DEIODINATION AND CONJUGATION OF T_3 AND OTHER IODOTHYRONINES

Dejodering en conjugatie van T_3 en andere jodothyronines

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PROF. DR. A.H.G. RINNOOY KAN

EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.

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PROMOTIECOMMISSIE

Promotor:	Prof. Dr. Ir. T.J. Visser
Overige leden:	Prof. Dr. H.G. van Eijk
	Prof. Dr. G. Hennemann
	Prof. Dr. G.J. Mulder

The studies reported in this thesis were carried out in the laboratory of the Thyroid Hormone Research Unit (head Prof. Dr. G. Hennemann) at the Department of Internal Medicine III and of Clinical Endocrinology (head Prof. Dr. J.C. Birkenhäger), Erasmus University Medical School, Rotterdam, The Netherlands.

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Aan Anky

CONTENTS

LIST OF ABBREVIATIONS

cAMP	cyclic adenosine 3′,5′-monophosphate
BHDB	butyl-4-hydroxy-3,5-diiodobenzoate
BSA	bovine serum albumin
BW	body weight
DCNP	2,6-dichloro-4-nitrophenol
DTT	dithiothreitol
G	glucuronide
GAM	galactosamine
HPLC	high performance liquid chromatography
IOP	iopanoic acid
IRD	inner ring deiodination
K_m	Michaelis constant
NTI	non-thyroidal illness
ORD	outer ring deiodination
PAPS	3′-phosphoadenosine-5′-phosphosulfate
PCP	pentachlorophenol
PR	production rate
PST	phenol sulfotransferase
PTU	6-propyl-2-thiouracil
RIA	radioimmunoassay
rT_3	3,3′,5′-triiodothyronine (reverse triiodothyronine)
S	sulfate
SAM	salicylamide
T_0	thyronine
T_1	monoiodothyronine
T_2	diiodothyronine
T_3	3,3′,5-triiodothyronine
T_4	thyroxine
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone (thyrotropin)
UDP	uridine diphosphate
UDPGT	UDP-glucuronyltransferase
UTP	uridine triphosphate
V_{max}	maximal velocity

CHAPTER 1

GENERAL INTRODUCTION

IA Thyroid hormone synthesis

Iodine enters the thyroid follicular cells as inorganic iodide and is incorporated through a series of metabolic steps into the main thyroid hormones thyroxine (T_4) and 3,3',5-triiodothyronine (T_3). Consecutive steps in this metabolic sequence are: 1) active transport of iodide by the thyroid follicular cell; 2) iodination of tyrosyl residues of thyroglobulin at the apical membrane; 3) coupling of mono- and diiodotyrosine molecules within thyroglobulin to form T_4 and T_3; 4) proteolysis of thyroglobulin, with release of T_4, T_3 and iodotyrosines; and 5) deiodination of iodotyrosines within the thyroid and reutilization of the liberated iodide. The iodothyronines are then secreted into the blood.

The production and secretion of iodothyronines by the thyroid are under control of thyroid stimulating hormone (TSH) secreted by the anterior pituitary gland. TSH binds to a specific plasma membrane receptor on the surface of the follicular cells and exerts its action mainly via the second messenger cAMP (1). The secretion of TSH is regulated by two interacting elements: neural control by the hypothalamus (stimulation by TRH, inhibition by somatostatin (2) and dopamine (3)) and negative feedback control by thyroid hormones (4-6).

The main product secreted by the thyroid is T_4 (structural formulas of the iodothyronines are shown in Fig. 1). In the normal adult the mean T_4 secretion rate is ~115 nmol/d per 70 kg body weight (BW). Thyroidal secretion of T_3 amounts to ~9 and of 3,3',5'-triiodothyronine (reverse T_3, rT_3) to ~2 nmol/d per 70 kg BW (for a review, see ref. 7). As the total production rates of T_3 (~43 nmol/d) and rT_3 (45-90 nmol/d) are much higher (7), it will be clear that the production of these iodothyronines occurs predominantly outside the thyroid. Contribution of thyroidal seretion to the circulating diiodothyronines 3,3'-T_2, 3,5-T_2 and 3',5'-T_2 is negligible (8,9).

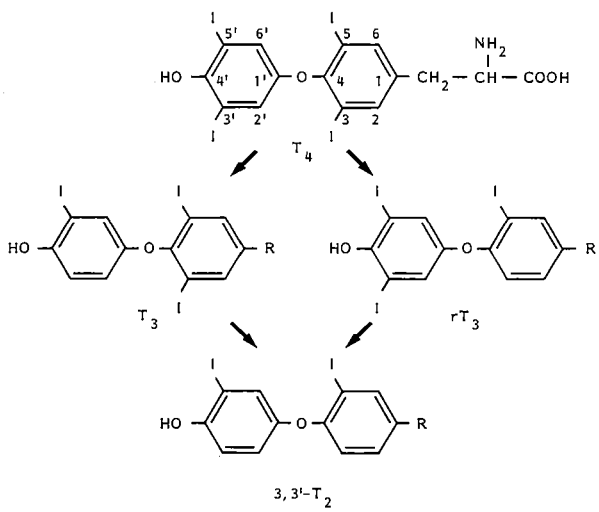

Figure 1
Structural formulas of iodothyronines.

IB Regulation of thyroid hormone bioavailability

In the hypothesis formulated by Robbins and Rall (10) it is proposed that the "free hormone concentration in serum governs hormone delivery to the cell and ultimately regulates hormonal action". This hypothesis is based on the assumption that 1) free but not protein-bound hormone is taken up by tissues; 2) the rate of tissue uptake and, hence, the availability of intracellular hormone are determined by the free hormone concentration in plasma; and 3) the biological effect of a hormone is positively correlated with its intracellular concentration. With respect to thyroid hormone, the free concentrations of T_4 and T_3 in plasma are undoubtly important determinants of the thyroid status of the organism. However, the existence of multiple regulatory mechanisms makes that this relationship becomes less obvious under different circumstances. In the subsequent paragraph a number of factors which are important in regulating thyroid hormone bioavailability are discussed. These are: 1) the free thyroid hormone concentration in serum; 2) active uptake of thyroid hormone into the tissues; 3) extrathyroidal metabolism of iodothyronines; and 4) free hormone gradients between intracellular compartments.

1: The free thyroid hormone concentration

The iodothyronines circulate in the blood largely bound to three specific plasma transport proteins. These are T_4-binding globulin (TBG), T_4-binding prealbumin (TBPA) and albumin which carry approximately 75, 15 and 10% of plasma T_4, respectively (for a review, see ref. 11). The distribution of T_3 over these proteins is less certain. Values range between 38-80% for TBG, 9-27% for TBPA and 11-35% for albumin (11). In normal serum free T_4 (FT_4) and FT_3 average approximately 0.02% and 0.2% of total T_4 and T_3 levels.

Several inherited variations involving each of the major transport proteins have now been described. Among these, the condition known as congenital TBA elevation, which was first described by Hennemann et al. (12) deserves special mention. In these euthyroid subjects total T_4 is increased due to an abnormally increased T_4 binding in the albumin region. However, FT_4 measured by equilibrium dialysis, total T_3 and the response of TSH to TRH are normal. Recent studies suggest that this 'abnormal' T_4-binding albumin (TBA) is also present in normal serum, although in a lesser amount (13).

2: Active uptake of thyroid hormone into tissues

Originally, it was postulated that thyroid hormones, by way of their lipophilic nature, would pass the lipid bilayer of the cell membrane by passive diffusion (14). Recently, however, evidence has been obtained that thyroid hormone is, at least in part, transported through the plasma membrane by an energy-dependent, carrier-mediated process (for a review, see ref. 15). These studies have demonstrated that the kinetics of uptake of T_4 and T_3 by rat hepatocytes consist of three components. The first, non-saturable process probably represents the partition of hormone in the lipid bilayer of the plasma membrane. The second component is thought to result from the interaction of T_4 and T_3 with binding sites on the external cell surface. The third process is involved with the transport of T_4 and T_3 across the cell membrane. It appears that T_4 and rT_3 share a common pathway to enter the cell that is different from that of T_3. More direct evidence for the presence of an active transport system for thyroid hormone was obtained with the use of a monoclonal antibody (ER-22) directed against the rat hepatocyte plasma membrane (16,17). Active transport systems for thyroid hormones have also been found in cultured GH_3 pituitary tumor cells and human fibroblasts (15). Preliminary experiments in our laboratory suggest the existence of active iodothyronine uptake systems in human liver cells (Krenning et al, unpublished work).

3: Extrathyroidal metabolism of iodothyronines

Iodothyronines undergo two principal metabolic reactions, i.e. deiodination of the tyrosyl (inner) or phenolic (outer) ring, and conjugation of the phenolic hydroxyl group with glucuronic acid or sulfuric acid (Fig. 2). They are also subject to deamination and decarboxylation of the amino acid side chain, and to ether link cleavage. These pathways are not mutually exclusive but are interrelated in a complex manner. Deiodination of iodothyronines is, at least in man, quantitatively the most important metabolic route and will be discussed here. Glucuronidation and sulfation will be the subject of chapter II. The remaining two metabolic pathways, deamination and ether link cleavage are least important and will not be discussed here (for reviews, see refs. 18,19).

Type I Iodothyronine deiodinase

Studies in rats have shown that there are at least three different iodothyronine-deiodinating enzymes: type I, II and III (for reviews, see

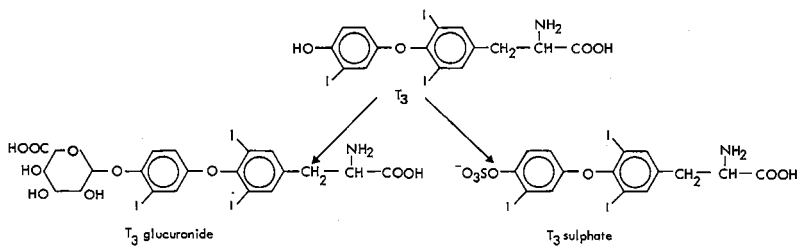

Figure 2
Glucuronidation (left) and sulfation (right) of T_3.

Table 1. THREE TYPES IODOTHYRONINE DEIODINASE

	I	II	III
	T_4 → T_3, rT_3 → T_2	T_4 → T_3, rT_3 → T_2	T_4 → rT_3, T_3 → T_2
Location	liver, kidney	brain, BAT, pituitary	brain, skin, placenta
Substrate preference	$rT_3 > T_4 - T_3$	$T_4 > T_3$	$T_3 > T_4$
Thiols	stimulation	stimulation	stimulation
PTU	inhibition	no effect	no effect
Hypothyroidism	decrease	increase	decrease
Hyperthyroidism	increase	decrease	increase

refs. 19-23). The characteristics of these enzymes will only be shortly discussed here. In rats high type I deiodinase activities are found in liver, kidney and thyroid, and low levels in many other tissues. The enzyme is an integral membrane protein that in liver appears to be associated with the endoplasmic reticulum while in kidney it is located in the plasma membranes (23). The physiological cofactor is unknown. Glutathione (GSH), thioredoxin and glutaredoxin have all been implied as possible cofactors, although they are much less potent in vitro than for instance the synthetic dithiol dithiotreitol (DTT). Other characteristics of the type I enzyme, which distinguish this enzyme from the other two deiodinases are 1) the capability to catalyse both inner ring deiodination (IRD) and outer ring deiodination (ORD) of iodothyronines (Table I); 2) the substrate preference for $rT_3 >> T_4 > T_3$; 3) the facilitated deiodination of iodothyronine sulfate conjugates (chapter IIC); and 4) the inhibition by the thyrostatic drug propylthiouracil (PTU).

The characteristics of the human type I deiodinase activities of thyroid (24), kidney (25,26) and liver (27,28) have recently been reported. It appears that these organs contain similar if not identical enzymes. In addition, evidence has been obtained that the human liver type I deiodinase is analogous to the rat liver type I enzyme (28). Further investigations into the similarity of the type I deiodinases of humans and rats awaits the purification of these enzymes and the elucidation of their aminoacid sequences.

Type II and III iodothyronine deiodinase

The type II enzyme catalysis the ORD of iodothyronines, while the type III enzyme is a specific inner ring deiodinase. Thus, the type II enzyme converts T_4 to T_3 and rT_3 to $3,3'-T_2$. The type III enzyme deiodinates T_4 to rT_3 and T_3 to $3,3'-T_2$. The central nervous system (CNS) and placenta contain both type II and III deiodinases, while the type II enzyme is also found in pituitary gland and brown adipose tissue. In addition, skin contains type III enzyme activity (29). Besides PTU-insensitivity there are two other unique properties that distinguish the enzymatic reactions catalyzed by the type II deiodinase from type I ORD. These are 1) Michaelis constants of iodothyronines that are 200-400 fold lower for the type II deiodinase than those for the type I enzyme; and 2) (a characteristic shared with the type I and III enzymes) the reciprocal regulation of enzyme activities by thyroid hormone, i.e. the activity of the type I deiodinase

is decreased and that of the type II enzyme is increased in hypothyroidism while the reverse is the case in hyperthyroidism. The activity of the type III deiodinase changes in parallel with type I enzyme activity according to thyroid status. This latter feature enables tissues with type II deiodinase activity to maintain intracellular T_3 levels within narrow limits despite changes in the availability of substrate. For example, in hypothyroidism, a decreased T_4 supply is counteracted by an increase in type II and a decrease in type III tissue levels. This leads on the one hand to an increased T_3 production and on the other hand to a diminished breakdown of this hormone (for a review, see ref. 20).

Monodeiodination of T_4

In man monodeiodination of T_4 accounts for ~80% of its disposal (for reviews, see refs. 7,18,19). Approximately equal proportions of T_4 are used for T_3 and rT_3 generation. T_4, which is generally considered to have little or no bioactivity, is converted by ORD to the metabolically active hormone T_3. Reverse T_3, which is devoid of thyromimetic activity, is produced by IRD of T_4. In this way 80% of plasma T_3 and >95% of plasma rT_3 are derived from peripheral deiodination of T_4, the remainder being secreted by the thyroid.

Reverse T_3 and T_3 production

Reverse T_3 is produced by type I and III IRD of T_4. However, as the efficiency of ORD of rT_3 by the type I enzyme is >900-fold higher than that of the production of rT_3 by type I IRD of T_4, it follows that rT_3 once produced is rapidly further deiodinated by this enzyme (20). Therefore, rT_3 produced by type I IRD of T_4 is rapidly further degraded by the same enzyme and does not contribute to plasma rT_3. It is therefore logical to assume that plasma rT_3 is predominantly derived from type III IRD of T_4 in CNS (20) and skin (29), while it is cleared mainly by type I ORD in liver and kidney (30). The latter reaction is inhibited by PTU (chapter III) or IOP (6), explaining the increased rT_3 plasma levels after treatment of rats and humans with these drugs (chapters VI & VII; ref. 31).

T_3 is produced by type I and II ORD of T_4. In euthyroid rats 60-70% of peripheral T_3 production is derived from the former pathway (32). At present the quantitative contribution of the individual human organs to the daily extrathyroidal T_3 and rT_3 production is unknown.

