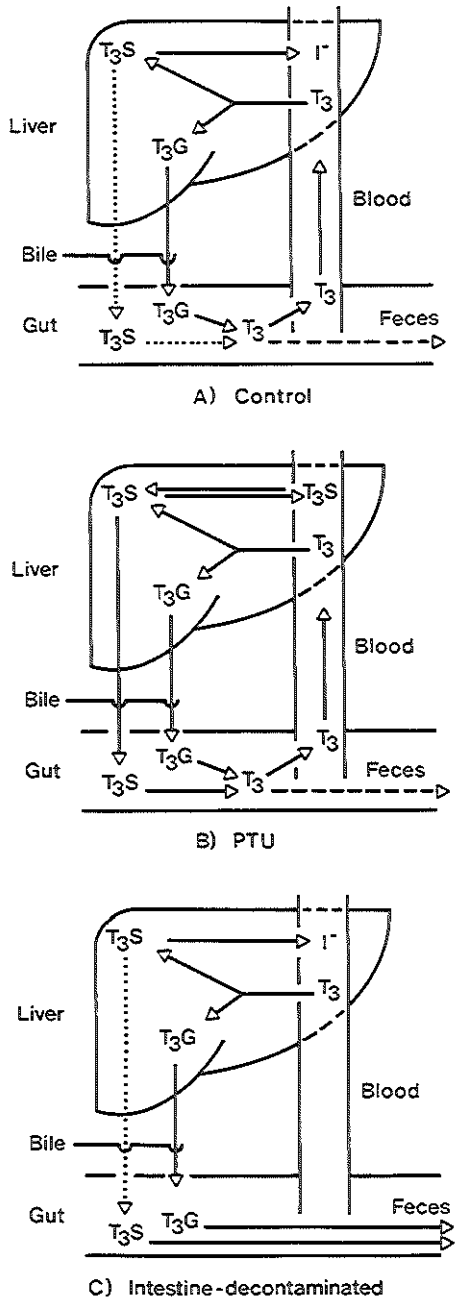


Figure 7. The enterohepatic circulation of T_3

levels are 3-fold lower at corresponding time points, illustrating that the presence of intestinal bacteria is crucial (Chapter VIII). Moreover, after iv administration of T_3G to rats with a completely interrupted EHC due to bile diversion, plasma T_3 was >12-fold lower than in intact control animals.

We furthermore demonstrated that a significant proportion of T_3 is reabsorbed from biliary-excreted T_3S after iv injection of this conjugate -although less than after T_3G injection- provided that T_3S is not deiodinated during hepatobiliary transport (Fig. 7B; Chapter VIII). Normally, the EHC of T_3 occurs via T_3G since this conjugate is largely responsible for biliary clearance of T_3, although the amount of T_3 involved in this EHC remains to be assessed.

Apart from deiodination, the major pathway of T_4 metabolism in rats is biliary clearance via T_4G (section 3.3). It is, however, still a matter of dispute if T_4 is engaged in an EHC. Only indirect evidence is available which favours (1,20,48,112,120,131) or negates its existence (34,71). Several of these studies indicate that between 10-37 % of the biliary-excreted metabolites undergo recycling, although the compounds have not been identified. DiStefano *et al.* (46,48) have presented evidence that the lumenal content of the rat intestine represent a physiologically significant pool of exchangable thyroid hormones.

Compared with rats, humans eliminate less thyroid hormone with feces (44[b],46,114), which may point to a smaller proportion of iodothyronines being conjugated or to a more extensive EHC. In cholecystectomized patients a biliary T_4 clearance of 10-30 ml/h was reported, corresponding to as much as 20-60 % of total T_4 clearance (113,130). Furthermore, the existence of an EHC of T_4 in humans, as already proposed by Myant in 1956 (130), has not been established yet (71) although T_4G is effectively hydrolysed by human intestinal bacteria (38,39,82). Likewise, since T_3 is sulfated in human liver (55,194,198) and T_3S is hydrolysed by human intestinal bacteria (39,82), an EHC involving T_3S is possible in humans under conditions of diminished type I deiodinase activity, such as in hypothyroidism. To judge the extent of T_4 conjugates involved in a putative EHC of T_4 in man, the contributions of T_4G and T_4S to thyroid hormone turnover have to be assessed in health and disease. This should include the effects of drugs such as antiepileptics and other microsomal enzyme inducers, which are known to stimulate glucuronidation (197).

REFERENCES

1 Albert A, Keating FR 1952 The role of the gastrointestinal tract including the liver in the metabolism of radiothyroxine. Endocrinology 51: 427-443

2 Andrea TA, Cavalieri RR, Goldfine ID, Jorgensen EC 1980 Binding of thyroid hormone analogues to the human plasma protein prealbumin. Biochemistry 19: 55-63

3 Arthur JR, Nicol F, Beckett GJ 1990 Hepatic iodothyronine 5'-deiodinase. The role of selenium. Biochem J 272: 537-540

4 Auf'mkolk M, Köhrle J, Hesch RD, Cody V 1986 Inhibition of rat liver iodothyronine deiodinase. Interaction of aurones with the iodothyronine ligand-binding site. J Biol Chem 261: 11623-11630

5 Back DJ, Breckenridge AM, Crawford FE 1980 Reduction of the enterohepatic circulation of norethisterone by antibiotics in the rat: correlation with changes in the gut flora. J Steroid Biochem 13: 95-100

6 Bastomsky CH 1973 The biliary excretion of thyroxine and its glucuronic acid conjugate in normal and Gunn rats. Endocrinology 92: 35-40

7 Bastomsky CH 1974 Effects of a polychlorinated biphenyl mixture (Aroclor 1254) and DDT on biliary excretion in rats. Endocrinology 95: 1150-1155

8 Bastomsky CH, Wyse JM 1975 Enhanced thyroxine metabolism following cutaneous application of microscopic immersion oil. Res Commun Chem Pathol Pharmacol 10: 725-733

9 Bastomsky CH 1977 Enhanced thyroxine metabolism and high uptake goiters in rats after a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Endocrinology 101: 292-296

10 Beckett GJ, MacDougall DA, Nicol F, Arthur JR 1989 Inhibition of type I and type II deiodinase activity in rat liver, kidney and brain by selenium deficiency. Biochem J 259: 887-892

11 Beetstra JB, van Engelen JGM, Karels P, van der Hoek HJ, de Jong M, Docter R, Krenning EP, Hennemann G, Brouwer A, Visser TJ 1991 Thyroxine and 3,3',5-triiodothyronine are glucuronidated in rat liver by different uridine diphosphate-glucuronyltransferases. Endocrinology 128: 741-746

12 Behne D, Kyriakopoulos A, Meinhold H, Köhrle J 1990 Identification of type I deiodinase as a selenoenzyme. Biochem Biophys Res Commun 173: 1143-1149

13 Berry MJ, Banu L, Larsen PR 1991 Type I deiodinase is a selenocysteine-containing enzyme. Nature 349: 438-440

14 Bianco AC, Nunes MT, Hell NS, Maciel RMB 1987 The role of glucucorticoids in the stress-induced reduction of extrathyroidal 3,5,3'-triiodothyronine generation in rats. Endocrinology 120: 1033-1038

15 Björkman U, Elkholm R 1990 Biochemistry of thyroid hormone formation and secretion. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 83-126

16 Bollman JL, Flock EV 1965 The role of the liver in the metabolism of ^{131}I-thyroid hormones and analogues. In: Taylor W (ed) The Biliary System. Blackwell, Oxford, p 345-365

17 Boye N 1986 Thyroxine monodeiodination in normal human kidney tissue in vitro. Acta Endocrinol 112: 536-540

18 Braverman LE, Ingbar SH 1962 Effects of propylthiouracil and thiouracil on the metabolism of thyroxine and several of its derivatives by rat kidney slices in vitro. Endocrinology 71: 701-712

19 Braverman LE, Ingbar SH, Sterling K 1970 Conversion of thyroxine (T_4) to triiodothyronine in athyreotic subjects. J Clin Invest 49: 855-864

20 Briggs FN, Taurog A, Chaikoff IL 1953 The enterohepatic circulation of thyroxine in the rat. Endocrinology 52: 559-567

21 Burchell B, Coughtrie MWH 1989 UDP-glucuronosyltransferases. Pharmacol Ther 43: 261-289

22 Burger A 1986 Nondeiodinative pathways of thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 255-276

23 Cavalieri RR, Pitt-Rivers R 1981 The effects of drugs on the distribution and metabolism of thyroid hormones. Pharmacol Rev 33: 55-79