Diiodothyronine (T_2) production

Three diiodothyronines are generated by the monodeiodination of T_3 and rT_3, i.e. 3',5'-T_2 by IRD of rT_3, 3,5-T_2 by ORD of T_3 and 3,3'-T_2 by both ORD of rT_3 and IRD of T_3. Little information is available about the possible occurence of the first two reactions. Estimates of the quantitative role of monodeiodination in the disposal of the triiodothyronines vary considerably in the literature, depending on the estimates of the serum T_2 concentrations (for a review, see ref. 18). Based on the means of the reported PR's of the T_2's ,T_3 and rT_3, Engler and Burger computed that 83% of the daily PR of the triiodothyronines is converted by monodeiodination and 17% is disposed of by other mechanisms. Using PRs of these iodothyronines determined in their own laboratory, these authors calculated that monodeiodination accounted for 46-69% and other pathways for 31-54% of triiodothyronines disposal. However, since part of the 3,3'-T_2, and perhaps also of the other T_2's, is rapidly further metabolized before release into the circulation, the calculation of their PR's represents a gross underestimation.

A detailed analysis of the origin of plasma 3,3'-T_2 in humans has recently been undertaken by Engler et al (33). From the plasma appearance of labeled 3,3'-T_2 following injection of radioiodinated rT_3, they calculated that only 15% of total 3,3'-T_2 is derived from ORD of plasma rT_3. Therefore, most plasma 3,3'-T_2 originates from the IRD of T_3, although only 25% of plasma T_3 was calculated to undergo IRD to 3,3'-T_2 (33). However, based on previous measurements of the MCR of 3,3'-T_2 (34) the plasma production rate of 3,3'-T_2 may amount to approximately 45 nmol/d per 70 kg BW. If indeed 85% of plasma 3,3'-T_2 is produced by IRD of T_3, then in combination with the known T_3 PR , it may be deduced that as much as 75% of plasma T_3 is metabolized to 3,3'-T_2. This would be in agreement with the study reported by Zucchelli et al (35), which indicated that the clearance of labeled T_3 injected into humans was almost completely accounted for by generation of radioactive 3,3'-T_2 in plasma.

The T_2's are further metabolized by stepwise deiodination to a 3- and 3'-monoiodothyronine (T_1) and finally thyronine (T_0).

Thyroid hormone changes in non-thyroidal illness

Non-thyroidal illness (NTI), stress, caloric deprivation and several medications (like PTU, propranolol, dexamethasone and X-ray contrast agents) influence thyroid hormone economy at various levels, i.e. secre-

tion, transport, metabolism and action at the cellular level (for reviews, see refs. 36-38). A continuum of changes in serum thyroid hormone levels relative to the severity of the NTI has been observed (39,40). Total T_4 levels correlate fairly well with clinical outcome; the highest mortality occuring in the patients with the lowest total T_4 levels (39,40). Total T_4 levels may be increased (in mild disease) or decreased (in more severe disease), but serum T_3 is consistently decreased and rT_3 levels are elevated. This is due to decreased production with unaltered clearance of T_3 and a decreased MCR with unaltered production of rT_3.

The cause of these changes in iodothyronine serum levels is probably multifactorial (39). As T_4 to T_3 conversion and rT_3 clearance are both catalyzed by type I ORD in the liver (and kidney), this enzyme has been implied as an important factor in the genesis of this syndrome. Recent reports, however, suggest that tissue 5'-deiodinase activity, at least in vitro, is not consistently decreased in experimental models of NTI, i.e. rats bearing the hypercalcemic Walker sarcoma 256 (41) and rats with an inflammation syndrome due to injection of turpentine oil (42). Another mechanism for the NTI-induced changes in thyroid hormone metabolism is through a diminished tissue uptake of iodothyronine substrates resulting from a) decreased intracellular ATP concentrations (e.g. shock, food deprivation); b) a circulating inhibitor, which interferes with the binding of thyroid hormone to serum transport proteins (42), but also blocks the entry of iodothyronines into hepatocytes (43,44; for a review, see ref. 15).

4: Free hormone gradients between intracellular compartments

T_3 regulates the expression of certain genes by binding to specific receptors in the cell nucleus of thyroid hormone responsive tissues. These specific T_3-binding sites have been found in several tissues of rat and man (for reviews, see refs. 45-47). The mechanism by which T_3 is transferred from the cytoplasm to the nucleus is not clear at the moment, but a recent report has suggested that a stereospecific, energy-dependent transport system is also involved in this translocation (43).

IC Scope of the present study

As described in the preceding paragraph plasma T_3 and rT_3 are mainly derived from extrathyroidal deiodination of T_4. The liver takes a central place in iodothyronine metabolism in that it is the prime site for the peripheral production of T_3 and the clearance of rT_3. Besides, conjugation with glucuronic acid and sulfate are, at least in rat, important processes for the hepatic T_3 metabolism.

In chapter II a review of two of the most important conjugation reactions, glucuronidation (chapter IIA) and sulfation (chapter IIB) will be given. In chapter IIC the role of conjugation in thyroid hormone metabolism and available evidence for the existence of an enterohepatic cycle will be discussed. In this chapter the results of our studies of rT_3 and T_3 metabolism in isolated rat hepatocytes (chapters III and V) are integrated. At the same time the importance of sulfation for the metabolism of T_3 in rats will be discussed (chapters V and VI). Finally, evidence will be presented that in man sulfation plays a role in T_3 metabolism, although this conjugation reaction seems less important than in rats (chapter VII).

The following chapters III–VII describe investigations related to the importance of glucuronidation and sulfation in the metabolism of T_3 and rT_3. Metabolism of rT_3 is the subject of chapter III. In chapter IV the development and characterisation of a radioimmunoassay for T_3S is described. With this RIA the metabolism of T_3 was studied in isolated rat hepatocytes (chapter V), in intact rats (chapter VI) and in man (chapter VII).

CHAPTER II

IIA Glucuronidation

Introduction

Glucuronidation represents one of the major conjugation reactions involved in the metabolic conversion of a variety of xenobiotics and endogenous compounds to polar water-soluble metabolites (48). The resulting glucuronides are frequently the end products of metabolism and are mainly excreted via the urine or the bile. Glucuronidation followed by excretion is one of the most important pathways of detoxication in man and most other mammals.

The first part of this chapter will focus on glucuronidation in general and in the second part glucuronidation of thyroid hormones will be described.

Regulation of glucuronidation

Numerous drugs and/or their metabolites are converted in vivo into glucuronide conjugates. Compounds with functional groups such as OH, COOH, NH_2 and SH may be glucuronidated directly (a so-called phase II reaction), whereas for relatively nonpolar substances introduction of polar groups by oxidation, reduction or hydrolysis (so-called phase I reactions) is a prerequisite for glucuronidation. UDP-glucuronyltransferases (EC 2.4.1.17) (UDPGT) are a family of enzymes which catalyze this conjugation reaction by transfer of glucuronic acid from UDP-glucuronic acid to the acceptor molecule (Fig. 1). The cosubstrate UDP-glucuronic acid is synthesized in vivo from UDP-glucose by the enzyme UDP-glucose dehydrogenase (EC. 1.1.1.22). UDP-glucose is formed from glucose-1-P and UTP, which are abundant in tissues (48). Therefore, depletion of UDP-glucuronic acid will not easily occur under normal conditions. However, steady-state rates of glucuronidation requiring 10 umol/g liver/h UDP-glucuronic acid have been observed in perfused livers. Under these conditions the pool of UDP-glucuronic acid must be resynthesized every 2 min in order to maintain intracellular concentrations above the K_m of UDPGT. This suggests that cofactor supply can become rate limiting when large doses of substrate are presented to the enzyme (49). In a variety of hepatic preparations the concentration of UDP-glucuronic acid is 0.3-0.5 mM. The K_m of UDPGT for UDP-glucuronic acid in native and detergent-activated microsomes from rat liver for substrates as 1-naphtol or p-nitrophenol varies between 0.16-0.39 mM (49), once again strengthening the above mentioned fact that cofactor

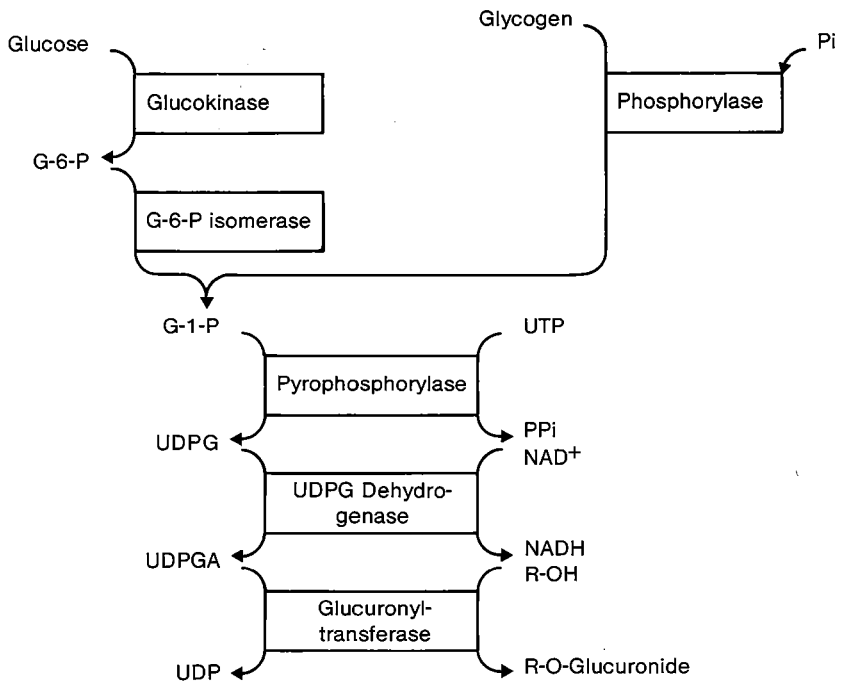

Figure 1
Intracellular glucuronidation pathway.
G-6-P = glucose 6-phosphate; G-1-P = glucose 1-phosphate; UDPG = uridine diphosphate glucose; UDPGA = uridine diphosphate glucuronic acid; NAD^+ = nicotinamide adenine dinucleotide; Pi = inorganic phosphate; PPi = inorganic pyrophosphate; R-OH = substrate for glucuronidation.

supply may become rate-limiting.

UDPGT's are located in the endoplasmic reticulum of many organs, particularly the liver, small intestine and kidneys. Glucuronidation rates are not evenly distributed throughout the liver, but glucuronyltransferase activities in pericentral hepatocytes exceed those in the periportal region threefold (50,51). Recently the complete aminoacid sequence of 2 isoenzymes of rat (52,53) and human UDPGT (54) has been deduced from complementary DNA clones.

Glucuronidation of drug metabolites has been studied primarily in isolated rat hepatocytes and in the perfused rat liver. From these studies substrate supply, among others, has been identified as one of the rate-controlling factors (55). Frequently, there is competition between sulfotransferases and UDPGT's for the conjugation of common substrates. While the contribution of sulfation is usually more important at low substrate levels, glucuronidation is general the predominant conjugation step at high substrate availability. Glucuronidation, therefore, can be characterized as a high-capacity, high-K_m process, whereas sulfation is a low-capacity, low-K_m reaction. The higher capacity of glucuronidation is, among others, the result of more rapid production of the cofactor UDP-glucuronic acid than of the cofactor for the sulfation reaction (chapter IIB).

Several drugs, like barbiturates, chloroquine and rifampicine, have been known to induce UDPGT activity, whereas conditions such as hyperthyroidism, starvation and experimental diabetes decrease enzyme activity (56). Galactosamine inhibits glucuronidation rates (chapter V) by trapping of UDP-glucose and inhibition of UDP-glucose pyrophos-phorylase (Fig. 2) (57).

β-Glucuronidase activity

Glucuronides are hydrolyzed by β-glucuronidases (EC 3.2.1.21) present in all animal tissues examined, and also in plants and bacteria (48). The enzyme accepts all known glucuronides as substrate, with only a few exceptions. The enzyme is located in lysosomes, but unlike most lysosomal enzymes, also in microsomes. β-Glucuronidase occurs in bile, intestinal juice, and salivary secretions. In the intestinal lumen it is very active, originating mainly from sloughed cells and in the lower reaches also from the microflora. As many glucuronides of endogenous and foreign compounds are excreted in the bile, the action of β-glucuronidase in promoting the liberation and possible reabsorption of aglycons (enterohepatic circu-

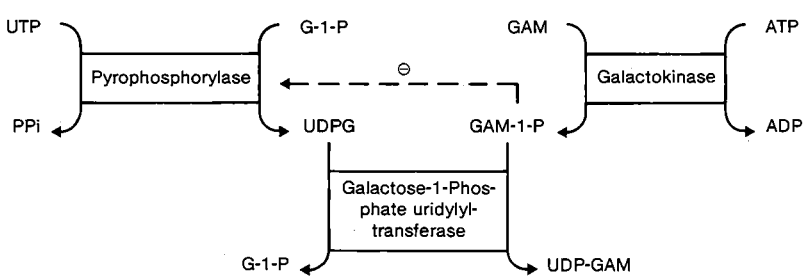

Figure 2
Mode of action of galactosamine (GAM).
1) Trapping of UDPG; and 2) Inhibition of pyrophosphorylase
PPi = inorganic pyrophosphate; G-1-P = glucose 1-phosphate;
UDPG = uridine diphosphate glucose; UDP-GAM = uridine diphosphate galactosamine

lation) is physiologically and pharmacologically important.

Glucuronidation of iodothyronines

Glucuronidation rates of different iodothyronines in liver vary considerably. T_4 and rT_3 are (next to deiodination) mainly glucuronidated (chapter IIC), while 3,3'-T_2 (chapter III, ref. 58) and 3'-T1 are mainly sulfated (Visser TJ, unpublished observations). T_3 is intermediate in this respect in that glucuronidation and sulfation rates are similar (chapter V). Isolation and characterization of the specific iodothyronine UDPGT(s) will be needed to further clarify this substrate specificity.

Hydrolysis of T_4G and T_3G by β-glucuronidase-producing bacteria in the human and rat intestine plays an important role in the enterohepatic circulation for thyroid hormone (for a review, see chapter IIC).

IIB Sulfation

Introduction

Sulfate conjugation is another important pathway in the biotransformation of drugs, xenobiotic compounds, hormones and other endogenous compounds. Phenol sulfotransferase (PST) (E.C.2.8.2.1), located in the cytosol, catalyzes the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a phenolic acceptor molecule (59). Knowledge of the biochemistry, regulation and function of PST in human tissues has increased significantly during the past decade. Especially the discovery of PST activity in human blood platelets (60), an easily accessible tissue, served as an important stimulus for the study of the regulation of this enzyme.

The first part of this chapter will be devoted to regulation of sulfation in general and in the second part sulfation of iodothyronines will be described.

Regulation of sulfation

Enzyme concentration, cofactor supply, substrate availability (see chapter IIA) and activity of sulfate-hydrolysing enzymes (sulfatases) influence the degree of sulfation in intact cells. There are at least 4 forms of rat liver PST that elute sequentially from an ion-exchange column (61-63). Forms I and II in rat liver catalyze the sulfate conjugation of simple phenols, while form IV has a higher affinity for catechol substrates. Form III in rat liver has not been well characterized.

All human tissues that have been studied in detail contain at least 2 forms of PST (64). These 2 forms differ with respect to their physical properties, substrate specificity, inhibitors and regulation. One form is relatively thermolabile, resistant to inhibition of 2,6-dichloro-4-nitrophenol (DCNP) and catalyzes the sulfate conjugation of micromolar concentrations of dopamine and other catecholamines. The other form is thermostabile, relatively sensitive to DCNP inhibition and catalyzes the sulfate conjugation of micromolar concentrations of "simple" phenols. Thermolabile and thermostable forms of PSTs display similar properties in different organs (65).

Regulation of cofactor supply

PAPS is the direct sulfate-donating co-substrate in the sulfation reaction. For a continuous PAPS supply, which is synthesized intra-

cellularly (Fig. 3), inorganic sulfate (and ATP) should be available. The intracellular inorganic sulfate concentration is dependent on 1) extracellular (serum) sulfate; 2) transport of sulfate across the cell membrane; 3) availability of sulfate precursors (cysteine); and 4) the rate of sulfate production from precursors.

L-cysteine is derived from catabolism of ingested proteins, but may also be generated from methionine via the cystathione pathway (59). Krijgsheld and Mulder calculated that the half-maximal concentration of inorganic sulfate in serum for sulfation in the intact animal was 0.4 mM (66). This suggests that under normal conditions serum sulfate concentrations (normal values in rat: 0.8-1.2 mM) are not rate-limiting. Quantitative data for humans, where the serum sulfate concentration is 0.2-0.3 mM, are not available. Enteral sulfate absorption has been demonstrated to be rapid and almost complete, unless diarrhea occurs. The serum sulfate concentration may increase 2 to 3-fold following oral administration of inorganic sulfate (59). The serum sulfate concentration is primarily regulated by the kidneys. Below a certain threshold inorganic sulfate is completely reabsorbed from the primary urine in the proximal tubule. If the serum sulfate levels increase above this threshold sulfate is excreted.

Sulfate transport across the liver cell membrane

Sulfate transport is mediated by a carrier process (67). Uptake of inorganic sulfate across the plasma membrane is very rapid (600 umol/g liver/h) and greatly exceeds the maximal rates of sulfation (2-6 umol/g/h) (49). The concentration of the cofactor PAPS in rat liver is about 30 uM (68), which may be an important rate determinant for sulfation, since the apperent K_m of sulfotransferases for PAPS ranges from 2-30 uM. Because of these low steady-state concentrations of PAPS, its turnover must be quite rapid during sulfation. In certain conditions the pool of PAPS in the liver was estimated to be resynthesized every 4 sec (49).

Sulfatases

Sulfatases are lysosomal and microsomal enzymes which catalyze the hydrolysis of sulfate esters ($ROSO_3^-$) according to the following reaction, where ROH can be an alcohol, a phenol, a carbohydrate or a steroid:

$$ROSO_3^- + 2H_2O \longrightarrow ROH + H_3O^+ + SO_4^{2-}$$

The best known of these enzymes are the aryl-sulfatases (EC 3.1.6.1), which catalyze the hydrolysis of a variety of arylsulfates and have been detected

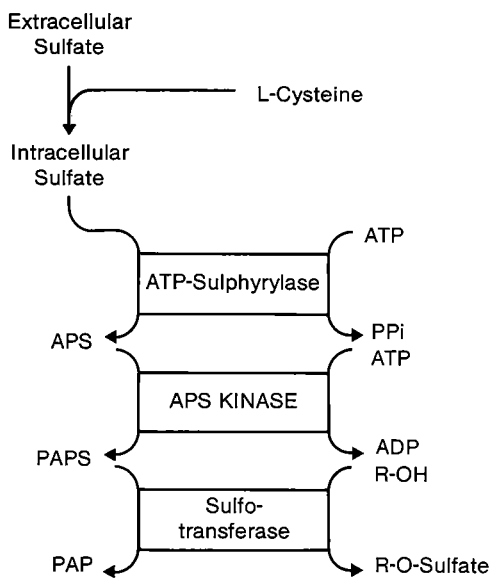

Figure 3
Intracellular sulfation pathway.
APS = adenosine 5'-phosphate; PAPS = 3-phosphoadenosine 5'-phosphosulfate;
PAP = adenosine 3',5'-diphosphate; PPI = inorganic pyrophosphate;
R-OH = substrate for sulfation.

in most animal tissues, bacteria and plants. The physiological function of these enzymes is difficult to understand. As described above, arylsulfates are produced by mammals as part of their detoxication mechanism. Once formed, subsequent hydrolysis of arylsulfates by sulfatases would reexpose the organism to the toxicity of the phenolic compound (for a review, see ref. 69). Although much has to be learned about their physiological function, these enzymes in general have recently attracted considerable attention because deficiency of several sulfatases has been implied to play a role in the genesis of a number of human genetic disorders, e.g. Hunter's syndrome. This disease is probably caused by the accumulation of dermatan sulfate primarily in the brain and bones due to the absence of the enzyme iduronate sulfatase (69).

Sublobular hepatic sulfotransferase and sulfatase distribution

In contrast to the preferentially pericentral distribution of UDPGT in human liver, PST activity was found predominantly in the periportal region. Sulfatases were evenly distributed across the liver lobule (51). The physiological significance of the different distribution of these enzymes has not yet been elucidated.

Sulfation of iodothyronines

Sulfation of iodothyronines has long been considered a relatively minor pathway. Renewed interest in this subject arose when Otten and Visser showed that sulfation of 3,3'-T_2 and T_3 was not an independent pathway, but facilitated deiodination (see chapter IIC) (58,70). The in vitro characterisation of iodothyronine PST activity of rat liver was carried out by Sekura et al (71). These workers reported that 3'-T1 and 3,3'-T_2 were good substrates for aryl sulfotransferase I and IV from rat liver. T_4 was not sulfated by these enzymes and activity towards T_3 was intermediate. This is in excellent agreement with the in vitro and in vivo findings reviewed in chapter IIC. The characterisation of human platelet iodothyronine sulfotransferase (72) should further facilitate investigations into the role of sulfation in iodothyronine metabolism in man.

Desulfation of T_3S by microsomal preparations from rat liver, brain and kidney has recently been described by Kung et al (73). Rat liver was 5- to 20-fold as active as kidney and brain. In isolated rat hepatocytes incubated with 7-54 uM T_3S, 1-1.5%. of substrate was hydrolysed after 3 h. As at these high concentrations the preferential pathway of T_3S metabo-

lism, i.e. deiodination, is saturated (chapter V), the physiological role of the T_3S sulfatase pathway remains to be elucidated.

As is described in chapter IIC, sulfatases in the intestinal microflora of rats and humans may play a role in the enterohepatic cycle of iodothyronines, although under normal conditions type I deiodination is the predominant route for the disposal of these conjugates.

IIC **Conjugation and deiodination of iodothyronines**

Little is known about the possible role of iodothyronine conjugation in man. This paragraph, therefore, represents mainly an overview of data obtained in rats concerning the importance of conjugation and deiodination for thyroid hormone metabolism. In succession, the metabolism of T_4, rT_3 and T_3 will be discussed in isolated rat hepatocytes, in the perfused rat liver, in intact rats and finally in man.

Thyroid hormone metabolism in vitro

Sato and Robbins investigated the metabolism of outer- and inner ring labeled ^{125}I-T_4 in isolated rat hepatocytes (74). At low substrate concentrations (<10 nM) I^- was the main product (45% of added T_4), which formation gradually diminished at increasing substrate concentrations. The ratio of $^{125}I^-$ released from inner ring-labeled ^{125}I-T_4 to that from outer ring-labeled ^{125}I-T_4 was ~1 at all substrate concentrations. Less than 10% of added T_4 was conjugated to T_4G and T_4S in a ratio of ~2:1. PTU nearly completely inhibited I^- production in these incubations with a resulting increase in T_4-conjugates to 20% of added T_4 without altering the relative distribution of T_4G and T_4S. These results suggest that IRD and ORD are equally important for the metabolism of T_4 in these cells, much more so than glucuronidation and especially sulfation.

Direct ORD is also the main degradative pathway of rT_3 in isolated rat hepatocytes (chapter III). Normally, $^{125}I^-$ is the main product in incubations with rT_3, which production is effectively inhibited by PTU. In accordance with the observations of Sato (74), conjugation of rT_3 to mainly rT_3G was only a minor pathway at substrate concentrations <10 nM. Reverse T_3 metabolism is not sulfate-dependent as it is not influenced by either sulfate depletion or addition of SAM and DCNP. However, compared with control incubations the relative distribution of labeled products in the medium differed considerably under these latter conditions, i.e. I^- production declined while 3,3'-T_2 accumulated. Normally, 3,3'-T_2 is not observed due to rapid sulfation of this product and subsequent ORD of 3,3'-T_2S. This underscores the previously mentioned fact that the PR of 3,3'-T_2 is underestimated by analysis of plasma kinetics in humans (chapter IB).

The metabolism of T_3 is quite different from that of T_4 and rT_3. In the studies described in chapter V, previous observations that sulfation of T_3 facilitates deiodination (75) were confirmed. Normally, I^- and T_3G are the main products after incubation of rat hepatocytes with physiological T_3

concentrations (i.e. <1-10 nM in the presence of bovine serum albumin (BSA)). The metabolism of T_3 is sulfate-dependent as it is lowered by sulfate depletion or addition of the sulfotransferase inhibitors DCNP and PCP. PTU, however, does not inhibit overall T_3 metabolism despite a near complete reduction in I^- production, due to the reciprocal accumulation of T_3S. With HPLC analysis, we also demonstrated the presence of 3,3'-T_2S in these latter incubations. This, in combination with the accumulation of 3,3'-T_2 and 3,3'-T_2G in sulfate-deplete incubations led us to hypothesize the presence of an enzyme which shares the characteristics of the type III deiodinase in that it catalyzes the IRD of T_3 only at low substrate concentrations and is not inhibited by PTU. In chapter V evidence is provided that such an enzyme is indeed present in adult rat liver.

So far, the type III enzyme has been demonstrated in rat brain (20), skin (29) and placenta (20), but never in adult liver. Fetal rat (76) and chicken liver (77), however, do contain a similar enzyme. Recent studies in our laboratory, however, show that type III-like deiodinase activity was not lower in old (300 g BW) than in young (100 g BW) rats (unpublished observations).

The metabolism of thyroid hormones in the isolated perfused rat liver was investigated by Flock and Owen (78). T_4 was metabolized less rapidly than rT_3 and T_3. With all three substrates tested I^- was always the main product in the perfusate. After 5h 12% of labeled T_4 was excreted into the bile, mainly as T_4G. Biliary excretion of metabolites of rT_3 was even lower, while that of T_3 metabolites, mainly as T_3G, amounted to 40% in 5 h.

Conjugation of rT_3 is apparently a minor pathway and its metabolism depends primarily on ORD (chapters IB & III). Therefore, in the following section we will only review in vivo data of conjugation and deiodination of T_4 and T_3.

In vivo metabolism of T_4 in rats

The secretion of T_4G in rat bile after injection of labeled T_4 was already demonstrated in 1955 by Taurog et al (79) and confirmed by others (80,81). It appears that, retrospectively, T_4S has also been detected by Flock et al in bile of thiouracil-treated rats, although this conjugate was not identified as such by these workers (80,82,83). This was due to the unrecognized property of T_4S to be resistant to the sulfatase Mylase P used by Flock.

Recently, Rutgers et al undertook a detailed analysis of the biliary

and fecal excretion of labeled T_4 in rats (84). Eight h after an iv injection of [^{125}I]T_4 biliary excretion of total radioactivity amounted to 14%, mainly as T_4G and smaller amounts of T_3G and rT_3G. In addition, a small but significant amount of T_4S was present in bile of untreated rats, the excretion of which was was increased 5-fold after PTU-treatment. In the latter rats, clearance of T_4 and production of T_3G were decreased and excretion of rT_3G increased. The PTU-induced >24-fold decrease in the T_3G/rT_3G ratio emphasizes the key role of the type I deiodinase for T_3 production and rT_3 clearance. Although apparently sulfation is a significant pathway of T_4 metabolism in intact rats, its precise contribution remains to be assessed.

Urinary and fecal radioactivity were also investigated in these rats. Within 15 h of ^{125}I-T_4 administration to normal rats 20% and 13% of injected radioactivity was excreted in the urine and feces, respectively. The urinary radioactivity consisted almost entirely of $^{125}I^-$ and fecal radioactivity consisted largely of T_4, T_3 and rT_3 in a ratio of 70:18:2. The absence of conjugates in the feces confirms previous studies from de Herder et al that the intestinal flora contains bacterial strains capable of hydrolysing iodothyronine conjugates (see below). In the PTU-treated rats urinary I^- excretion diminished and fecal radioactivity excretion increased (in a ratio of T_4: T_3: rT_3 of 68: 7: 6) as was also observed by others (85).

In vivo metabolism of T_3 in rats

As shown in chapter V, glucuronidation and sulfation are important pathways in the metabolism of T_3. T_3G is excreted into the bile (78,80) and T_3S is rapidly deiodinated by the type I deiodinase (chapter V). Biliary excretion of T_3S is low, but not absent in normal rats, and is increased after treatment with butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) (80,86) and PTU (87).

Plasma T_3G levels are virtually undetectable, which can be explained by the fact that 1) extrahepatic production of T_3G is low (86); 2) hepatic clearance of T_3G is very efficient (88, 89); and 3) T_3G produced by the liver is preferentially transported to the bile rather than plasma (78).

T_3S was identified for the first time by Roche et al in plasma and bile of thyroidectomized rats after iv injection of [^{125}I]T_3 (90-93). Normally, plasma T_3S is low in the rat and increases after treatment of the animals with the type I deiodinase inhibitors PTU (chapter VI, refs. 89,94)

or IOP (31). Recent evidence explains why Roche et al were able to detect T_3S in plasma of rats not treated with these inhibitors. In their studies they used thyroidectomized rats, a condition which leads to a decrease in type I enzyme activity (6) with a resultant inhibition of T_3S metabolism.

A detailed analysis of plasma T_3S was recently undertaken by Rutgers et al (94). In rats iv injected ^{125}I-T_3S was cleared more rapidly than iv injected ^{125}I-T_3 despite increased binding to plasma proteins. Treatment of rats with PTU decreased plasma T_3S clearance with 81%. Biliary T_3S excretion was markedly enhanced in the latter situation (94). The very rapid plasma clearance of T_3S may explain, at least in part, the low steady state plasma T_3S levels in rats (chapter VI). The high capacity of isolatred rat hepatocytes for T_3 sulfation suggests that the liver is a possible source for circulating T_3S. However, significant extrahepatic T_3S production has been observed in dogs (86). The rapid clearance of T_3S by type I deiodinase containing tissues thus ensures low plasma levels of this conjugate in normal rats.