24 Cavalieri RR 1986 Effects of drugs on thyroid hormone metabolism. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 359-382
25 Cheng SY, Wilchek M, Cahnmann HJ, Robbins J 1977 Affinity labeling of human serum prealbumin with N-bromoacetyl-L-thyroxine. J Biol Chem 252: 6076-6081
26 Chopra IJ 1981 Triiodothyronines in health and disease. In: Gross F, Grumbach MM, Labhart A, Lipsett MB, Mann T, Samuels LT, Zander J (eds) Monographs on Endocrinology, vol 18. Springer-Verlag, New York
27 Chopra IJ, Boado RJ, Geffner DL, Solomon DH 1988 A radioimmunoassay for measurement of thyronine and its acetic acid analog in urine. J Clin Endocrinol Metab 76: 480-487
28 Chopra IJ, Wu SY, Chua Teco G, Santini F 1991 Measurement of 3,5,3'-triiodothyronine sulfate (T_3S) in thyroidal and nonthyroidal diseases, pregnancy, and neonatal life. Thyroid 1 (Suppl 1): Abstract 79
29 Closon J, 1964 La sulfoconjugaison de la triiodothyronine et la destineé de son produit. Editions Arscia, Bruxelles
30 Cody V 1980 Thyroid hormone interactions: Molecular conformation, protein binding, and hormone action. Endocrine Rev 1: 140-166
31 Cody V 1988 Thyroid hormone structure-activity relationships: molecular structure of 3,5,3'-triiodothyropropionic acid. Endocrine Res 14: 165-176
32 Cooper DS 1991 Substances that affect thyroid function or thyroid hormone metabolism. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 323-344
33 Cooper JAD, Beringer ED 1955 Metabolism of labeled thyroxine and triiodothyronine in bile fistula dogs. Federation Proc 14: 197
34 Cottle WH 1964 Biliary and fecal clearance of endogenous thyroid hormone in cold-acclimated rats. Am J Physiol 207: 1063-1066
35 Crossley DN, Ramsden DB 1979 Serum tetraiodothyroacetate (T_4A) levels in normal healthy euthyroid individuals determined by gas chromatography-mass fragmentography (GC-MF). Clin Chim Acta 94: 267-272
36 Curran PG, DeGroot LJ 1991 The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. Endocrine Rev 12: 135-150
37 DeGroot LJ, Larsen PR, Refetoff S, Stanbury JB (eds) 1984 The thyroid and its diseases. Wiley, New York
38 De Herder WW, Hazenberg MP, Pennock-Schröder AM, Hennemann G, Visser TJ 1986 Hydrolysis of iodothyronine glucuronides by obligately anaerobic bacteria isolated from human fecal flora. FEMS Microbiol Lett 35: 249-253
39 De Herder WW, Hazenberg MP, Pennock-Schröder AM, Hennemann G, Visser TJ 1986 Rapid and bacteria-dependent in vitro hydrolysis of iodothyronine conjugates by intestinal contents of humans and rats. Med Biol 64: 31-35
40 De Herder WW, Bonthuis F, Rutgers M, Hazenberg MP, Visser TJ 1988 Effects of inhibition of type I iodothyronine deiodinase and phenol sulfotransferase on the biliary clearance of triiodothyronine in rats. Endocrinology 122: 153-157
41 De Herder WW, Hazenberg MP, Pennock-Schröder AM, Oosterlaken AC, Rutgers M, Visser TJ 1989 On the enterohepatic cycle of triiodothyronine in rats; importance of the intestinal microflora. Life Sci 45: 849-856
42 De Herder WW, van der Hul RL, Bonthuis F, Jansen PLM, Oude Elferink RPJ, Visser TJ 1989 Pathways for the biliary excretion of T_3 conjugates. Ann Endocrinol 50: 98 (Abstract)
43 De Herder WW 1990 Enterohepatic circulation of triiodothyronine. Thesis, Erasmus University Rotterdam
44[a] DiStefano JJ, Yang M, Kaplan MM 1985 Optimized kinetics of reverse-triiodothyronine distribution and metabolism in the rat: dominance of large, slowly exchanging tissue pools for iodothyronines. Endocrinology 116: 446-456
44[b] DiStefano JJ 1986 Modeling approaches and models of the distribution and disposal of thyroid hor-

mones. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 39-76
45 DiStefano JJ, Sapin V 1987 Fecal and urinary excretion of six iodothyronines in the rat. Endocrinology 121: 1742-1750
46 DiStefano JJ 1988 Excretion, metabolism and enterohepatic circulation pathways and their role in overall thyroid hormone regulation in the rat. Am Zool 28: 373-387
47 DiStefano JJ, Feng D 1988 Comparative aspects of the distribution, metabolism and excretion of 6 iodothyronines in the rat. Endocrinology 123: 2514-2525
48 DiStefano JJ, Sternlicht M, Harris DR 1988 Rat enterohepatic circulation and intestinal distribution of enterally-infused thyroid hormones. Endocrinology 123: 2526-2539
49 Docter R, Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 233-254
50 Dowling RH 1972 The enterohepatic circulation. Gastroenterology 62: 122-135
51 Dutton GJ 1980 Glucuronidation of drugs and other compounds. CRC Press, Boca Raton, Florida
52 Eelkman Rooda SJ, van Loon MAC, Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. J Clin Invest 79: 1740-1748
53 Eelkman Rooda SJ, van Loon MAC, Bonthuis F, Heusdens FA, Kaptein E, Rutgers M 1988 Effects of iopanoic acid (IOP) on the metabolism of T_3 in rats. Ann Endocrinol 49: 182 (Abstract)
54 Eelkman Rooda SJ, Kaptein E, Rutgers M, Visser TJ 1989 Increased plasma 3,5,3'-triiodothyronine sulfate in rats with inhibited type I iodothyronine deiodinase activity, as measured by radioimmunoassay. Endocrinology 124: 740-745
55 Eelkman Rooda SJ, Kaptein E, Visser TJ 1989 Serum triiodothyronine sulfate in man measured by radioimmunoassay. J Clin Endocrinol Metab 61: 552-556
56 Eelkman Rooda SJ, Otten MH, van Loon MAC, Kaptein E, Visser TJ 1989 Metabolism of triiodothyronine in rat hepatocytes. Endocrinology 125: 2187-2197
57 Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocrine Rev 5: 151-184
58 Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240: 889-895
59 Eyssen H, Van Eldere J 1984 Metabolism of bile acids. In: Coates ME, Gustafsson BE (eds) The germ-free animal in biomedical research, Laboratory Animal Handbooks 9. Laboratory animals Ltd, London, p 291-316
60 Fauvert R, Roche J, Michel R, Thiéblemont P, Gruson M 1958 Mise en évidence, dans le plasma et la bile de l'homme, de l'ester sulfurique de la 3:5:3'triiodo-L-thyronine. Rev Franç Etudes Clin Biol 53: 587-593
61 Flock EV, Bollman JL 1955 The metabolism of thyroxine and triiodothyronine in the eviscerated rat. J Biol Chem 214: 709-721
62 Flock EV, Bollman JL, Grindlay JH, Orvis AL 1956 Metabolism of L-thyroxine and L-triiodothyronine in the absence of the liver. Am J Physiol 187: 407-414
63 Flock EV, Bolmann JL, Grindlay JH, McKenzie BF 1957 Metabolites of radioactive L-thyroxine and L-triiodothyronine. Endocrinology 61: 461-473
64 Flock EV, Bollman JL, Grindlay JH 1960 Conjugates of triiodothyronine and its metabolites. Endocrinology 67: 419-429
65 Flock EV, Bollman JL 1962 The effect of thiouracil on the metabolism of L-thyroxine. Biochem J 84: 621-626
66 Flock EV, Bollman JL, Stobie GHC 1962 Metabolic pathways of tetraiodothyroacetic acid, triiodothyroacetic acid, tetraiodothyropropionic acid and triiodothyropropionic acid. Biochem Pharmacol 11: 627-637
67 Flock EV, Bollman JL 1964 Effect of butyl 4-hydroxy-3,5-diiodobenzoate on the metabolism of L-thyroxine and related compounds. Endocrinology 75: 721-732
68 Flock EV, Bollman JL, Owen CA, Zollman PE 1965 Conjugation of thyroid hormones and analogs by the Gunn rat. Endocrinology 77: 303-314
69 Földes O, Langer P, Straussová K, Brozmanova H, Gschwendtová K 1982 Direct quantitative estimation of several iodothyronines in rat bile by radioimmunoassay and basal data on their biliary