Biliary excretion of T_3 and its metabolites was recently investigated by De Herder et al (89). Within 4 h after injection of ^{125}I-T_3 to normal rats, 22.4% of the tracer was excreted in the bile with 74% of radioactivity as T_3G, 8% as T_3S and <1% as 3,3'-T_2S. In the same time period biliary excretion of rats treated with PTU amounted to 36% of the total dose due to a dramatic increase in T_3S and 3,3'-T_2S excretion, while T_3G production was not affected. The enhanced excretion of 3,3'-T_2S in the latter situation may be explained by extrahepatic T_3 to 3,3'-T_2 conversion (e.g. by PTU-insensitive type III IRD in the brain and skin), clearance of 3,3'-T_2 by the liver (30) and subsequent sulfation (58). The product 3,3'-T_2S is excreted in the bile, as of course ORD of 3,3'-T_2S is also inhibited by PTU. Evidence for this route was obtained by Rutgers et al (94). However, as described above, the presence of a type III-like deiodinase in the liver also contributes to the excretion of 3,3'-T_2S in bile.

Metabolism of T_4 and T_3 in man

T_4G was demonstrated in human bile by Myant in 1956 (95), but less is known about the quantitative role of this pathway nor of the possible existence of a sulfation pathway for T_4 in humans. The presence of T_3G has been demonstrated in bile, and T_3S in plasma and bile of a hypothyroid patient after iv injection of [^{125}I]T_3 (96). In chapter VII we used the RIA for T_3S to study the importance of sulfation for the metabolism of T_3 in

man. Normally, T_3S levels are at or below the detection limit of this RIA (<0.1 nM). Serum T_3S became detectable by treatment of volunteers with replacement doses of T_3. Treatment in addition with PTU and especially with IOP increased T_3S levels 2.5 to 3-fold. However, the serum T_3S/T_3 ratio is at least 10-fold lower in humans than in rats, suggesting that sulfation is a less important route for T_3 metabolism in humans than in rat. However, as described in chapter VII other possible explanations have not been excluded to elucidate these differences.

Enterohepatic cycle

As described above glucuronidated compounds are excreted in the urine as well as in the bile. Whereas renal excretion of a glucuronide invariably results in the irreversible elimination of the substance, this is not necessarily true for biliary excretion. After passage to the intestinal lumen glucuronides may become substrates for the β-glucuronidases produced by bacterial strains of the microflora. The liberated compound may then be reabsorbed.

Enterohepatic cycle for thyroid hormone

The existence of an enterohepatic cycle for thyroid hormone was previously postulated but only recently made plausible for the rat (for a review, see ref. 97). Evidence for this enterohepatic cycle is based on the following: 1) strains of anaerobic bacteria, belonging to the major residents of the intestinal flora, have been isolated and identified, which are able to hydrolyse iodothyronine conjugates; 2) In normal rats orally administered ^{125}I-T_3G was recovered as plasma radioactive T_3 in similar quantities as that observed after oral administration of labeled T_3. In feces of these rats no T_3G was detectable; and 3) In rats decontaminated with antibiotics little hydrolysis of orally administered T_3G was observed and T_3G was excreted unaltered into the feces. In these rats resorption of T_3 from administered T_3G was strongly diminished, although plasma T_3 after oral T_3 administration was actually enhanced in decontaminated rats. Quantitative data are needed to fully establish the role of the enterohepatic cycle for T_3 and T_4.

Sulfatase-producing strains have also been identified in the intestinal micoflora of rats and humans (97). However, since biliary excretion of iodothyronine sulfates is normally low, intestinal hydrolysis of these conjugates contributes little to the pool of free iodothyronines available

for resorption. In pathological situations, however, e.g. as in hypothyroidism, this pathway may increase in significance due to the rise in biliary T_3S excretion (90-93).

Apart from the above-mentioned identification of iodothyronine glucuronidase-producing microflora, only indirect data on the existence of an enterohepatic cycle for thyroid hormone in humans are available. Biliary clearance of T_4 amounts to about one third of total body clearance, of which perhaps 30-50% is reabsorbed (98). Pancreatic disease, the ingestion of soy flour or cholestyramine disrupt this cycle and has been shown to increase T_4 turnover appreciably (98). Development of techniques for the biosynthetic preparation of T_3G and T_4G (99) will undoubtedly stimulate further research into this area.

Quantitative data for the glucuronidation pathway

The importance of the glucuronidation pathway for thyroid hormone metabolism in vivo was studied in the homozygous Gunn rat, a species which carries an hereditary defect in hepatic UDPGT activity. This defect is expressed as congenital hyperbilirubinaemia. Various changes in thyroid hormonal status have been described in this strain. Serum T_4 is elevated 100-102) and serum T_3 is decreased (101,103) or equal (100) compared to normal Wistar or heterozygous (i.e. phenotypically normal) Gunn rats. Biliary clearance of plasma ^{125}I-T_4 was decreased 3-fold, while excretion of iv injected labeled T_4G was not affected in the Gunn rat. This suggests that indeed deficiency of thyroid hormone UDPGT(s) is the cause of the reduced glucuronide excretion (104).

Flock and coworkers undertook a detailed analysis of the biliary and renal clearance of iv injected ^{131}I-T_4 or ^{131}I-T_3 in Gunn and normal rats (80). After administration of these iodothyronines, biliary excretion of T_4G or T_3G was 4 and 3-fold lower, respectively, in the Gunn rats, while renal excretion of T_4 and T_3 metabolites was increased 20% and 40%, respectively. Taken together, these findings suggest that due to diminished hepatic glucuronidation, renal excretion of T_4 and T_3 metabolites is increased. In order to compensate for this (irreversible) renal loss and to maintain near normal serum T_3 levels, thyroid T_4 production is stimulated by increased TSH levels and serum T_4 is elevated. Diminished hepatic T_3 production, which might be due to the high endogenous levels of unconjugated bilirubin, may play an additional pathophysiological role in these rats (103).

In patients with minor abnormalities of glucuronide conjugation, such as Gilbert's disease, there is a reduction of conjugated T_4 in the bile without evident repercussions on the thyroid function (105). No data are available concerning thyroid hormone metabolism in persons suffering from the Crigler-Najjar syndrome, the human equivalent of the homozygous Gunn rat.

Summary of the hepatic metabolism of iodothyronines in rats

The data in the preceding paragraphs on the metabolism of iodothyronines in rat liver are summarized in Fig. 4. Iodide, released in the various deiodinative steps, is released into the circulation (not shown). An active transport system is involved in the passage of thyroid hormones from the plasma compartment across the liver cell membrane. T_4 and rT_3 share the same pathway, which differs from that of T_3. Intracellularly, T_4 is converted by type I deiodination to either T_3 or rT_3, or glucuronidated to T_4G, which product is excreted into the bile. Since little is known of the precise role of sulfation in the metabolism of T_4, this pathway is omitted here.

The major part of intrahepatically formed T_3 is released into the circulation. The remainder, in combination with T_3 taken up from the plasma, is subjected to three pathways. The first pathway is glucuronidation, and the product T_3G is excreted into the bile. Secondly, T_3 is sulfated to T_3S, which is mainly further metabolized by type I IRD to 3,3'-T_2S, but also partly excreted into the bile. The third pathway comprises direct IRD by a type III-like deiodinase to 3,3'-T_2.

Intrahepatic rT_3, which is formed by IRD of T_4 or cleared from the plasma, is rapidly degraded by type I ORD to 3,3'-T_2. Glucuronidation and sulfation of rT_3 are relatively minor pathways and are omitted.

As described above 3,3'-T_2 is formed intracellularly via two pathways, i.e. by IRD of T_3 and ORD of rT_3. It is sulfated to 3,3'-T_2S, which product is further metabolized by type I ORD.

The biliary excretion products T_4G, T_3G and T_3S are hydrolyzed by β-glucuronidase and sulfatase producing anaerobic strains in the intestinal microflora. The liberated hormones may then be partly reabsorbed, partly preserving these hormones for the body.

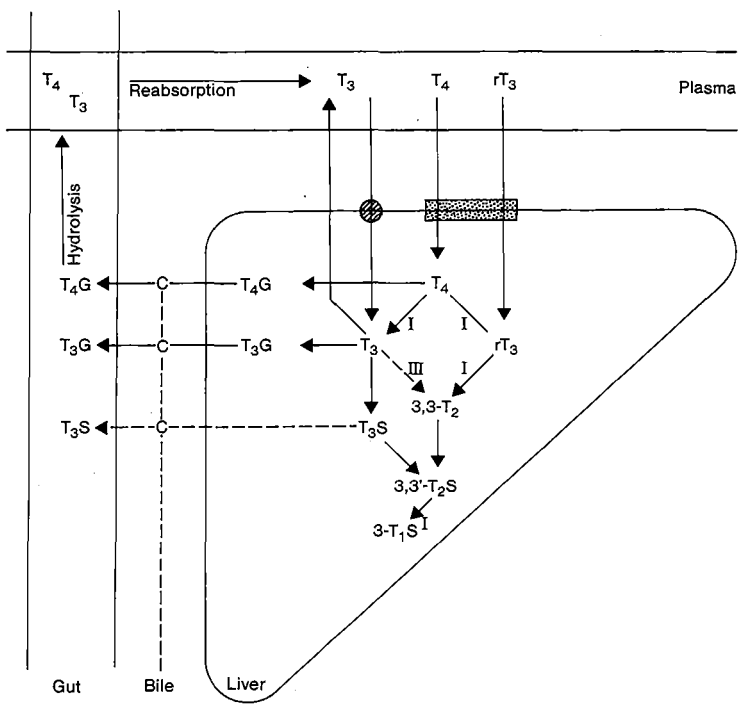

Figure 4
Metabolism of iodothyronines.

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CHAPTER III

METABOLISM OF REVERSE TRIIODOTHYRONINE BY ISOLATED RAT HEPATOCYTES

Metabolism of Reverse Triiodothyronine by Isolated Rat Hepatocytes

Sebo Jan Eelkman Rooda, Marla A. C. van Loon, and Theo J. Visser
Department of Internal Medicine III and Clinical Endocrinology, Erasmus University Medical School, Rotterdam, The Netherlands

Abstract

Reverse triiodothyronine (rT_3) is metabolized predominantly by outer ring deiodination to 3,3'-diiodothyronine (3,3'-T_2) in the liver. Metabolism of rT_3 and 3,3'-T_2 by isolated rat hepatocytes was analyzed by Sephadex LH-20 chromatography, high performance liquid chromatography, and radioimmunoassay, with closely agreeing results. Deiodinase activity was inhibited with propylthiouracil (PTU) and sulfotransferase activity by sulfate depletion or addition of salicylamide or dichloronitrophenol. Normally, little 3,3'-T_2 production from rT_3 was observed, and $^{125}I^-$ was the main product of both 3,[3'-^{125}I]T_2 and [3',5'-^{125}I]rT_3. PTU inhibited rT_3 metabolism but did not affect 3,3'-T_2 clearance as explained by accumulation of 3,3'-T_2 sulfate. Inhibition of sulfation did not affect rT_3 clearance but 3,3'-T_2 metabolism was greatly diminished. The decrease in I^- formation from rT_3 was compensated by an increased recovery of 3,3'-T_2 up to 70% of rT_3 metabolized. In conclusion, significant production of 3,3'-T_2 from rT_3 by rat hepatocytes is only observed if further sulfation is inhibited.

Introduction

In euthyroid subjects the main secretory product of the thyroid is thyroxine (T_4).[1] Some 3,3',5-triiodothyronine (T_3) is secreted as well, but thyroidal production of 3,3',5'-triiodothyronine (rT_3) is negligible. More than 97.5% of plasma rT_3 and ~ 80% of plasma T_3 originate from peripheral deiodination of T_4 (1). As thyroid hormone bioactivity is exerted largely through T_3, it is important to understand the regulatory mechanisms of iodothyronine metabolism.

In rats, three types of iodothyronine-deiodinating enzymes have been identified (2). Most likely, the type I deiodinase of liver catalyzes both inner ring deiodination (IRD) and outer ring deiodination (ORD) of iodothyronines (2). A similar enzyme is present in kidney and thyroid (2). Type II deiodinase has been localized in brain, pituitary, brown adipose tissue, and placenta. It deiodinates only the outer ring of substrates such as T_4 and rT_3 (2–4). Type III enzyme is found in brain, placenta, and skin, and it is a specific inner ring deiodinase (2, 5). Type I deiodinase is inhibited by 6-propylthiouracil (PTU), while types II and III are PTU insensitive (2). Thus, there may be two sources of plasma rT_3, namely type I or type III IRD of T_4. There are also two pathways of rT_3 deiodination, i.e., type I and type II ORD to 3,3'-diiodothyronine (3,3'-T_2).

Considering the high rate of rT_3 ORD by the type I deiodinase (2), it is likely that little rT_3 produced from T_4 in the liver is released into the circulation. It would seem, therefore, that most plasma rT_3 is derived from type III deiodination of T_4, while it is cleared mainly by the liver. This hypothesis is substantiated by measurements of arterio-venous gradients of rT_3 across the liver in patients with mild liver failure.[2]

In vivo studies in normal rats have demonstrated that the type I deiodinase is the predominant site for the peripheral production of T_3 (6). Opposite variation in plasma T_3 and rT_3 concentrations has been observed in a number of clinical situations, in which changes are due to a decrease in both the production of plasma T_3 and the clearance of plasma rT_3 (7). To investigate the potential importance of changes in type I deiodinase activity for the regulation of thyroid hormone metabolism, we initiated studies of the deiodination of rT_3 by isolated rat hepatocytes.

Initial results, using outer ring ^{125}I-labeled rT_3, showed that radioiodide was the main product, but little production of 3,3'-T_2 from unlabeled rT_3 could be detected by radioimmunoassay (RIA) (8). Further investigations have demonstrated rapid metabolism of added 3,3'-T_2 in rat hepatocytes by sulfation and subsequent ORD of the 3,3'-T_2 sulfate (3,3'-T_2S) formed (9). 3,3'-T_2S is a far better substrate for the type I deiodinase than 3,3'-T_2 itself (9). This may be the reason for our failure to detect significant production of 3,3'-T_2 from rT_3 by liver cells. If so, the yield of 3,3'-T_2 produced by this pathway should increase if its further sulfation is inhibited. This hypothesis was tested in the present study using rat hepatocytes with diminished phenol sulfotransferase activity.

Methods

The materials used are essentially the same as described previously (10). Carrier-free 3,[3'-^{125}I]T_2 and [3',5'-^{125}I]rT_3 were prepared in our laboratory by radioiodination of 3-iodothyronine or of 3,3'-T_2 (Henning GmbH, Berlin, Federal Republic of Germany) using the chloramine T method (11, 12) and purified by Sephadex LH-20 chromatography. Salicylamide (SAM) and 2,6-dichloro-4-nitrophenol (DCNP) were purchased from Riedel-de Haën AG, Hannover, Federal Republic of Germany. All other chemicals were of the highest quality commercially available.

Hepatocytes. Rat hepatocytes were prepared by collagenase perfusion (10). Monolayers of hepatocytes were obtained by seeding 10^6 cells in 2 ml culture medium (10) into uncoated 3.5-cm wells of plastic 6-well dishes (Nunc, Roskilde, Denmark). The plates were kept for 4 h at 37°C

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Address correspondence to Dr. Visser, Dept. of Internal Medicine III, Erasmus U. Medical School, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

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1. *Abbreviations used in this paper:* DCNP, 2,6-dichloro-4-nitrophenol; G, glucuronide; IRD, inner ring deiodination; ORD, outer ring deiodination; PTU, propylthiouracil; rT_3, 3,3',5'-triiodothyronine; S, sulfate; SAM, salicylamide; T_2, diiodothyronine, T_3, 3,3',5-triiodothyronine; T_4, thyroxine.

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in a culture stove under atmospheric conditions. Before each experiment, cell viability was tested by trypan blue exclusion and exceeded 85%. Nonviable cells were removed by aspiration of the medium.

General incubation procedures. Incubations were done for 60 min in 2 ml of Dulbecco's balanced salt solution which contained 1 mM $MgSO_4$, 0.1% bovine serum albumin (BSA), 2 mM glutamine, and 1 mM vitamin C. Experiments were carried out in triplicate under atmospheric conditions at 37°C. The dishes were placed on a slightly angled, slowly rotating plate. Substrate levels were 10 nM rT_3 or 3,3'-T_2 with or without 0.1 μCi [^{125}I]rT_3 or [^{125}I]3,3'-T_2, respectively. Sulfation was inhibited with 100 μM SAM or 100 μM DCNP and deiodination was inhibited with 10 μM PTU.

Sulfate depletion. Hepatocytes were preincubated for 30 min at 37°C in Dulbecco's solution which contained 1 mM $MgCl_2$, 2 mM glutamine, and 1 mM vitamin C with or without 100 μM SAM. Incubations were done as described above in medium without SO_4^{2-}. Controls were preincubated and incubated in medium containing $MgSO_4$.

Analysis of incubation medium. Incubation media with unlabeled rT_3 and 3,3'-T_2 were analyzed before and after hydrolysis with specific RIAs (11, 12). Hydrolysis of eventual S conjugates was achieved by addition of 250 μl 1 N HCl to 100-μl samples and treatment for 1 h at 80°C (13). Thereafter, 300 μl 1 N NaOH was added. The nonhydrolyzed samples were treated the same way but without heating. RIA was done in duplicate on 50-μl aliquots of the mixtures.

Incubation media with labeled rT_3 and 3,3'-T_2 were assayed by column chromatography. An equal volume of 1 N HCl was added to 500-μl samples and the mixtures were applied to small (bed volume 0.75 ml) Sephadex LH-20 columns equilibrated in 0.1 N HCl. Stepwise elution was done by successive application of 2 × 1 ml 0.1 N HCl, 6 × 1 ml H_2O, 6 × 1 ml 0.1 N NaOH-ethanol (9:1, vol/vol), and 3 × 1 ml 0.1 N NaOH-ethanol (1:1, vol/vol).

High performance liquid chromatography (HPLC). Analysis of rT_3 and 3,3'-T_2 conjugates and native iodothyronines was accomplished by reverse-phase HPLC. For this we used a 10 × 0.3-cm CP Spher C18 column (Chrompack, Middelburg, The Netherlands), a model 6000 A solvent delivery system and a model 440 fixed wavelength detector (Waters Assoc., Millipore Corp., Milford, MA). Flow was 0.6 ml/min and absorbance was measured at 254 nm. For separation of conjugates a 20:80 vol/vol mixture of acetonitrile and 0.02 M ammonium acetate (pH 4) was used, and for separation of rT_3 and 3,3'-T_2 a 55:45 vol/vol mixture of methanol and 0.02 M ammonium acetate (pH 4) was employed.

To obtain the conjugates for HPLC analysis, the water fractions of the LH-20 chromatography were pooled, acidified, and rechromatographed. The columns were washed with 2 ml 0.1 N HCl, 0.5 ml H_2O, and 0.5 ml 0.1 M ammonia in ethanol. Conjugates were then collected in a subsequent fraction of 1 ml 0.1 M ammonia in ethanol. The solvent was evaporated at 50°C under a stream of N_2. To obtain the iodothyronine fraction, medium was processed as above and LH-20 chromatography was modified as follows. After the H_2O fractions, columns were washed with 0.5 ml 0.1 M ammonia-ethanol (1:1 vol/vol), and iodothyronines were collected in 1 ml 0.1 M ammonia in ethanol. The solvent was evaporated as above.

Enzymatic analysis of rT_3 and 3,3'-T_2 conjugates. Another method by which the conjugates were identified was by enzymatic hydrolysis with glucuronidase and sulfatase in the presence or absence of saccharic acid lactone (10). Hydrolysis was quantified by LH-20 chromatography and the liberated iodothyronines were identified by HPLC.

Analysis of cell content. Cell-associated radioactivity was extracted after removing the medium by immediate addition of 1 ml 0.1 N NaOH. After centrifugation, 0.5 ml of supernatant was chromatographed on LH-20 as described above for medium.

Miscellaneous. The influence of SAM (10–1000 μM) and DCNP (1–100 μM) on the type I deiodinase was tested by measurement of their effects on the release of $^{125}I^-$ from 10 nM [^{125}I]rT_3 in incubations with rat liver microsomes (14) in 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA, and 5 mM dithiothreitol (DTT). The free fractions of rT_3 and 3,3'-T_2 in incubation medium were determined by equilibrium dialysis. Cellular ATP content was measured according to the method described by Jaworek et al. (15).

Data analysis. In each experiment, I^- production was corrected for the amount of I^- recovered from control incubations, while the 3,3'-T_2 production from rT_3 was corrected for the slight contamination of the 3,3'-T_2 fraction with rT_3. Production of unlabeled 3,3'-T_2 was corrected for rT_3 crossreactivity (0.03%) in the 3,3'-T_2 RIA. Statistical analysis was done by Student's t test for unpaired data.

Results

Sephadex LH-20 chromatography. The Sephadex LH-20 chromatographic pattern of I^-, 3,3'-T_2S, 3,3'-T_2, and rT_3 is depicted in Fig. 1. More than 97% of $^{125}I^-$ activity was found in fractions 1–4. Recovery of 3,3'-T_2S, eluting in the H_2O fractions, was > 97.5%. All other 3,3'-T_2 and rT_3 conjugates eluted also in fractions 5–9. Approximately 97% of 3,3'-T_2 was found in fractions 10–15, while fractions 16–18 contained at least 95% of rT_3. Thus, there was little overlap of fractions containing the different metabolites, although some 4% of rT_3 eluted in the 3,3'-T_2 fractions. This was not due to contamination of rT_3 tracer with 3,3'-T_2 as checked by HPLC.

Time dependence of 3,3'-T_2 and rT_3 metabolism. Fig. 2 shows the LH-20 analysis of the main products in the medium generated from 3,3'-T_2 and rT_3 as a function of time of incubation in the absence or presence of SAM. In 3,3'-T_2 incubations I^-

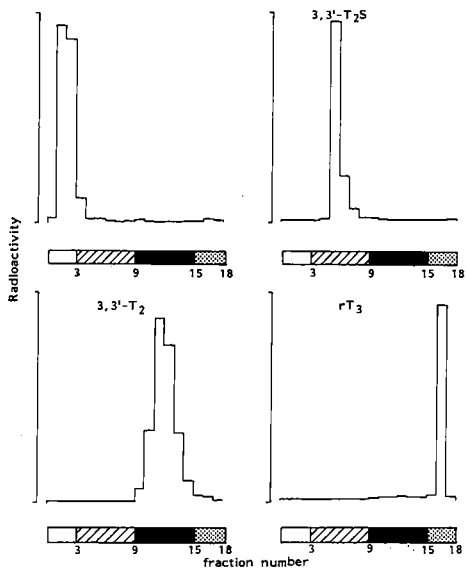

Figure 1. Sephadex LH-20 chromatography of iodide, 3,3'-T_2S, 3,3'-T_2, and rT_3. Each ^{125}I-labeled compound was applied separately in 1 ml 0.5 N HCl to a small Sephadex LH-20 column with a bed volume of 0.75 ml which was equilibrated in 0.1 N HCl. Subsequently, columns were eluted using 2 ml 0.1 N HCl (□), 6 ml H_2O (▩), 6 ml ethanol-0.1 N NaOH (1:9 vol/vol) (■), and finally 3 ml ethanol-0.1 N NaOH (1:1 vol/vol) (□). Each 1-ml fraction was counted for radioactivity.

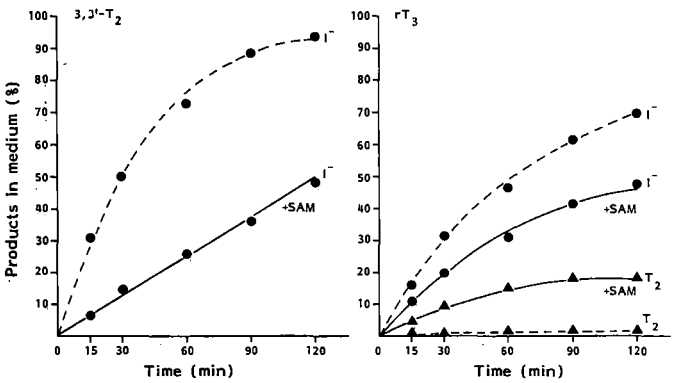

Figure 2. Time dependence of 3,3'-T_2 and rT_3 metabolism. 10 nM 3,3'-T_2 or rT_3 with 0.1 μCi [^{125}I]3,3'-T_2 or [^{125}I]rT_3 were incubated with 10^6 hepatocytes in 2 ml S-supplemented Dulbecco's medium without (*dashed line*) or with (*solid line*) 100 μM SAM. After 15, 30, 60, 90, and 120 min incubation was stopped by removing the medium that was analyzed by Sephadex LH-20 chromatography. Production of I^- from 3,3'-T_2 (*left*) and of I^- and 3,3'-T_2 from rT_3 (*right*) are expressed as percentage of total radioactivity in medium. Note that no correction was made for the fact that the specific radioactivity of the I^- and 3,3'-T_2 produced is only half of that of added rT_3. Data are taken from a representative experiment with results that were reproduced on at least two other occasions.

was the main metabolite, the amount of which was inversely correlated with that of remaining 3,3'-T_2. The semi-logarithmic plot of the latter against time demonstrated that the disappearance of 3,3'-T_2 followed first order kinetics with a rate constant of 0.031 min^{-1}. Addition of SAM strongly inhibited I^- formation. In the presence of this inhibitor 3,3'-T_2 clearance remained a first order process with a rate constant of 0.007 min^{-1}.

In rT_3 incubations I^- was also the main metabolite found, while 3,3'-T_2 formation was undetectable under control conditions. Addition of SAM resulted in the inhibition of I^- release with a reciprocal accumulation of 3,3'-T_2. Clearance of rT_3 was also a first order process, characterized by a rate constant of 0.014 min^{-1}, which was not influenced by SAM. The identity of the 3,3'-T_2 produced was further tested by HPLC analysis (Fig. 3). Little or no 3,3'-T_2 formation could be detected in control incubations by HPLC, while in the presence of SAM 3,3'-T_2 accumulation was substantial. Relative to the quantity of rT_3 remaining after 30, 60, and 120 min, 3,3'-T_2 accumulation amounted to 11, 33, and 80%, respectively.

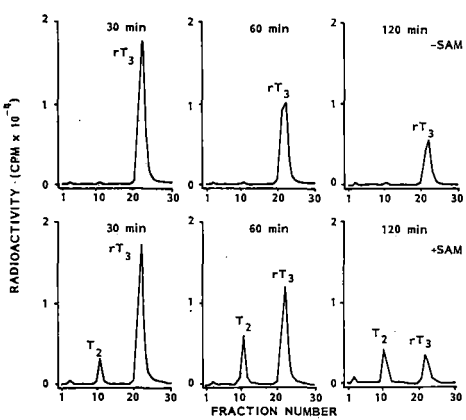

Figure 3. HPLC analysis of iodothyronines recovered from incubations of rT_3 with hepatocytes. Parallel samples of the same incubations described in Fig. 2 were chromatographed on Sephadex LH-20 modified such that 3,3'-T_2 and rT_3 were eluted collectively with 0.1 M ammonia in ethanol (see Methods). Solvent was evaporated, the residue taken up in a 55:45 vol/vol mixture of methanol and 0.02 M ammonium acetate (pH 4) and subjected to HPLC using the same solvent at a flow of 0.6 ml/min. Fractions of 0.3 ml were collected and counted for radioactivity. The elution position of 3,3'-T_2 and rT_3 was determined by monitoring absorbance at 254 nm after application of pure synthetic substances.

Dose-dependent effects of SAM (Fig. 4). As previously shown (10), addition of 10 μM PTU greatly inhibited $^{125}I^-$ production in incubations with 3,3'-T_2, but did not affect the clearance of this compound. Most radioactivity recovered from the medium now eluted in the water fractions of the LH-20 chromatography, which was shown to consist mainly of 3,3'-T_2S (see below). Addition of PTU also led to an increase in cell-associated radioactivity, from < 5% in the absence to 22% in the presence of the inhibitor. This radioactivity was predominantly in the form of conjugates and presumably represented the accumulation of 3,3'-T_2S within the cells. Addition of 10–1,000 μM SAM resulted in a progressive decrease in the clearance of 3,3'-T_2 independent of PTU. This was indicated by the parallel decrease of I^- formation without PTU and of 3,3'-T_2S accumulation with PTU. With the SAM-induced inhibition of 3,3'-T_2 clearance there was an increase in cell-associated 3,3'-T_2, irrespective of PTU.

In contrast to 3,3'-T_2, rT_3 metabolism was almost completely blocked by PTU but was not affected by even 1,000 μM SAM. In the absence of SAM, little 3,3'-T_2 production was observed in the medium once again, and no 3,3'-T_2 was detectable in the cells. With increasing SAM concentrations, 3,3'-T_2 accumulation in the medium was accompanied by a rise in cellular radioactivity from 5 to 12%, part of which was in the form of 3,3'-T_2. Based on the dose-effect relationship for SAM on the metabolism of both 3,3'-T_2 and rT_3, 100 μM was chosen as the near-maximal sulfotransferase inhibitory concentration.