excretion. Biochim Biophys Acta 716: 383-390
70 Galton VA, Pitt-Rivers R 1959 The identification of the acetic acid analogues of thyroxine and triiodothyronine in mammalian tissues. Biochem J 72: 319-321
71 Galton VA, Nisula BC 1972 The enterohepatic circulation of thyroxine. J Endocrinol 54: 187-193
72 Gavin LA, Livermore BM, Cavalieri RR, Hammond ME, Castle JN 1980 Serum concentration, metabolic clearance, and production rates of 3,5,3'-triiodothyroacetic acid in normal and athyreotic man. J Clin Endocrinol Metab 51: 529-534
73 Glazenburg EJ, Jekel-Halsema IMC, Scholtens E, Baars AJ, Mulder GJ 1983 Effects of variation in the dietary supply of cysteine and methionine on liver concentration of glutathione and "active sulfate" (PAPS) and serum levels of sulfate, cystine, methionine and taurine: relation to the metabolism of acetaminophen. J Nutr 113: 1363-1373
74 Goldstein JA, Taurog A 1968 Enhanced biliary excretion of thyroxine glucuronide in rats pretreated with benzpyrene. Biochem Pharmacol 17: 1049-1065
75 Green WL, Ingbar SH 1961 The peripheral metabolism of tri- and tetraiodothyroacetic acids in man. J Clin Endocrinol Metab 21: 1548-1565
76 Greer MA (ed) 1990 The Thyroid Gland. Raven Press, New York
77 Gregus Z, Klaassen CD 1986 Enterohepatic circulation of toxicants. In: Rozman R, Hänninen O (eds) Gastrointestinal Toxicology. Elsevier Science Publishers BV, Amsterdam, p 57-118
78 Hardeveld C van 1986 Effects of thyroid hormone on oxygen consumption, heat production, and energy economy. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 579-608
79 Hardy JJ, Thomas CL, Utiger RD 1986 Characteristics of thyroxine 5'-deiodinase activity in human liver. Am J Med Sci 292: 193-197
80 Hays MT 1988 Thyroid hormone and the gut. Endocrine Res 14: 203-224
81 Hays MT, Hsu L 1988 Equilibrium dialysis studies of plasma binding of thyroxine, triiodothyronine and their glucuronide and sulfate conjugates in human and cat plasma. Endocrine Res 14: 51-58
82 Hazenberg MP, de Herder WW, Visser TJ 1988 Hydrolysis of iodothyronine conjugates by intestinal bacteria. FEMS Microbiol Rev 54: 9-16
83 Hennemann G (ed) 1986 Thyroid Hormone Metabolism. Marcel Dekker, New York
84 Hennemann G 1986 Thyroid hormone deiodination in healthy man. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 277-295
85 Hennemann G, Docter R 1990 Plasma transport proteins and their role in tissue delivery of thyroid hormone. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 221-232
86 Hesch RD, Köhrle J 1986 Intracellular pathways of iodothyronine metabolism. In: Ingbar SH, Braverman LE (eds) The Thyroid. Lippincott, Philadelphia, p 154-200
87 Hill MJ 1986 Microbial metabolism in the digestive tract. CRC Press, Boca Raton, Florida
88 Hillier AP 1972 Transport of thyroxine glucuronide into bile. J Physiol 227: 195-200
89 Hommel E, Faber F, Kirkegaard C, Siersbaek-Nielsen K, Friis T 1985 Urinary excretion of unconjugated and conjugated 3,5-diiodothyronine. Horm Metab Res 17: 90-92
90 Irvine CHG 1969 Qualitative and quantitative aspects of thyroxine metabolism in sheep. Endocrinology 85: 622-673
91 Ishi H, Inada M, Tanaka K, Mashio Y, Naito K, Imura H 1981 Triiodothyronine generation from thyroxine in human thyroid: enhanced conversion in Graves' thyroid disease. J Clin Endocrinol Metab 52: 1211-1217
92 Jakoby WB, Sekura RD, Lyon ES, Marcus J, Wang JL 1980 In: Jakoby WB (ed) Enzymatic basis of detoxication. Academic Press, Orlando, p 199-228
93 Jansen PLM, Peters WHM, Lamers WH 1985 Hereditary chronic conjugated hyperbilirubinemia in mutant rats caused by defective hepatic anion transport. Hepatology 5: 573-579
94 Jansen PLM, Oude Elferink RPJ 1988 Hereditary hyperbilirubinemias: a molecular and mechanistic approach. Semin Liver Dis 8: 168-178
95 Jones SL, Van Middlesworth L 1960 Normal I^{131} L-thyroxine metabolism in the presence of potassium perchlorate and interrupted by propylthiouracil. Endocrinology 67: 855-861

96 Jorgensen EC 1978 Thyroid hormones and analogs II. Structure-activity relationships. In: Li CH (ed) Hormonal Proteins and Peptides, vol VI. Academic Press, New York, p 106-204
97 Kaiser CA, Seydoux J, Giacobino JP, Girardier L, Burger AG 1988 Increased plasma clearance rate of thyroxine despite decreased 5'-monodeiodination: Study with a peroxisome proliferator in the rat. Endocrinology 122: 1087-1093
98 Kaplan MM 1984 The role of thyroid hormone deiodination in the regulation of hypothalamo-pituitary function. Neuroendocrinology 38: 245-260
99 Kaplan MM 1986 Regulatory influences on iodothyronine deiodination in animal tissues. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 231-254
100 Kent TH, Fischer LJ, Marr R 1972 Glucuronidase activity in intestinal contents of rat and man and relationship to bacterial flora. PSBEM 140: 590-594
101 Kieffer JD, Larsen PR 1991 Photoaffinity labeling of rat type I iodothyronine deiodinase. Endocrinology 129: 1042-1048
102 Klaassen CD, Watkins JB 1984 Mechanisms of bile formation, hepatic uptake, and biliary excretion. Pharmacol Rev 36: 1-67
103 Kleiman de Pisarev DL, Sancovich HA, Ferramola de Sancovich AM 1989 Enhanced thyroxine metabolism in hexachlorobenzene-intoxicated rats. J Endocrinol Invest 12: 767-772
104 Köhrle J, Hesch RD 1984 Biochemical characteristics of iodothyronine monodeiodination by rat liver microsomes: The interaction between iodothyronine substrate analogs and the ligand binding site of the iodothyronine deiodinase resembles that of the TBPA-iodothyronine ligand binding. Horm Metabol Res 14 (Suppl): 42-55
105 Köhrle J, Auf'mkolk M, Rokos H, Hesch RD, Cody V 1986 Rat liver iodothyronine monodeiodinase. Evaluation of the iodothyronine ligand binding site. J Biol Chem 261: 11613-11622
106 Köhrle J, Brabant G, Hesch RD 1987 Metabolism of thyroid hormones. Hormone Res 26: 58-78
107 Köhrle J, Kaiser C, Rokos H, Hesch RD, Leonard JL 1987 Affinity labeling of the substrate binding subunit(s) of type I and II 5'-deiodinases. Ann Endocrinol 48: 132 (Abstract)
108 Krenning EP, Docter R 1986 Plasma membrane transport of thyroid hormone. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 107-132
109 Kuipers F, Havinga R, Bosschieter H, Toorop GP, Hindriks FR, Vonk RJ 1985 Enterohepatic circulation in the rat. Gastroenterology 88: 403-411
110 Lang S, Premachandra BN 1963 Propylthiouracil and hepatic clearance of thyroid hormones. Am J Physiol 204: 133-136
111 Langer P, Kokesová H, Michajlovskii N, Gschwendtová K, Hrcka R, Bukovská M 1977 Rapid disappearance of loading doses of thyroxine from the blood and their excretion by the bile in rats. Acta Endocrinol 85: 531-540
112 Langer P, Földes O, Straussová K, Gschwendtová K 1982 Preliminary observations on the absorption of biliary iodothyronines from the intestine in vivo in rats. Endocrinol Exp 16: 117-127
113 Langer P, Moravec R, Ohradka B, Földes O 1988 Iodothyronines in human bile. Endocrinol Exp 22: 35-39
114 Larsen PR, Atkinson AJ, Wellman HN, Goldsmith RE 1970 The effect of diphenylhydantoin on thyroxine metabolism in man. J Clin Invest 49: 1266-1279
115 Larson FC, Albright EC 1958 Distribution of 3:5:3'-triiodothyroacetic acid in the rat. Endocrinology 63: 183-190
116 Leonard JL, Visser TJ 1984 Selective modification of the active center of renal iodothyronine 5'-deiodinase by iodoacetate. Biochim Biophys Acta 787: 122-130
117 Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 189-229
118 Leonard JL 1990 Identification and structure analysis of iodothyronine deiodinase. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 285-306
119 Matsui M, Hakozaki M 1979 Discontinuous variation in hepatic uridine diphosphate glucuronyltransferase toward androsterone in Wistar rats. Biochem Pharmacol 28: 411-415
120 Miller LJ, Gorman CA, Go VW 1978 Gut-thyroid interrelationships. Gastroenterology 75: 901-911