3,3'-T_2 and rT_3 metabolism; correlation between LH-20 and RIA. Table I shows the effects of 100 μM SAM or DCNP or 10 μM PTU on 3,3'-T_2 and rT_3 metabolism as measured by LH-20 chromatography or by RIA. To compare the results obtained by these two methods it has to be taken into account that (*a*) LH-20 data are not corrected for cell-associated radioactivity

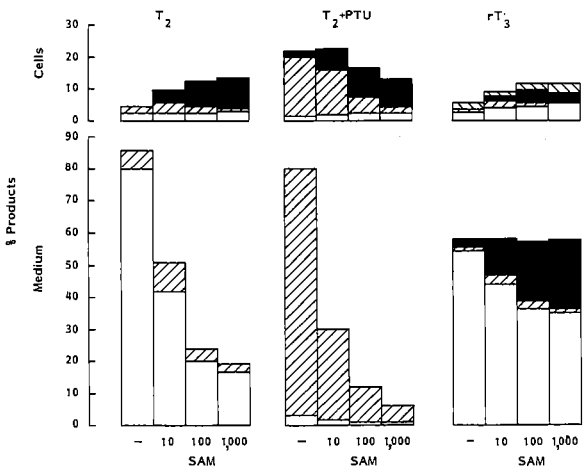

Figure 4. Dose-effect relationship of SAM on the metabolism of 3,3'-T_2 and rT_3. Hepatocytes were incubated with 10 nM [^{125}I]3,3'-T_2 or [^{125}I]rT_3 in S-supplemented Dulbecco's medium. SAM was added at concentrations of 10, 100, and 1,000 μM, and PTU at 10 μM. After 60 min medium was removed and cells were extracted immediately by adding 1 ml 0.1 N NaOH. Medium samples of 0.5 ml or supernatant obtained after centrifugation of the cellular extracts were acidified with 0.5 ml 1 N HCl and chromatographed on LH-20. Products in medium are given as percentage of radioactivity in medium, while products in cells are expressed as percentage of total radioactivity added. Completion of medium radioactivity to 100% represents the proportion of unaltered substrate. No correction is made for difference in specific radioactivity between substrate and products in rT_3 incubations. Typical results are shown from one out of three closely agreeing experiments. (□) I^-; (▨) conjugates; (■) 3,3'-T_2; and (▫) rT_3.

(Fig. 4), and (*b*) the specific radioactivity of the 3,3'-T_2 produced is half that of added rT_3. With this in mind there exists a good correlation between the two methods.

After incubation for 60 min with [^{125}I]3,3'-T_2, 80% of radioactivity in the medium was recovered as $^{125}I^-$. SAM, DCNP, and PTU reduced I^- formation by 64, 57, and 95%, and 3,3'-T_2 clearance by 59, 54, and 10%, respectively. If remaining 3,3'-T_2 in medium was measured by RIA, inhibition of the clearance of 3,3'-T_2 by these treatments was 52, 62, and 1%, respectively. The 3,3'-T_2S accumulating in the presence of PTU as observed in the incubations with [^{125}I]3,3'-T_2 was largely recovered as immunoreactive 3,3'-T_2 after hydrolysis of medium from parallel incubations with unlabeled 3,3'-T_2. No 3,3'-T_2 was liberated by hydrolysis in all other experimental conditions.

Table I. Comparison of RIA and Sephadex LH-20 Analyses of the Effects of SAM, DCNP, and PTU on the Metabolism of 3,3'-T_2 and rT_3 by Rat Hepatocytes

	Products in medium								
	LH-20					RIA			
Condition	*n*	I^-	Conjugates	3,3'-T_2	rT_3	*n*	3,3'-T_2(−)	3,3'-T_2(+)	rT_3
3,3'-T_2 30 min									
Control	9	48.2±4.0	3.9±0.6	44.5±4.4		6	44.9±2.9	48.8±2.5	
SAM	8	11.5±1.9*	2.3±0.2†	83.5±1.9*		4	67.7±2.2*	68.8±2.2*	
DCNP	6	17.4±2.1*	2.2±0.3†	77.5±2.3*		3	63.8±0.9‡	64.6±3.3‖	
PTU	4	4.7±1.3*	26.3±5.3*	65.6±6.5‖		3	45.8±2.4	63.4±1.3‖	
3,3'-T_2 60 min									
Control	10	79.0±2.4	5.7±1.2	12.9±1.6		6	12.7±1.0	16.1±1.3	
SAM	9	28.3±3.3*	4.6±0.8	63.4±3.5*		5	54.9±3.3*	55.9±4.2*	
DCNP	6	33.7±4.9*	5.6±0.9	58.5±5.8*		3	45.9±8.5*	48.5±5.8*	
PTU	5	4.4±1.3*	68.9±2.4*	21.8±3.2‖		3	13.7±2.6	52.8±3.8*	
rT_3 60 min									
Control	11	47.7±3.9	1.4±0.2	0.6±0.1	43.9±3.3	6	1.8±0.4	1.9±0.6	53.8±3.9
SAM	10	34.5±3.2‖	1.8±0.3	14.5±0.5*	42.7±2.9	5	24.7±2.5*	24.9±2.3*	53.9±4.6
DCNP	6	44.5±4.3	2.2±0.4	16.9±1.9*	33.1±6.0	3	24.6±3.1*	25.9±1.9*	30.9±6.7‖
PTU	5	1.8±0.6*	5.3±0.7*	−0.4±0.3‡	87.0±2.0*	3	2.1±0.8	1.7±0.9	84.8±3.0‡

10 nM labeled or unlabeled 3,3'-T_2 or rT_3 were incubated for 30 or 60 min at 37°C with 10^6 rat hepatocytes in 2 ml S-supplemented Dulbecco's medium with or without 100 μM SAM or DCNP or 10 μM PTU. Radioactive products were separated on Sephadex LH-20 and unlabeled 3,3'-T_2, and rT_3 were measured by RIA before (−) and after (+) hydrolysis as described in Methods. Since acid treatment did not change rT_3 levels, mean values of these two measurements are given. RIA values are expressed as percentage of iodothyronines measured after incubations without cells. The results are given as the mean±SE of the number of observations indicated. Statistical analysis of differences between experimental and control conditions was done by Student's unpaired *t* test: * $P < 0.001$. ‡ $P < 0.005$. ‖ $P < 0.01$. ‖ $P < 0.025$. † $P < 0.05$.

After incubation for 60 min with [^{125}I]rT_3, 48% of radioactivity in the medium was accounted for by $^{125}I^-$, while the sum of the conjugate and 3,3'-T_2 fractions was only 2%. Again, rT_3 disappearance was not changed by SAM, but I^- formation was decreased by 13.2% (~ 2.6 nM I^-) while 3,3'-T_2 accumulation was increased by 13.9% (~ 2.8 nM 3,3'-T_2). These results correspond with an increase of immunoassayable 3,3'-T_2 by 23.0% (~ 2.3 nM 3,3'-T_2). On the basis of the results obtained by LH-20 chromatography and by RIA, it was calculated that with SAM 51 and 54%, respectively, of the amount of rT_3 cleared was recovered as medium 3,3'-T_2. Similar results were obtained with DCNP, except that the acceleration of rT_3 metabolism seen with this inhibitor was different. PTU inhibited rT_3 disappearance from the medium by 77 and 67% as estimated by LH-20 and RIA, respectively.

S dependence of 3,3'-T_2 and rT_3 metabolism. As discussed above, 3,3'-T_2 was metabolized predominantly by successive sulfation and ORD. The small amount of conjugates found after incubation of 3,3'-T_2 in S-containing medium (Fig. 5) was shown by HPLC to consist of similar proportions of 3,3'-T_2 glucuronide (3,3'-T_2G) and 3,3'-T_2S (Fig. 6). In the presence of PTU, I^- formation was again strongly suppressed (Fig. 5), while radioactivity in the conjugate fraction almost entirely eluted in the position of 3,3'-T_2S with no change in the absolute amount of 3,3'-T_2G recovered (Fig. 6). If cells were preincubated with S-free medium and incubated with 3,3'-T_2 in this medium plus PTU, conjugation and clearance were diminished by 55%. A further reduction in 3,3'-T_2 conjugation to 30% was observed if SAM was added to the preincubation, while the experiment was otherwise conducted under the same S-free conditions (Fig. 5). HPLC analysis of the conjugate fraction demonstrated that 3,3'-T_2S formation was virtually blocked, and that now most radioactivity eluted in the position of 3,3'-T_2G (Fig. 6). In the absence of PTU, I^- release was decreased from 74 to 38% by omission of S from the medium, and further to 17% after addition of SAM to the preincubation. However, conjugates represented an increasing proportion of the radioactivity in the medium, i.e., 7, 10, and 15%, respectively (Fig. 5). HPLC revealed that this increase was totally accounted for by a rise in 3,3'-T_2G (Fig. 6).

Clearance of rT_3 was not S dependent. Iodide production, however, decreased from 48% in the presence of S to 38% in the absence of S, and further to 33% after preincubation with SAM. The loss of I^- was completely compensated for by the accumulation of 3,3'-T_2, which amounted to 1, 9, and 14% under these conditions, respectively (Fig. 5). In S-supplemented incubations the conjugate fraction contained small amounts of rT_3G, 3,3'-T_2G, and 3,3'-T_2S. S depletion induced an increase in conjugates mostly in the form of 3,3'-T_2G, although even after preincubation with SAM the conjugate fraction comprised only 5% of medium radioactivity. Inhibition of deiodination by PTU augmented the appearance of conjugates in S-replete incubations (Table I, Fig. 6). In S-deplete cultures, addition of PTU led to a decrease in T_2G and an increase in rT_3G without a change in the total amount of conjugate formed (Fig. 6).

Effect of substrate concentration on rT_3 metabolism (Fig. 7). In S-replete incubations, progressive increases in unlabeled rT_3 concentration from 1 to 1,000 nM led to minimal increments in the recovery of immunoassayable 3,3'-T_2 from the medium. Medium 3,3'-T_2 ranged from undetectable after incubation with 1 nM rT_3 until maximally 1.9 pmol per dish (0.1%) at 1,000 nM rT_3. After addition of SAM, accumulation of 3,3'-T_2 rose from 0.8 pmol/dish (38%) at 1 nM rT_3 to 29.7 pmol/dish (1.5%) at 1,000 nM rT_3. The results indicated that 3,3'-T_2 accumulation under these conditions was a saturable process; it was half-maximal between 10 and 100 nM rT_3. Cell-associated 3,3'-T_2 was similarly dependent on substrate concentration.

Effects of SAM and DCNP on free substrate levels, cellular integrity, and deiodinase activity (Table II). In the absence of inhibitors, the free fraction of 3,3'-T_2 and rT_3 in Dulbecco's medium containing 0.1% BSA was 8.5 and 4.9%, respectively. Up to 1,000 μM, SAM did not influence the free 3,3'-T_2 and rT_3 fractions. DCNP had no effect on free 3,3'-T_2, while there was a dose-dependent increase in free rT_3 up to 7.8% at 100 μM DCNP.

Up to 100 μM, SAM did not inhibit rT_3 deiodination by rat liver microsomes, but a 25% reduction of I^- release was observed at 1,000 μM SAM. In contrast, 100 μM DCNP resulted in a 65% lowering of deiodinase activity, while only slight inhibition was noted at 10 μM.

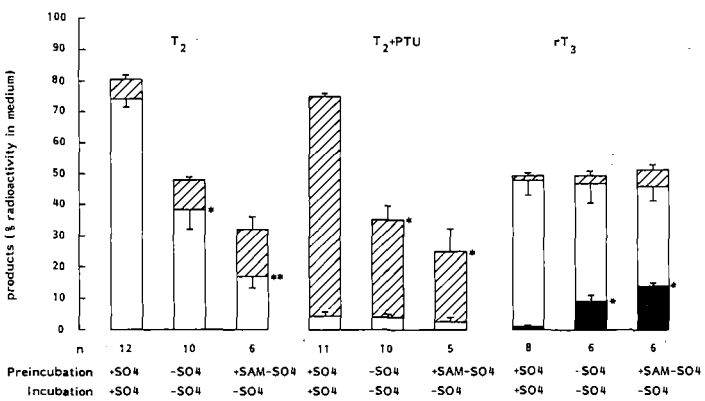

Figure 5. S dependence of 3,3'-T_2 and rT_3 metabolism. Monolayers of 10^6 hepatocytes were preincubated for 30 min in S-supplemented medium or in S-free medium plus or minus 100 μM SAM. Subsequently, incubations were performed for 60 min with 10 nM [^{125}I]3,3'-T_2 or [^{125}I]rT_3 in S-replete or -deplete medium. PTU was added at 10 μM. Medium was analyzed by Sephadex LH-20 chromatography and data are expressed as mean±SE of the number of observations indicated. Completion of medium radioactivity to 100% represents the proportion of unaltered substrate. No correction is made for difference in specific radioactivity between substrate and products in rT_3 incubations. (□) I^-; (▨) conjugates; and (■) 3,3'-T_2. * Significant difference with preincubation $+SO_4^{2-}$, $P < 0.001$. ** Significant difference with preincubation $-SO_4^{2-}$, $P < 0.05$.

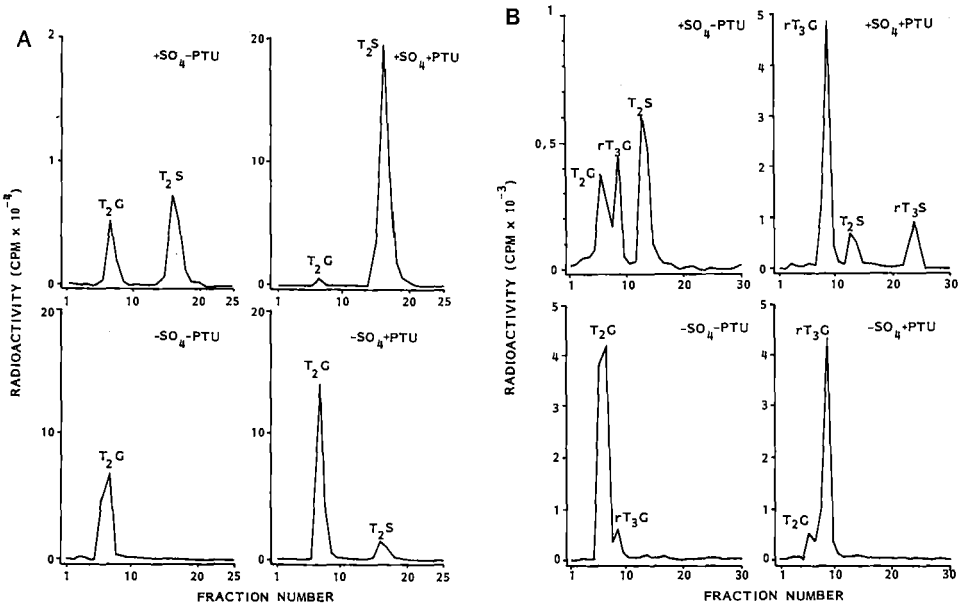

Figure 6. HPLC analysis of conjugates generated in incubations of (*A*) 3,3'-T_2 and (*B*) rT_3 with hepatocytes. Incubations were done with S-replete or -deplete cells after preincubation in medium with S or in medium without S plus 100 μM SAM as described in the legend to Fig. 5. Conjugates were isolated on Sephadex LH-20, concentrated on a second LH-20 column, and collected in ammonia-ethanol as described in Methods. After evaporation, the residue was analyzed by HPLC using a 20:80 vol/vol mixture of acetonitrile and 0.02 M ammonium acetate (pH 4) at a flow of 0.6 ml/min. Fractions of 0.3 ml were collected and counted for radioactivity. Elution position of S conjugates was determined using synthetic standards and conjugates were further identified by enzymatic liberation of 3,3'-T_2 or rT_3 after treatment with sulfatase or glucuronidase. Radioactivity eluting in the position of rT_3S was not hydrolyzed by sulfatase, in agreement with previous observations using synthetic rT_3S (13).

Cell viability as assessed by ATP measurements was not affected by SAM. However, a 35% reduction of cellular ATP was found after incubation with 100 μM DCNP.

Discussion

The occurrence of rT_3 and 3,3'-T_2 in rat thyroid as well as in rat plasma has already been reported by Roche et al. in 1955 (16–18). These workers also provided evidence for the conversion of both T_3 and rT_3 to 3,3'-T_2 in vivo by demonstration of the presence of radioactive 3,3'-T_2 in the kidney after administration of labeled T_3 or rT_3 to thyroidectomized rats (19). In humans, most plasma 3,3'-T_2 originates from IRD of T_3 (20, 21). Plasma 3,3'-T_2[2] (22) and rT_3[2] are cleared predominantly by the liver, which indicates that they are derived from extrahepatic IRD of T_3 and T_4, respectively. It is likely that these processes are catalyzed by the type III deiodinase, which specifically acts on the inner ring of iodothyronines and which has been localized in tissues such as brain and skin but not in liver (2, 5). In our studies we focused on the role of the type I deiodinase in the hepatic clearance of rT_3.

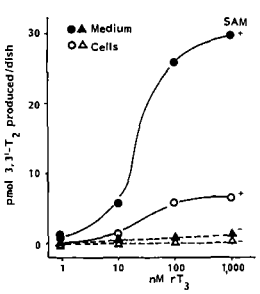

Figure 7. Conversion of rT_3 to 3,3'-T_2 by rat hepatocytes as a function of substrate concentration. Monolayers of 10^6 hepatocytes were incubated for 60 min with 1–1,000 nM unlabeled rT_3 in Dulbecco's medium containing S with or without 100 μM SAM. 3,3'-T_2 content was measured by RIA in duplicate in 50-μl aliquots of medium or 0.1 N NaOH extracts of cells. Data are taken from a representative experiment repeated on three different occasions with closely agreeing results.

Numerous studies have appeared on the characteristics of the deiodination of different iodothyronines by homogenates and subcellular fractions of rat liver (for a review, see reference 2). These studies have established the existence of a so-called type I deiodinase in the endoplasmic reticulum, which is an integral membrane protein and requires thiols for deiodinase activity. The enzyme is a nonspecific deiodinase that is capable of removing iodines from either ring of iodothyronine substrates (2). However, the enzyme is most effective in the ORD of rT_3 (2, 23). To test the possible regulatory function of this enzyme in peripheral thyroid hormone metabolism, we have therefore initiated studies of the breakdown of rT_3 by isolated rat hepa-

Table II. Effects of SAM and DCNP on Medium Free rT_3 and 3,3'-T_2 Levels, on Microsomal Deiodinase Activity, and on Cellular ATP Content

Treatment		Free fraction (%)*		Deiodinase‡ activity	ATP§
		rT_3	3,3'-T_2		
				Control (%)	*Control (%)*
Control		4.9	8.5	100	100
SAM	10 μM	4.4	8.7	107	100
	100 μM	4.6	8.4	103	85
	1,000 μM	4.8	8.7	77	94
DCNP	10 μM	6.0	8.8	89	97
	100 μM	7.8	9.2	35	67

* Free iodothyronine levels in medium were determined in triplicate by equilibrium dialysis.

‡ Deiodinase activity was measured in triplicate in mixtures of 10 nM [^{125}I]rT_3, 2 or 5 μg microsomal protein/ml, and 5 mM DTT in 0.1 M phosphate (pH 7.2) and 2 mM EDTA (14). After incubation for 15 min at 37°C, reactions were halted by addition of serum, and $^{125}I^-$ released was measured by TCA precipitation. Data are expressed as the means of the six observations relative to iodide production in the absence of inhibitors.

§ Cells were incubated for 1 h at 37°C with Dulbecco's medium plus S and the indicated concentrations of SAM and DCNP. ATP was measured in 0.2-M perchloric acid extracts (0.5 ml/10^6 cells) after centrifugation and neutralization with 2 M KOH according to the method of Jaworek et al. (15). Data are taken from a representative experiment repeated under different conditions with similar results.

tocytes. In spite of the high rate of rT_3 to 3,3'-T_2 conversion with isolated microsomes, and the stability of the product in such incubations (23), surprisingly little production of 3,3'-T_2 from rT_3 was observed in incubations with intact liver cells (8). That nevertheless rapid ORD took place in this system was demonstrated by the large amounts of radioiodide released from outer ring labeled rT_3 (8). This implies that any 3,3'-T_2 formed would have undergone further deiodination, as substantiated by analysis of the metabolism of added 3,3'-T_2. Similar rapid degradation of both rT_3 and 3,3'-T_2 with extensive I^- formation was observed by Flock et al. (24) in isolated rat liver perfusions. The unexpected rapid deiodination of 3,3'-T_2 in rat liver cells in contrast to microsomes was subsequently shown to be due to the efficient sulfation of 3,3'-T_2 in hepatocytes yielding 3,3'-T_2S, a highly effective substrate for the type I deiodinase (10). A similar facilatory effect of S conjugation has been described for the IRD of T_3 and T_4 (25, 26).

In the present study, we tested the hypothesis that it is possible to demonstrate production of 3,3'-T_2 from rT_3 in this system if further metabolism, especially sulfation, of 3,3'-T_2 is prevented. In experiments with isolated rat hepatocytes three methods are available in principle to manipulate the sulfotransferase activity of the cells. Firstly, it has been demonstrated that there is a rapid equilibrium between medium and cellular inorganic S levels (27–29). Rat liver cells also have the capacity to generate S by oxidation of the sulfur-containing amino acids methionine and cysteine (28). Therefore, considerable S depletion is obtained by keeping cells in medium without S and possible precursors. Secondly, sulfation of phenolic substances may be prevented by competitive substrates (30). SAM has been shown to undergo extensive sulfation and at higher concentrations also glucuronidation in rat liver cells. For instance, Koike et al. (29) have reported that in isolated rat hepatocytes, sulfation of SAM is characterized by an apparent K_m value of 6 μM with a V_{max} value of 2 nmol/min per 10^6 cells at 1.2 mM inorganic S in the medium. This level of S is similar to that used in our system (1 mM), providing near-maximal rates of S conjugation (9, 10). Glucuronidation of SAM became more pronounced if the competing pathway of sulfation was saturated at high SAM levels or prevented by S depletion (29). G conjugation occured with a K_m value of 0.2 mM and V_{max} of 1.3 nmol/min per 10^6 cells as determined under S-depleted conditions. Thirdly, specific inhibition of phenol sulfotransferase activity may be obtained using DCNP, a compound which in itself is not sulfated (31, 32). All three abovementioned ways of intervention with the further sulfation of 3,3'-T_2 have been applied to our study of the deiodination of rT_3 by rat hepatocytes.

Previous studies from our laboratory have demonstrated the feasibility of using small Sephadex LH-20 columns for separation of 3,3'-T_2 and its main metabolites, 3,3'-T_2S and I^- (10). We have adapted this method in the present investigation for the analysis of rT_3 metabolites. With this modification it was possible to separate fractions containing I^-, conjugates, 3,3'-T_2, and rT_3, respectively. Since the fraction eluted with water may contain various conjugates, and also because there is a slight contamination of the 3,3'-T_2 fraction with rT_3, these analyses were extended by HPLC and RIA determinations. Despite the limitations of the Sephadex LH-20 method, a good correlation between 3,3'-T_2 production rates as measured by the three different techniques was observed.

Time-course studies of the metabolism of 3,3'-T_2 and rT_3 demonstrated that under the conditions used, i.e., 10-nM substrate levels and S-supplemented cells, clearance of both substances follows first-order rate kinetics (Fig. 2). Such kinetics were also observed in the presence of 100 μM SAM, which did not affect the rate constant for rT_3 but lowered that for 3,3'-T_2 by 77%. These results show that conjugation is an important, rate-limiting step in the metabolism of 3,3'-T_2, but not of rT_3. SAM does not only inhibit degradation of added 3,3'-T_2, but apparently also that of 3,3'-T_2 produced from rT_3. No evidence was obtained by HPLC for the generation of iodothyronines other than 3,3'-T_2 such as 3',5'-T_2 or 3'-iodothyronine (Fig. 3).

The 100-μM SAM concentration used mostly in our experiments was shown to have near-maximal effects on the clearance of 3,3'-T_2. The generation of conjugates observed in the presence of PTU is inhibited to only a slightly greater extent at 1,000 μM SAM, while 10 μM of this drug already provided 60% inhibition. This value is in excellent agreement with the Michaelis constant (K_m) value for SAM sulfation of 6 μM, which was reported by Koike et al. (29). Note that after incubation of 3,3'-T_2 in the presence of PTU, substantial radioactivity in the form of 3,3'-T_2S remains associated with the cells, which suggests that exit of cellular 3,3'-T_2S is a relatively slow process. If the amount of cell-bound 3,3'-T_2S is taken into account is is obvious that PTU does not affect clearance of 3,3'-T_2, whereas metabolism of rT_3 is strongly impaired (see below). In the absence of PTU, SAM induces a dose-dependent decrease in I^- formation from 3,3'-T_2 that parallels the decrease of 3,3'-T_2S in incubations with PTU. However, I^- production is not completely suppressed with even 1,000 μM SAM, suggesting that direct ORD of 3,3'-T_2 may occur to some extent if sulfation is inhibited. Direct ORD of 3,3'-T_2 has also been observed with isolated rat liver microsomes, albeit with only 2% of the efficiency of 3,3'-T_2S ORD (9). Therefore,

with unimpeded sulfation, direct deiodination is a negligible pathway.

Also concerning the accumulation of 3,3′-T_2 produced from rT_3, 1,000 μM SAM shows only slightly greater effects than 100 μM of this drug, while roughly a half-maximal effect is observed with 10 μM SAM. At the highest SAM concentration, 3,3′-T_2 represents approximately one-third and I^- approximately two-thirds of the rT_3 metabolites, which corresponds to a 70% protection against further degradation of the 3,3′-T_2 produced. It should be stressed that clearance of rT_3 is not affected by even 1,000 μM SAM. If added to deiodinase assay mixtures of rT_3 with microsomes, inhibition was observed only with 1,000 μM SAM (Table II). The differential effects of 1,000 μM SAM on rT_3 deiodination by intact cells, and that by isolated microsomes, indicate that intracellular SAM levels are substantially lower than those added to the medium. The extremely rapid metabolism of SAM by both sulfation and glucuronidation (29) may be an important factor contributing to this difference. The lack of effect of SAM on medium free iodothyronine levels and cellular ATP (Table II) further underscores the usefulness of this compound in studies of the conjugation of thyroid hormone.

Table I illustrates that similar effects on the metabolism of 3,3′-T_2 and rT_3 are obtained with 100 μM DCNP as with 100 μM SAM. However, DCNP also exhibits some unwanted side effects. DCNP has been shown by Mulder and co-workers to be a specific inhibitor of sulfation in vivo as well as in vitro without being sulfated itself (31, 32). This compound does not affect glucuronidation and was reported to have no major toxic effects, which is in contrast with our findings that show a substantial decrease in cellular ATP by 100 μM DCNP. This effect on ATP may be related to the toxic effects on oxidative phosphorylation generally observed with phenols (33, 34) which may be promoted by the relative deficiency of nutrients and oxygen in our system. A further disadvantage of DCNP is that it may inhibit deiodinase activity directly, as demonstrated by the influence of DCNP in incubations with microsomes (Table II). Similar to SAM, however, intracellular DCNP concentrations may be substantially lower than the total concentrations added to the medium. One possible reason for this is that DCNP binds extensively to BSA (31). The decreased availability of DCNP to the cells, therefore, prevents direct inhibition of the deiodinase, and metabolism of rT_3 is even stimulated by 100 μM DCNP. This latter phenomenon is probably due to displacement of rT_3 from BSA (Table II), which causes a shift of rT_3 into the intracellular compartment. Nevertheless, results obtained with DCNP are in confirmation of the experiments with SAM which underscore the importance of sulfation for the metabolism of 3,3′-T_2 but not of rT_3.

The Sephadex LH-20 method used in this study does not permit the distinction of the different conjugates of 3,3′-T_2 and rT_3. Previous findings have indicated that sulfate conjugates do not crossreact in RIAs of non–conjugated iodothyronines (13). The RIA data presented in Table I are, therefore, consistent with the results obtained by LH-20 insofar as they indicate that clearance of 3,3′-T_2 is not inhibited by PTU. If the product that is formed from 3,3′-T_2 in the presence of PTU is subjected to acid hydrolysis, similar amounts of 3,3′-T_2 are recovered as those measured after incubation with SAM or DCNP. Since iodothyronine sulfates are prone to acid hydrolysis (13), while glucuronides are much more resistant (Oosterlaken, T., S. J. Eelkman Rooda, and T. J. Visser, unpublished observations), this indicates accumulation of 3,3′-T_2S in the presence of PTU. However, most direct evidence for the identity of the conjugates is provided by HPLC in combination with enzymatic analysis using sulfatase or glucuronidase. These experiments were made on samples from incubations with cells in different degrees of S depletion.

S-deplete hepatocytes were prepared by preincubation with medium lacking inorganic S and by using the same medium for incubation with substrate. The results obtained with 3,3′-T_2 in the presence of PTU showed that under these conditions S depletion was not complete, and led to some residual 3,3′-T_2S formation. Further exhaustion of cellular S stores was obtained by preincubation with SAM. A similar approach has been followed by other investigators to reveal the significance of the conjugation reactions in the elimination of drugs (35, 36). Thus, substantial lowering of hepatic UDP–glucuronic acid and adenosine 3′-phosphate 5′-phosphosulfate levels has been achieved by in vivo treatment with SAM due to the consumption of these co-factors in the glucuronidation and sulfation of the drug (35, 36). Application of a SAM load induces a prolonged lowering of body S stores with pronounced decrements in plasma S levels (35), but depletion of tissue UDP–glucuronic acid is only transient, and co-factor levels are rapidly restored (36). We also obtained evidence for the reversible nature of these effects. After preincubation with SAM, 3,3′-T_2 sulfation rates after readdition of SO_4^{2-} and T_3 glucuronidation rates (Eelkman Rooda, S. J., and T. J. Visser, unpublished observations) were completely normal.