121 Mol JA, Docter R, Hennemann G, Visser TJ 1984 Modification of rat liver iodothyronine 5'-deiodinase activity with diethylpyrocarbonate and rose bengal; evidence for an active site histidine residue. Biochem Biophys Res Commun 120: 28-36
122 Mol JA, Docter R, Kaptein E, Jansen G, Hennemann G, Visser TJ 1984 Inactivation and affinity-labeling of rat liver iodothyronine deiodinase with N-Bromoacetyl-3,3',5'-triiodothyronine. Biochem Biophys Res Commun 124: 475-483
123 Mol JA, Visser TJ 1985 Rapid and selective inner ring deiodination of T_4 sulfate by rat liver deiodinase. Endocrinology 117: 8-12
124 Mol JA, van den Berg TP, Visser TJ 1988 Partial purification of the microsomal rat liver iodothyronine deiodinase I. Solubilization and ion-exchange chromatography. Mol Cell Endocrinol 55: 149-157
125 Mol JA, van den Berg TP, Visser TJ 1988 Partial purification of the microsomal rat liver iodothyronine deiodinase II. Affinity chromatography. Mol Cell Endocrinol 55: 159-166
126 Morreale de Escobar G, Escobar del Rey F 1967 Extrathyroid effects of some antithyroid drugs and their metabolic consequences. Recent Prog Horm Res 23: 87-106
127 Mulder GJ 1981 Sulfation of drugs and related compounds. CRC Press, Boca Raton, Florida
128 Mulder GJ 1986 Kinetics of sulfation in the rat in vivo and in perfused rat liver. Federation Proc 45: 2229-2234
129 Myant NB 1956 Biliary excretion of thyroxine in humans. Clin Sci 15: 227-237
130 Myant NB 1956 Enterohepatic circulation of thyroxine in humans. Clin Sci 15: 551-555
131 Myant NB 1957 Faecal clearance rate of endogenous thyroid hormone in rats. J Physiol 136: 198-202
132 Myant NB 1966 Excretion of glucuronide of thyroxine in cat bile. Biochem J 99: 341-346
133 Nakamura Y, Chopra IJ, Solomon DH 1978 An assessment of the concentration of acetic acid and propionic acid derivatives of 3,5,3'-triiodothyronine in human serum. J Clin Endocrinol Metab 46: 91-97
134 Nicholls RG, Roy AB 1971 Arylsulfatases. In: Boyer PD (ed) The enzymes, volume V. Academic Pres, New York, p 21-41
135 Nikodem VM, Petty KJ, Mitsuhashi T, Desvergne B 1990 Structure and mechanism of action of thyroid hormone receptors. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 307-322
136 Oppenheimer JH, Samuels HH 1983 Molecular basis of thyroid hormone action. Academic press, New York
137 Oppenheimer JH, Schwartz HL 1986 Thyroid hormone action at the nuclear level. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 383-415
138 Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NCW, Freake HC 1987 Advances in our understanding of thyroid hormone action at the cellular level. Endocrine Rev 8: 288-308
139 Otten MH, Mol JA, Visser TJ 1983 Sulfation preceeding deiodination of iodothyronines in rat hepatocytes. Science 221: 81-83
140 Otten MH, Hennemann G, Docter R, Visser TJ 1984 Metabolism of 3,3'-diiodothyronine in rat hepatocytes: interaction of sulfation with deiodination. Endocrinology 115: 887-894
141 Pardridge WM, Landaw EM 1987 Steady state model of 3,5,3'-triiodothyronine transport in liver predicts high cellular exchangeable hormone concentration relative to in vitro free hormone concentration. Endocrinology 120: 1059-1068
142 Pardridge WM 1987 Plasma protein-mediated transport of steroid and thyroid hormones. Am J Physiol 252: E157-E164
143 Pinchera A, MacGillivray MH, Crawford JD, Freeman AG 1965 Thyroid refractoriness in an athyreotic cretin fed soybean formula. New Engl J Med 1273: 83-87
144 Pittman CS, Buck MV, Chambers JB 1972 Urinary metabolites of ^{14}C-labeled thyroxine in man. J Clin Invest 51: 1759-1766
145 Pittman CS, Shimuzu T, Burger A, Chambers JB 1980 The nondeiodinative pathways of thyroxine

metabolism: 3,5,3',5'-tetraiodothyroacetic acid turnover in normal and fasting subjects. J Clin Endocrinol Metab 50: 712-716
146 Pontecorvi A, Robbins J 1989 The plasma membrane and thyroid hormone entry into cells. Trends Endocrinol Metab 1: 90-94
147 Ramsden DB, Crossley DN 1986 Serum concentrations of 3,5,3',5'-tetraiodothyroacetate (T_4A) in subjects with hypo-, hyper- and euthyroidism. Acta Endocrinol 112: 1912-1916
148 Renwick AG 1986 Gut bacteria and the enterohepatic circulation of foreign compounds. In: Hill MJ (ed) Microbial metabolism in the digestive tract. CRC Press, Boca Raton, Florida, p 135-153
149 Roche J, Michel R, Tata J 1953 Sur la présence dans le sang de glycuroconjugués de la triiodothyronine et de la thyroxine après ligature du canal cholédoque. CR Soc Biol 147: 1574-1576
150 Roche J, Michel R, Tata J 1953 Sur l'excrétion biliaire et la glycuroconjugaison de la 3:5:3'-triiodothyronine. Biochim Biophys Acta 11: 543-547
151 Roche J, Michel O, Michel R, Tata J 1954 Sur l'élimination biliaire de la triiodothyronine et de la thyroxine et sur leur glycuroconjugaison hépatique. Biochim Biophys Acta 13: 471-479
152 Roche J, Michel R 1955 Nature, biosynthesis and metabolism of thyroid hormones. Physiol Rev 35: 583-610
153 Roche J, Michel R, Jouan P, Wolf W 1956 The recovery of 3:5:3'-triiodothyroacetic acid and 3:3'-diiodothyronine from rat kidney after injection of 3:5:3'-triiodothyronine. Endocrinology 59: 425-432
154 Roche J, Michel R, Closon J, Michel O 1958 Sur la sulfoconjugaison de l'acide 3:5:3'-triiodothyroacétique (TA_3), produit de dégradation de la 3:5:3'-triiodo-L-thyronine (T_3). Bull Soc Chim Biol 40: 2125-2142
155 Roche J, Michel R, Closon J, Michel O 1959 Sur la sulfoconjugaison hépatique de la 3,5,3'-triiodothyronine et la présence d'un ester sulfurique de cette hormone dans la bile et la plasma. Biochim Biophys Acta 33: 461-468
156 Roche J, Michel R, Nunez J, Jaquemin C 1959 On the metabolism of 3,3'-diiodothyronine and 3,3',5'-triiodothyronine. Endocrinology 65: 402-407
157 Roche J, Michel R 1960 On the peripheral metabolism of thyroid hormones. Ann N Y Acad Sci 86: 454-478
158 Roche J, Michel R, Closon J, Michel O 1960 Sur le métabolisme du sulfoconjugué de la 3,5,3'-triiodothyronine chez le rat. Biochim Biophys Acta 38: 325-332
159 Roche J, Turpin R, Michel R, Salabè G, Cruveiller J 1961 Métabolisme rénal des hormones thyroïdiennes. II. Etude des métabolites de la 3:5:3'-triiodo-l-thyronine et de la L-thyroxine prenant naissance dans le rein de chien. CR Soc Biol 155: 2246-2249
160 Robbins J, Bartalena L 1986 Plasma transport of thyroid hormones. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 3-38
161 Roy AB 1971 The hydrolysis of sulfate esters. In: Boyer PD (ed) The enzymes volume V. Academic Pres, New York, p 1-9
162 Rutgers M, Bonthuis F, de Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80: 758-762
163 Rutgers M, Heusdens FA, Bonthuis F, de Herder WW, Hazenberg MP, Visser TJ 1989 Enterohepatic circulation of triiodothyronine (T_3) in rats: Importance of the microflora for the liberation and reabsorption of T_3 from biliary T_3 conjugates. Endocrinology 125: 2822-2830
164 Rutgers M, Heusdens FA, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. I: Deiodination of TA_3 and TA_3 sulfate by microsomes. Endocrinology 125: 424-432
165 Rutgers M, Heusdens FA, Bonthuis F, Visser TJ 1989 Metabolism of triiodothyroacetic acid (TA_3) in rat liver. II: Deiodination and conjugation of TA_3 by rat hepatocytes and in rats in vivo. Endocrinology 125: 433-443
166 Rutgers M, Pigmans IGAJ, Bonthuis F, Docter R, Visser TJ 1989 Effects of propylthiouracil on the biliary clearance of thyroxine (T_4) in rats: Decreased excretion of 3,5,3'-triiodothyronine glucuronide and increased excretion of 3,3',5'-triiodothyronine glucuronide and T_4 sulfate. Endocrinology 125: 2175-2186

167 Rutgers M, Heusdens FA, Bonthuis F, Eelkman Rooda SJ, Visser TJ 1990 Identification of 3,3'-diiodothyroacetic acid sulfate: a major metabolite of 3,3',5-triiodothyronine in propylthiouracil-treated rats. Endocrinology 127: 1617-1624
168 Rutgers M, Heusdens FA, Visser TJ 1991 Deiodination of iodothyronine sulfamates by the type I deiodinase of rat liver. Endocrinology 129: 1375-1381
169 Sakane S, Chopra IJ 1990 Isolation of a hepatic iodothyronine 5'-monodeiodinase by nondenaturating agarose gel electrophoresis. Endocrinology 127: 2709-2715
170 Samuels HH, Forman BM, Horowitz ZD, Ye ZS 1988 Regulation of gene expression by thyroid hormone. J Clin Invest 81: 957-967
171 Sato K, Robbins J 1981 Thyroid hormone metabolism in primary cultured rat hepatocytes. Effects of glucose, glucagon, and insulin. J Clin Invest 68: 475-483
172 Schoenmakers CHH, Pigmans IGAJ, Hawkins HC, Freedman RB, Visser TJ 1989 Rat liver type I deiodinase is not identical to protein disulfide isomerase. Biochem Biophys Res Commun 162: 857-867
173 Sekura RD, Sato K, Cahnmann HJ, Robbins J, Jakoby WB 1981 Sulfate transfer to thyroid hormones and their analogs by hepatic aryl sulfotransferases. Endocrinology 108: 454-456
174 Shulkin BL, Bolger MB, Utiger RD 1988 Thyroid hormone analog inhibition of hepatic 5'-iodothyronine deiodinase activity. J Endocrinol Invest 11: 657-661
175 Silva JE, Gordon MB, Crantz FR, Leonard JL, Larsen PR 1984 Qualitative and quantitative differences in the pathways of extrathyroidal triiodothyronine generation between euthyroid and hypothyroid rats. J Clin Invest 73: 898-907
176 Silva JE, Larsen PR 1986 Regulation of thyroid hormone expression at the prereceptor and receptor levels. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York, p 441-488
177 Takai NA, Rapoport B, Yamamoto M 1980 Biliary excretion of iodothyronines in rats as determined by high pressure liquid chromatography: effect of starvation. Endocrinology 107: 176-182
178 Taurog A 1955 Conjugation and excretion of the hormone. In: Brookhaven Symposia in Biology, 7: The Thyroid, p 111-136
179 Taurog A 1986 Hormone synthesis: thyroid iodine metabolism. In: Ingbar SH, Braverman LE (eds) The Thyroid. Lippincott, Philadelphia, p 53-97
180 Tephly T, Green M, Puig J, Irshaid Y 1988 Endogenous substrates for UDP-glucuronosyltransferases. Xenobiotica 18: 1201-1210
181 Tephly TR, Burchell B 1990 UDP-glucuronosyltransferases: a family of detoxifying enzymes. Trends Pharmacol Sci 11: 276-279
182 Tomita K, Lardy HA, Larson FC, Albright EC 1957 Enzymatic conversion of thyroxine to tetraiodothyroacetic acid and triiodothyronine to triiodothyroacetic acid. J Biol Chem 224: 387-397
183 Tomita K, Lardy HA 1960 Enzymic conversion of iodinated thyronines to iodinated thyroacetic acids. J Biol Chem 235: 3292-3297
184 Van Doorn J, van der Heide D, Roelfsema F 1983 Sources and quantity of 3,5,3'-triiodothyronine in several tissues of the rat. J Clin Invest 72: 1778-1792
185 Van Doorn J, Roelfsema F, van der Heide D 1983 The effect of propylthiouracil and methimazole on the peripheral conversion of thyroxine to 3,5,3'-triiodothyronine in athyreotic thyroxine maintained rats. Acta Endocrinol 103: 509-520
186 Van Eldere J, Parmentier G, Robben J, Eyssen H 1987 Influence of an estrone-desulfating intestinal flora on the enterohepatic circulation of estrone-sulfate in rats. J Steroid Biochem 26: 235-239
187 Van der Heyden JTM, Docter R, van Toor H, Wilson JHP, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251: E156-E163
188 Vanotti A 1959 Hepatic regulation of thyroxine metabolism. Ciba Foundation Colloquia on Endocrinology 10: 215-219
189 Visser TJ, Fekkes D, Docter D, Hennemann G 1979 Kinetics of enzymatic reductive deiodination of iodothyronines. Effect of pH. Biochem J 179: 489-495

190 Visser TJ, Mol JA, Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112: 1547-1549
191 Visser TJ, Docter R, Krenning EP, Hennemann G 1986 Regulation of thyroid hormone bioactivity. J Endocrinol Invest 9 (Suppl 4): 17-26
192 Visser TJ 1988 Role of glutathione and other thiols in thyroid hormone metabolism. In: Dolphin D, Poulson R, Avramovic O (eds) Glutathione: Chemical, Biochemical, and Medical Aspects, part B. John Wiley & Sons, New York, p 571-611
193 Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RJB, Van der Molen HJ (eds) Hormones and Their Action, Part I. Elsevier, Amsterdam, p 81-103
194 Visser TJ, Kaptein E, Terpstra OT, Krenning EP 1988 Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 67: 17-24
195 Visser TJ, Rutgers M, de Herder WW, Eelkman Rooda SJ, Hazenberg MP 1988 Hepatic metabolism, biliary clearance and enterohepatic circulation of thyroid hormone. Acta Med Austriaca 15 (Suppl 1): 14-17
196 Visser TJ 1990 The role of glutathione in the enzymatic deiodination of thyroid hormone. In: Viñã J (ed) Glutathione: Metabolism and Physiological Functions. CRC Press, Boca Raton, p 317-333
197 Visser TJ 1990 Importance of deiodination and conjugation in the hepatic metabolism of thyroid hormone. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, p 285-306
198 Visser TJ, van Buuren JCJ, Rutgers M, Eelkman Rooda SJ, de Herder WW 1990 The role of sulfation in thyroid hormone metabolism. Trends Endocrinol Metab 1: 211-218
199 Visser TJ 1991 Recent advances in the characterization of type I iodothyronine deiodinase. In: Gordon A, Gross J, Hennemann G (eds) Progress in Thyroid Research. AA Balkema, Rotterdam, The Netherlands, p 27-31
200 Visser TJ, Kaptein E, Harper ES 1991 Differential expression and ciprofibrate induction of hepatic UDP-glucuronyltransferase for thyroxine and triiodothyronine in Fischer rats. Biochem J Pharmacol 42: 444-446
201 Wakabayashi M 1970 ß-Glucuronidases and metabolic hydrolysis. In: Fishman WH (ed) Metabolic Conjugation and Metabolic Hydrolysis, volume II. Academic Press, New York, p 519-602
202 Watkins JB, Klaassen CD 1983 Chemically-induced alteration of UDP-glucuronic acid concentration in rat liver. Drug Metab Dispos 11: 37-40
203 Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM 1986 The c-erb-A gene encodes a thyroid hormone receptor. Nature 324: 641-646
204 Weitering JG, Krijgsheld KR, Mulder GJ 1979 The availability of inorganic sulphate as a rate limiting factor in the sulphate conjugation or xenobiotics in the rat. Biochem Pharmacol 28: 757-762
205 Yoshida K, Sakurada T, Kitaoka H, Fukazawa H, Kaise N, Kaise K, Yamamoto M, Suzuki M, Saito S, Yoshinaga K, Kimura S, Yamanaka M 1982 Monodeiodination of thyroxine to 3,3',5'-triiodothyronine and 3,3',5-triiodothyronine in human kidney homogenate. Folia Endocrinol Jpn 58: 199-209
206 Young WF, Gorman CA, Weinshilboum RM 1988 Triiodothyronine: A substrate for the thermostable and thermolabile forms of human phenol sulfotransferase. Endocrinology 122: 1816-1824

SUMMARY

Thyroid hormone is of vital importance for the development and energy metabolism of vertebrates. The thyroid gland produces all circulating thyroxine (T_4) which is a prohormone since it lacks hormonal activity. Only 20 % of the bioactive hormone 3,3',5-triiodothyronine (T_3) is of thyroidal origin; most T_3 is generated outside the thyroid by outer ring deiodination (ORD) of T_4. Inner ring deiodination (IRD) of T_4 results in the inactive 3,3',5'-triiodothyronine (rT_3). Both ORD of rT_3 and IRD of T_3 result in formation of 3,3'-diiodothyronine (3,3'-T_2). The key enzyme in the activation of T_4 as well as the inactivation of T_3 is the type I deiodinase, catalyzing both ORD and IRD.

Chapter I describes, after a general introduction, the different pathways in the iodothyronine metabolism of which deiodination and conjugation are the most important. Deiodination in this thesis concerns primarily the conversion by the type I deiodinase of rat liver, which is important for production of plasma T_3 and clearance of plasma rT_3 in healthy individuals. Sulfation of iodothyronines stimulates their deiodinative degradation, while conjugation with glucuronic acid is a reversible process representing a first step in the enterohepatic circulation of T_4 and T_3. In addition, iodothyronines are metabolized by oxidative deamination and decarboxylation yielding the acetic acid side chain analogs. These iodothyroacetic acids are further degraded also by deiodination and conjugation.

In Chapter II the substrate behavior of synthetic N-sulfonated iodothyronines (T_iNS) for the type I deiodinase was compared with that of the naturally occuring 4'-O-sulfonated iodothyronines (T_iS). These latter sulfates are deiodinated 40-200 times more efficiently than the native iodothyronines. In contrast to T_4S, which is converted only by IRD, T_4NS underwent both IRD and ORD, similar to T_4 but much more rapidly. The type I deiodination efficiencies of T_4NS IRD and ORD, T_3NS IRD and 3,3'-T_2NS ORD are 4-17 fold higher than corresponding iodothyronines, mainly due to decreases in apparent K_m values. The less dramatic effects of N-sulfonation of iodothyronines compared with 4'-O-sulfonation indicates the importance of the site of sulfonation for efficient type I deiodination.

In vivo, we examined the effect of the type I deiodinase inhibitor 6-propyl-2-thiouracil (PTU) on plasma T_3S levels (Chapter III). Our studies clearly show that successive sulfation and deiodination of T_3 is a significant pathway in the rat. PTU treatment of rats receiving iv [^{125}I]T_3 or [^{125}I]T_3S effectively reduced plasma radioiodide levels, decreased the clearance of injected T_3S by 81 %, did not affect plasma T_3 clearance, but increased plasma T_3S by 4-fold in T_3-injected rats. Thus, if the type I deiodination is inhibited, the intermediate T_3S accumulates in plasma (Chapter III; Ref. 53), but also in bile as demonstrated in parallel studies (see Ref. 40) .

In Chapter IV the type I deiodination of 3,3',5-triiodothyroacetic acid (TA_3) and 3,3'-diiodothyroacetic acid (3,3'-TA_2) was investigated in rat liver microsomes. Their

sulfated conjugates TA_3S and 3,3'-TA_2S were also tested as a substrate. We demonstrated that (1) TA_3 and 3,3'-TA_2 are better substrates than T_3 and 3,3'-T_2 for the type I deiodinase of rat liver, (2) the IRD of TA_3 and ORD of 3,3'-TA_2 are markedly accelerated by sulfation similar to the parent iodothyronines, and (3) TA_3S is very rapidly deiodinated, being the best known substrate for IRD due to its very high affinity for the type I deiodinase (apparent K_m value: 4 nM).

This latter finding prompted us to perform detailed studies of the hepatic metabolism of TA_3, a naturally occurring T_3 metabolite, using isolated rat hepatocytes *in vitro* as reported in Chapter V. Normally, TA_3 glucuronide (TA_3G) and I^- were the main TA_3 products from added TA_3. PTU nullified I^- production while TA_3S increased from 2 to 14 %, indicating that part of TA_3 is normally metabolized via successive sulfation and deiodination like T_3. In sulfate-depleted cells direct deiodination of TA_3 clearly occurred, since the glucuronide of 3,3'-TA_2 appeared. If both sulfation and deiodination were inhibited, TA_3 was efficiently cleared by glucuronidation, unlike T_3 which greatly depends on sulfation for its metabolic degradation.

The additional *in vivo* experiments (Chapter V) showed that PTU treatment of [^{125}I]TA_3-injected rats reduced plasma radioiodide 6-fold, increased plasma sulfate conjugates nearly 3-fold, did not affect plasma TA_3 clearance, and stimulated the total biliary-excreted radioactivity 1.5 times. These PTU-induced changes are similar to those seen in T_3-injected animals. Notably, the biliary clearance rate of TA_3 is 2.5 times higher than that of T_3, irrespective of PTU treatment. Therefore, TA_3 is effectively metabolized in rat liver by glucuronidation and subsequent rapid biliary excretion of TA_3G. Some TA_3 is also deiodinated by the type I deiodinase, either directly or after prior sulfation.

Chapter VI deals with the identification of the sulfate conjugate of 3,3'-TA_2 (3,3'-TA_2S) which was encountered in plasma of PTU-treated, T_3-injected rats, but not in controls. The marked accumulation of plasma 3,3'-TA_2S is explained by its reduced deiodinative clearance induced by PTU. How much 3,3'-TA_2S contributes to the T_3 metabolism or how it is exactly generated from T_3 remains to be established. However, relatively high plasma 3,3'-TA_2S levels even in unanesthetized animals underscore the physiological relevance of this T_3 metabolite.

Since little was known about the formation of T_4 sulfate (T_4S) *in vivo*, we examined the effects of PTU on the excretion of T_4G, T_4S, T_3G and rT_3G in bile, 17-18 h after iv [^{125}I]T_4 injection (Chapter VII). PTU treatment increased plasma T_4 retention by 50 %, reduced urinary radioiodide by 74 %, increased fecal radioactivity by 47 %, and doubled the total biliary-excreted radioactivity without affecting the glucuronidative T_4 clearance. Continuous collection of bile for 8 h (between 0-8 h or 18-26 h after T_4 administration, demonstrated that in control rats the excretion rate of T_3G was lower and that of rT_3G higher, likely due to prolonged experimental stress. This limits the use of the biliary T_3G/rT_3G ratio as a sensitive indicator of type I deiodinase inhibition. However,

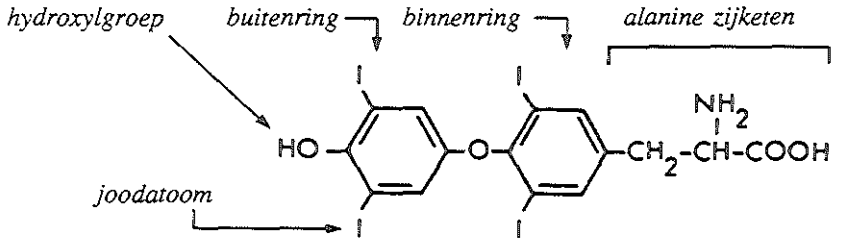
regardless of the period of bile collection, PTU induced a >23-fold decrease in the T_3G/rT_3G ratio and a 5-fold increase in T_4S excretion, which clearly proves that T_4 undergoes significant sulfation in rats *in vivo*. Thus, biliary excretion of T_4S is enhanced if its deiodination is inhibited.

In Chapter VIII we investigated the fate of T_3G, being the major T_3 metabolite excreted in bile, and its role in a putative enterohepatic circulation (EHC) of T_3 in rats. Intravenous injection of $[^{125}I]T_3G$ resulted in an almost quantitative excretion of T_3G in bile within 1 h and collected feces consisted of >90 % of T_3, indicating effective intestinal hydrolysis in conventional (CV) rats. However, in intestine-decontaminated (ID) animals most fecal radioactivity still consisted of T_3G, since these rats are largely incapable of intestinal deconjugation because of the lack of the necessary microflora. Within 4 to 7 hours after iv injection of $[^{125}I]T_3G$, which was cleared very rapidly from the circulation, plasma radioactivity reappeared only in CV rats but not in ID rats, and consisted of mainly T_3 and I^- (no T_3G). In these CV rats, peak plasma T_3 was 3 and 12 times higher than that in T_3G-injected, ID and bile-diverted rats, respectively. This demonstrates that a significant EHC of T_3 occurs in normal rats via T_3G, in which bacterial hydrolysis of the biliary-excreted T_3 conjugate is a prerequisite for the reabsorption of T_3.

Chapter IX discusses the studies described in Chapters II-VIII, including relevent data published elsewhere, linking the pathways in iodothyronine metabolism. The efficacy and specificity of the type I deiodination by rat liver microsomes of various synthetic and natural occurring iodothyronine derivatives is summarized (IX.1). Our structure-activity studies confirm that the presence of a negatively charged 4'-substituent and/or a negatively charged side chain stimulate the type I deiodination. Part of the sections on the metabolism of thyroid hormones in isolated rat hepatocytes *in vitro* (IX.2) and in rats *in vivo* (IX.3) are focused on the comparison of T_3 and TA_3. Furthermore, we present a scheme of the multiple metabolic routes of T_4 occuring *in vivo*. The significance of an EHC of thyroid hormones in rats and humans is finally discussed (IX.4).

VERKLARENDE WOORDENLIJST

cel	- microscopisch kleine, elementaire bouwsteen van weefsels
conjugatie	- chemische koppeling
conjugaat	- een met sulfaat of glucuronide gekoppelde verbinding
dejodase	- enzym dat joodatomen afsplitst
dejodering	- afsplitsing van joodatomen
derivaat	- afgeleide verbinding
enzym	- eiwit dat de snelheid van een biochemische reactie verhoogd
G	- glucuronide (een suikerachtige verbinding)
HPLC	- high pressure liquid chromatography (hoge druk vloeistof chromatografie): methode om een mengsel van stoffen te scheiden
jodide (I^-)	- afgesplitst joodatoom met een negatieve lading
metaboliet	- omzettingsprodukt
metabolisme	- stofwisseling: de verwerking van voedingsstoffen, hormonen, medicijnen e.d. in het lichaam door middel van opname, omzetting en uitscheiding
molekuul	- chemische verbinding opgebouwd uit atomen
prohormoon	- stof waaruit door omzetting een werkzaam hormoon ontstaat
PTU	- propylthiouracil: stof die de werking van het dejodase enzym remt
S	- sulfaat (een zwafel-zuurstof verbinding)
stofwisseling	- zie metabolisme
T_3	- tri-jodothyronine = biologisch actief schildklierhormoon
T_4	- tetra-jodothyronine = thyroxine = biologisch inactief schildklierhormoon (prohormoon)
in vitro	- kunstmatig, buiten het lichaam
in vivo	- in een levend organisme



Structuur Thyroxine (T_4)

SAMENVATTING VOOR NIET-VAKGENOTEN

Voor diegenen die niet of weinig bekend zijn met biochemisch onderzoek, volgt hieronder een toelichting op de in dit proefschrift beschreven studies over de vorming en afbraak van schildklierhormonen.

INLEIDING

De schildklier ligt in de hals aan weerszijden van de luchtpijp net onder het strottehoofd en produceert het schildklierhormoon thyroxine, dat wordt afgegeven aan de bloedbaan en zich zo verspreidt door het lichaam. Schildklierhormoon is van vitaal belang voor de groei en ontwikkeling van elk individu, en bepaalt de basale stofwisselingssnelheid, dat wil zeggen het energieverbruik van onze lichaamscellen.

In Nederland lijden circa 500.000 mensen aan een bepaalde vorm van schildklier aandoening, veelal veroorzaakt door een ontregelde hormoonproduktie. Indirect kunnen ook 'niet-schildklier ziekten' en het gebruik van bepaalde medicijnen de schildklierfunctie verstoren.

Het thyroxine-molekuul (chemische naam: tetra-jodothyronine, afgekort T_4) bestaat uit een thyronine skelet plus 4 joodatomen (zie Structuur op blz. 132). Dit T_4 is zelf nauwelijks biologisch actief en wordt alleen door de schildklier gemaakt. Daarentegen wordt het meeste van het biologisch werkzame schildklierhormoon tri-jodothyronine (afgekort T_3) uit T_4 gevormd in andere weefsels dan de schildklier. T_4 wordt daarom beschouwd als een prohormoon. In gezonde mensen vindt deze omzetting van T_4 naar T_3 vooral plaats in de lever, die rijk is aan het dejodase enzym. Dit dejodase splitst een joodatoom van de buitenring van T_4 af, waarbij jodide (I^-) vrijkomt; vervolgens wordt het meeste van het gevormde T_3 aan de bloedbaan afgegeven.

In het bloed is meer dan 99 % van T_4 en T_3 gebonden aan eiwitten. Deze schildklierhormoon-bindende eiwitten zijn van essentieel belang voor het normale transport van de schildklierhormonen naar lichaamsweefsels die T_4 of T_3 nodig hebben. De weefselcellen kunnen de schildklierhormonen selectief opnemen uit het bloed. Door binding van het werkzame T_3 aan zogenaamde receptoren in de kern van de cel, wordt een reeks van biochemische processen gestart, waaronder synthese van eiwitten.

Het in mijn proefschrift beschreven onderzoek betreft biochemische processen buiten de schildklier, die te maken hebben met de vorming en afbraak van schildklierhormoon: het schildklierhormoon-metabolisme. Een beter inzicht in de samenhang van deze metabole processen zal bijdragen aan het begrip hoe bepaalde verstoringen in de schildklierhormoon-huishouding tijdens ziekte worden veroorzaakt.

In het schildklierhormoon-metabolisme zijn dejodering en conjugatie het belangrijkste.

1. Dejodering. Naast de vorming van het werkzame hormoon T_3 uit T_4 (= activatie) is het dejodase enzym ook betrokken bij de afbraak van schildklierhormoon. T_4 kan voorgoed onwerkzaam worden gemaakt (= inactivatie) door een joodatoom van de binnenring af te halen, zodat het "omgekeerde" T_3 ontstaat (reverse T_3, afgekort rT_3). Evenzo kan T_3 worden geinactiveerd door dejodering van de binnenring.
2. Conjugatie. Schildklierhormonen kunnen via de hydroxyl (OH) groep van de buitenring worden gekoppeld met een sulfaat ("S") of suikerachtige groep (glucuronide,"G"). Het gevormde conjugaat wordt aangeduid met b.v. T_3S of T_3G. Door deze zogeheten sulfatering of glucuronidering van het hormoon onstaat een goed in water oplosbare verbinding. Hierdoor kunnen de nieren en de lever het veranderde hormoon beter in de urine, respectievelijk de gal (faeces) uitscheiden. Deze sulfatering en glucuronidering vinden vooral plaats in de lever, maar ook in de nieren met behulp van complexe enzymsystemen, die behoren tot het normale afbraak en ontgiftingssysteem van zowel lichaamseigen als lichaamsvreemde stoffen (medicijnen en gifstoffen).
3. Oxidatieve deaminering. Via zogenaamde "oxidatieve deaminering en decarboxylering" kunnen schildklierhormonen ook worden afgebroken, maar de hoeveelheid is beduidend minder in vergelijking met dejodering of conjugatie. Door oxidatieve verwijdering van de stikstofgroep (NH_2) en afsplitsing van kooldioxide (CO_2) wordt de alanine zijketen korter en krijgt een "zuur" karakter (zie Structuur op blz. 132). De ontstane verbinding noemen we jodothyroazijnzuur en korten we af met TA_n, waar n het aantal joodatomen aangeeft.

Al deze verschillende biochemische omzettingen leiden tot een grote verscheidenheid aan schildklierhormoon metabolieten.

EXPERIMENTELE METHODIEKEN

Het metabolisme van schildklierhormoon is onderzocht in de rat als proefdier, waarbij meerdere experimentele modelsystemen zijn gebruikt, die verschillen in complexiteit van metabole routes:
1) Met behulp van homogenaten van rattelevers die werkzaam dejodase bevatten, is in de reageerbuis de dejoderingssnelheid bestudeerd van schildklierhormoon en afgeleide verbindingen (derivaten). (Enzym kinetiek in hoofdstukken II en IV.)
2) Intacte cellen, geisoleerd uit de rattelever, kunnen kunstmatig in leven worden gehouden in schaaltjes met speciaal voedingsmedium ("celkweek"). In zo'n *in vitro* systeem is de samenhang onderzocht tussen dejodering en conjugatie: hoe groot is de bijdrage van deze omzettingen aan de totale afbraak? Door de samenstelling van het medium te manipuleren kunnen metabole routes selectief worden uitgeschakeld. Toevoeging van PTU remt het dejodase, terwijl sulfaatconjugatie wordt voorkomen door geen sulfaat in het medium op te lossen (Hoofdstuk IV).
3) Metabolisme vindt ook plaats in andere organen dan de lever. We hebben daarom in ratten onderzocht, welke metabolieten van schildklierhormoon in de gal of het bloed

terechtkomen. Bloed is afgenomen via de staartader en gal is afgetapt uit een tevoren geplaatste galdrain, alles onder algehele verdoving. Dit zogeheten *in vivo* onderzoek staat beschreven in Hoofdstukken III, V-VIII.

De joodatomen van de buitenring van schildklierhormoon kunnen radioactief worden gemerkt met het ^{125}I isotoop. Een dergelijk ^{125}I gemerkt molekuul kan met het dejodase enzym in een reageerbuis worden geïncubeerd, of in de bloedbaan van een rat worden gespoten. Vervolgens kan de produktie van het afgesplitste "vrije" $^{125}I^-$ relatief eenvoudig worden gemeten. Kwantificering van de andere ^{125}I-gemerkte metabolieten vereist een goede scheidingstechniek. Met behulp van een moderne analytisch-chemische techniek, de zogenaamde 'hoge druk vloeistof chromatografie' (afgekort HPLC) in combinatie met een radioactiviteitsmeter, is het mogelijk om complexe monsters te scheiden in de verschillende individuele componenten. Om ook bloed, gal, urine en faeces met HPLC te analyseren moeten deze monsters worden voorbewerkt, zodat storende eiwitten en vetten worden verwijderd. Ik heb specifieke HPLC-methoden ontwikkeld of aangepast om de verschillende schildklierhormoon-derivaten te kunnen identificeren. Voor illustraties van zogeheten chromatogrammen, waarin elke "piek" een specifieke verbinding voorstelt, verwijs ik naar de Figuren 1 in de Hoofdstukken V, VII en VIII.

RESULTATEN

Het dejodase vervult een belangrijke dubbelrol in het metabolisme van T_4 en T_3, namelijk activatie tegenover inactivatie. Het is daarom van fundamenteel belang om de biochemische werking van dit enzym beter te karakteriseren.

De grootte en (electrische) lading van het schildklierhormoon-molekuul kunnen chemisch worden veranderd, zodat geconjugeerde en azijnzure varianten ontstaan die ook van nature voorkomen in bijvoorbeeld de rat, hond en mens. Deze schildklierhormoon-derivaten zijn geïncubeerd met rattelever-homogenaat om te bepalen welke soort verbinding met de grootste voorkeur door het dejodase enzym wordt omgezet. Een overzichtstabel van dergelijke structuur-activiteit studies staat in Hoofdstuk IX (Tabel 1), waar de 'V_{max}/K_m ratio' de efficiëntie van de omzetting aangeeft (vijfde kolom).

Gesulfateerde schildklierhormonen worden 40 tot 200 maal sneller omgezet door het dejodase dan de onveranderde schildklierhormonen. Ook is het effect onderzocht van de kunstmatige koppeling van een sulfaatgroep aan de stikstof (N) in de zijketen van het hormoon (NS-derivaat) in plaats van aan de OH-groep van de thyronine buitenring (natuurlijk sulfaatconjugaat). De dejodering van dergelijke synthetische NS-derivaten bleek 18-20 maal te zijn toegenomen, wat beduidend minder is in vergelijking met natuurlijke sulfatering. De plaats van de sulfaatgroep is dus van grote invloed op de dejoderingssnelheid (Hoofdstuk II).

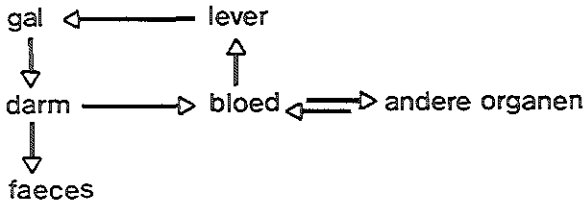
De in dit proefschrift beschreven experimenten met ratten tonen aan dat sulfatering ook een belangrijke rol speelt in het *in vivo* metabolisme van T_3 en T_4 (Hoofdstukken

III en VII). Gesulfateerde hormonen kunnen alleen in bloed of gal worden aangetoond als de snelle afbraak van deze metabolieten wordt voorkomen door de proefdieren vooraf te behandelen met de dejodase remmer PTU. Conclusie: een deel van het schildklierhormoon wordt "indirect" gedejodeerd, dat wil zeggen na voorafgaande sulfatering.

De omzetting van het azijnzure derivaat van T_3, tri-jodothyroazijnzuur (afgekort TA_3), verloopt grotendeels via dezelfde metabole routes als T_3: dejodering, sulfatering en glucuronidering. Dit is uitgebreid beschreven in de Hoofdstukken IV en V. Het gesulfateerde TA_3 blijkt extreem goed te worden gedejodeerd, namelijk bijna 1000 maal beter dan T_3 zelf. Verder hebben we in ratten aangetoond, dat het TA_3 minstens zo snel als T_3 uit de bloedbaan verdwijnt, doordat het zeer efficiënt wordt geglucuronideerd. De uitscheiding van TA_3G in de gal is bijna 2.5 maal groter dan die van T_3G.

Als gevolg van deze intensieve bestudering van de overeenkomsten en verschillen in het metabolisme van TA_3 en T_3 werd een tot nu toe onbekende metaboliet van T_3 ontdekt. Dit niet eerder beschreven produkt troffen we aan in het bloed van ratten, die met dejodase remmers waren behandeld. Wij identificeerden dit als het sulfaatconjugaat van di-jodothyroazijnzuur (afgekort TA_2S; Hoofdstuk VI). Hoe het precies uit T_3 ontstaat is nog onbekend.

De schildklierhormoon glucuronides worden niet gedejodeerd, maar door de lever als zodanig met de gal uitgescheiden. Dit hoeft niet te betekenen dat deze conjugaten vervolgens het lichaam verlaten. In de darm van mens en rat leven bacteriën die de glucuronide groep weer kunnen afsplitsen ("hydrolyseren"). Alleen het vrijgekomen schildklierhormoon kan vervolgens via de darmwand weer in het bloed worden opgenomen, terwijl de rest met de faeces word uitgescheiden. Deze "enterohepatische kringloop" is aangetoond door radioactief ^{125}I-T_3G te volgen in de rat (hoofdstuk IX).



Hiervoor zijn normale ratten bestudeerd in vergelijking met ratten waarin deze kringloop kunstmatig was onderbroken door, of de gal uit het lichaam te laten wegstromen via een drain, of de bacteriën in de darm te doden met behulp van antibiotica in het drinkwater.

De uitvoering van bovengenoemde studies zou ondenkbaar zijn geweest zonder de geweldige inzet en accuratesse van "mijn" analisten Frank Heusdens en Ingrid Pigmans en de inspirerende begeleiding van (pro)motor Theo Visser.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 3 augustus 1958 te Arnhem. Aldaar is in 1976 het Atheneum-B diploma behaald aan het Christelijk Atheneum (het huidige Van Lingen college).

Aansluitend hierop studeerde zij Biologie (orientatie Cel) aan de Landbouwhogeschool te Wageningen waar in 1983 het ingenieurs-examen werd afgelegd. Voor het hoofdvak 'Toxicologie' in de doctoraalstudie werkte zij op de vakgroep Toxicologie (Prof.Dr. J.H. Koeman; begeleider Ir. J.C.M. van der Hoeven) en vervolgens bij het Shell Toxicology Laboratory (Dr. B.J. Dean), Sittingbourne Research Centre in Sittingbourne, Engeland. Het tweede doctoraalvak 'Immunologie en Celbiologie' is uitgevoerd bij de vakgroep Experimentele Diermorfologie en Celbiologie (Prof.Dr. W.B. van Muiswinkel; begeleider Dr. E. Egberts).

Na een tijdelijke aanstelling bij laatstgenoemde vakgroep van de Landbouwhogeschool trad zij in september 1984 in dienst als onderzoekmedewerker van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO; Stichting MEDIGON). Hiervoor is tot maart 1988 onderzoek gedaan op de afdeling Interne Geneeskunde III en Klinische Endocrinologie van het Academisch Ziekenhuis Dijkzigt te Rotterdam, waarvan de resultaten zijn bewerkt tot dit proefschrift.

Vanaf oktober 1988 werkt zij bij het Nederlands Kanker Instituut (Antoni van Leeuwenhoekhuis) te Amsterdam op de afdelingen Experimentele Therapie (Prof.Dr. L.A. Smets) en Nucleaire Geneeskunde (Dr. C.A. Hoefnagel) in samenwerking met het Emma Kinderziekenhuis/Het Kinder AMC (Prof.dr. P.A. Voûte). Tot april 1992 werd het betreffende projekt gesubsidieerd door de Nederlandse Kankerbestrijding (voormalig Koningin Wilhelmina Fonds), waarna de financiering tot 1994 is overgenomen door de Stichting Kindergeneeskundig Kankeronderzoek.