In S-replete cells, sulfation is by far the predominant pathway of 3,3′-T_2 metabolism, although little 3,3′-T_2S is recovered in the absence of PTU due to further deiodination. However, in S-deplete cells the contribution of the glucuronidation pathway increases, and results in augmented accumulation of 3,3′-T_2G from added 3,3′-T_2, as well as from added rT_3. The latter findings indicate that glucuronidation does not facilitate deiodination of 3,3′-T_2, which is in contrast to the stimulatory effects of sulfation. Although ORD is the foremost step in the hepatic metabolism of rT_3, some glucuronidation of this metabolite is observed, especially in the presence of PTU. The low rate of rT_3 sulfation in comparison with the highly efficient sulfation of 3,3′-T_2 is in agreement with data of the structure–activity relationship for the sulfation of iodothyronines by isolated phenol sulfotransferases (37).

The effects of substrate concentration on the conversion of rT_3 to 3,3′-T_2 by rat liver cells indicate that half-maximal 3,3′-T_2 production is obtained between 10 and 100 nM rT_3. This is in the same concentration range as the K_m value determined for rT_3 ORD in reactions with microsomes and DTT (23). However, the rT_3 levels in the culture medium are largely protein bound and certainly do not reflect intracellularly available substrate concentrations. Together with the uncertainty about the saturation kinetics of the type I deiodinase in its natural environment it is, therefore, impossible to interpret these findings in terms of a possible uphill gradient of rT_3 across the cell membrane (38).

In conclusion, our results demonstrate that in isolated rat hepatocytes, rT_3 is metabolized almost exclusively by ORD. However, the immediate product of this reaction, 3,3′-T_2, is not observed unless its further metabolism by successive sulfation and deiodination is inhibited. Evidence for formation of 3′,5′-T_2 in these studies was not obtained. In different clinical situations, plasma T_3 and rT_3 levels change in opposite directions due to parallel alterations in the rate of T_3 production and rT_3 degradation. This is understandable, as both processes concern ORD reactions mediated by common enzymes among which the type I deiodinase of the liver plays an important function. It is expected, therefore, that a detailed knowledge of the ORD

of rT_3 by intact liver cells will deepen our insight into the regulation of the bioavailability of thyroid hormone.

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CHAPTER IV

DEVELOPMENT OF A RADIOIMMUNOASSAY FOR TRIIODOTHYRONINE SULFATE

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DEVELOPMENT OF A RADIOIMMUNOASSAY FOR TRIIODOTHYRONINE SULFATE

Sebo Jan Eelkman Rooda, Ellen Kaptein, Marla A.C. van Loon and Theo J. Visser. Department of Internal Medicine III and Clinical Endocrinology, Erasmus University Medical School, 3015 GE Rotterdam, The Netherlands.

ABSTRACT

This paper is the first description of a radioimmunoassay (RIA) for triiodothyronine sulfate (T_3S). Rabbits were immunized against T_3S coupled to bovine serum albumin using carbodiimide. All animals produced antibodies to T_3S but also even higher titers of T_3 antibodies. Ka values for binding of T_3 and T_3S to these antisera varied between $2x10^{10}$ and $8x10^{10}$ M^{-1}. One of the antisera (#8193) was selected for use in the T_3S RIA because of a high titer of T_3S antibodies (final dilution 1 : 50,000), a high sensitivity to T_3S (<2.5 fmol/tube), and a low crossreactivity by T_3 (0.4%). This RIA provides a tool for the study of the importance of sulfation as a metabolic pathway for T_3. (KEY WORDS: thyroid hormone, triiodothyronine sulfate, radioimmunoassay)

INTRODUCTION

Triiodothyronine (T_3) is the bioactive form of thyroid hormone. Roughly 20% of circulating T_3 is derived from thyroidal secretion while the majority is produced by phenolic ring deiodination of thyroxine (T_4) in peripheral tissues, especially the liver (1,2). In rats, T_3 is metabolized by deiodination of the tyrosyl ring and by conjugation of the phenolic hydroxyl group

with sulfate or glucuronic acid (3,4). Although direct deiodination of T_3 occurs in extrahepatic tissues (2,3), sulfation enhances T_3 deiodination in the liver (5,6). In contrast, T_3 glucuronide (T_3G) is a stable conjugate. Thus, after incubation of T_3 with isolated rat hepatocytes, iodide and T_3G are the major products (5). Accumulation of T_3 sulfate (T_3S) in these cultures occurs only after inhibition of deiodinase activity, for instance with propylthiouracil (PTU) (5). To further investigate the role of sulfation in T_3 metabolism we decided to develop a radioimmunoassay (RIA) for T_3S. Here we report the characteristics of this RIA.

METHODS

Production of Antiserum. The T_3S-protein conjugate used for immunization was prepared as follows. To 100 mg anhydrous T_3 (Sigma) and 1 μCi [^{125}I]T_3 (Amersham) was added 0.5 ml of a mixture of chlorosulfonic acid and di-methylformamide (1/4, v/v) at 0 C (7). After reaction for 40 h at room temperature, T_3S was precipitated by addition of this mixture to 5 ml H_2O at 0 C. The pellet was dissolved in 1 ml 2 M NH_4OH and reprecipitated with 5 ml 1 M HCl. The pellet was further washed by repeated suspension in 3 ml 0.1 M HCl. Analysis of this material by HPLC (7) indicated that it consisted of T_3S and only 1% T_3. The T_3S was dissolved in 5 ml dimethylformamide and added to a solution of 100 mg bovine serum albumin (BSA) in 20 ml H_2O. After adjusting the mixture to pH 5 with 0.1 M NaOH, 50 mg 1-ethyl-3-(3-dimethylaminopropyl)-

carbodiimide (Sigma) was added in 5 ml H_2O (8). This was repeated after 3 h, and the mixture was further stirred for 16 h at room temperature. The product was dialysed at 4 C against successively 2x2 1 H_2O, 2 1 0.1 M NaOH and 3x2 1 H_2O, changed twice daily. Analysis of remaining radioactivity indicated a low degree of T_3S incorporation (<5%), corresponding to <5 mol T_3S/mol BSA. The immunogen was stored at -20 C in a concentration of 2 mg protein/ml.

Four New Zealand white rabbits were immunized by s.c. injections with 1 mg conjugate in 1 ml of a 1 : 1 mixture of water and complete Freund´s adjuvant at multiple sites in the back. This was repeated after 2 and 4 months and subsequently at irregular intervals of 1-6 months. High-titer antibodies to T_3 and T_3S were detected in serum of all animals from the second immunization onwards. Two rabbits (#8190 and #8193) produced high-affinity T_3S antibodies, and the characteristics of antisera obtained 2 weeks after the 10^{th} injection (15 months after start of immunization) are described here.

Radioimmunoassay. Radioactive T_3S was prepared by reaction of [^{125}I]T_3 with chlorosulfonic acid and purified as previously described (7). In general, approximately 25,000 cpm (~8 fmol) ^{125}I-labeled T_3 or T_3S were incubated for at least 48 hours at 4 C with appropriate dilutions of antiserum #8190 or #8193 in a final volume of 1 ml RIA buffer (0.06 M barbital, 0.15 M NaCl, 0.1 % BSA, pH 8.6). Preliminary results had shown that this incubation period was necessary to obtain equilibrium, and that tracer binding was constant over a broad pH range. Antibody-bound

radioactivity was precipitated by addition of goat anti-rabbit IgG antiserum and polyethylene glycol 6000 (2.5 % final concentration). After incubation for 1 h at 4 C, the tubes were centrifuged, the supernatants were aspirated and the pellets were counted for radioactivity. Reference iodothyronines were obtained from Henning, Berlin, FRG, and sulfate conjugates were synthesized in our laboratory (7). Solutions of standards and analogs were prepared in 0.01 M NaOH and assayed in duplicate in 50-100 µl aliquots.

RESULTS

Figure 1 shows the binding of T_3 and T_3S tracers to progressive dilutions of antisera #8190 and #8193. Both antisera contained high titers of antibodies to T_3 and T_3S. However, higher dilutions of antiserum could be used to obtain the same degree of T_3 binding compared with T_3S. Binding of biosynthetic T_3G tracer to even low dilutions of antiserum was negligible (not shown). As a suitable condition for the RIA roughly 35% binding of T_3 tracer to either antiserum occured at a final dilution of 1 : 800,000 and in case of T_3S tracer at 1 : 50,000-60,000.

Using these antiserum dilutions the characteristics of T_3 and T_3S binding were further investigated. Significant displacement of T_3S tracer was observed with <2.5 (#8193) or 5 (#8190) fmol unlabeled T_3S per tube (Fig. 2). In case of the T_3 RIA lower limits of detection amounted to 3-5 fmol T_3 per tube. Logit-log

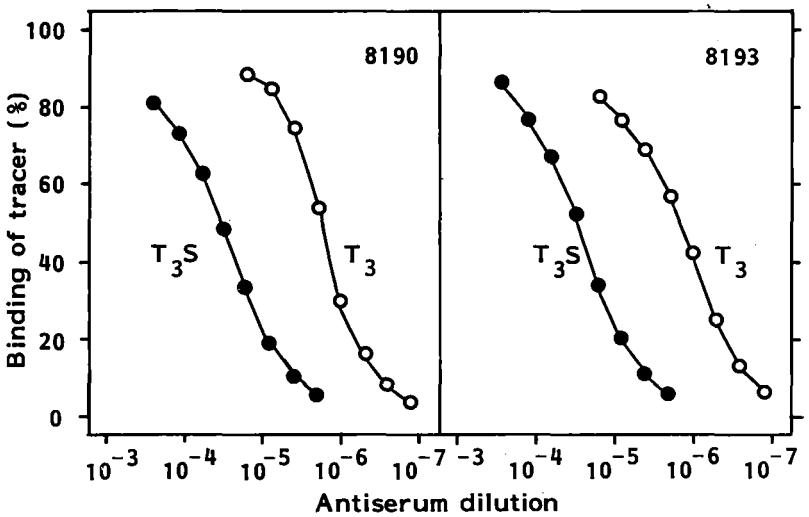

FIGURE 1. Binding of [^{125}I]T_3S (●) or [^{125}I]T_3 (o) to progressive dilutions of antiserum #8190 (left) or #8193 (right).

plots of the standard curves showed excellent fits ($r>0.99$). Linear transforms were also constructed using the method of Scatchard (9) with r values >0.95, which permitted estimation of average binding parameters of the antisera. Binding of T_3S to antisera #8190 and #8193 was characterised by Ka values of $2x10^{10}$ and $8x10^{10}$ M^{-1} and by values for the maximal binding capacity (MBC) in undiluted antiserum of 1.7 and 0.4 μM, respectively. In case of T_3, Ka values were $8x10^{10}$ and $3x10^{10}$ M^{-1} and MBC values 8 and 15 μM, respectively.

Not only the affinity but also the specificity of the binding of T_3S was different between the antisera (Fig. 2 and Table 1). Binding of T_3S to antiserum #8190 showed a high degree of

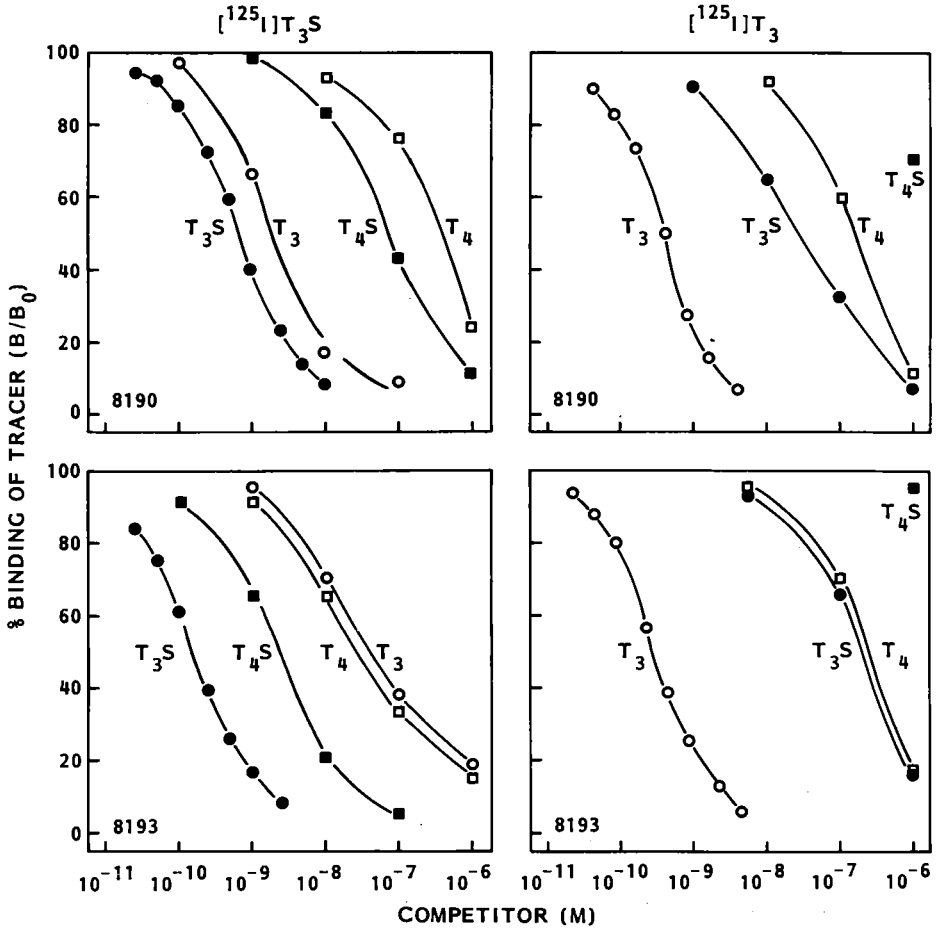

FIGURE 2. Inhibition of the binding of $[^{125}I]T_3S$ (left) to antiserum #8190 (1 : 60,000) or #8193 (1 : 50,000) or that of $[^{125}I]T_3$ (right) to antiserum #8190 or #8193 (both 1 : 800,000) by increasing concentrations of unlabeled T_3S (●), T_3 (○), T_4 (□) or T_4S (■). Results with antiserum #8190 are given in the upper panels and those with antiserum #8193 in the lower panels.

TABLE 1

Specificity of Binding of T_3S and T_3 to Antisera #8190 and #8193

Competitor	Crossreactivity (%)			
	$[^{125}I]T_3S$		$[^{125}I]T_3$	
	#8190 (1:60,000)	#8193 (1:50,000)	#8190 (1:800,000)	#8193 (1:800,000)
T_4	0.2	0.6	0.2	0.2
T_4S	0.9	9	<0.1	<0.01
T_3	31	0.4	100	100
T_3S	100	100	0.9	0.2
rT_3	<0.01	<0.01	<0.01	<0.01
rT_3S	<0.1	0.2	<0.01	<0.01
$3,5\text{-}T_2$	2	<0.01	0.6	0.4
$3,3'\text{-}T_2$	0.4	<0.01	0.3	0.5
$3,3'\text{-}T_2S$	2	0.5	<0.01	<0.01
$3\text{-}T_1$	<0.01	<0.01	<0.1	<0.01
$3'\text{-}T_1$	<0.01	<0.01	<0.01	<0.1
T_0	<0.01	<0.01	<0.01	<0.01

Percentage crossreactivity is defined as the ratio (x100) of the concentration of ligand divided by the concentration of competing substance that each produce 50 % displacement of tracer. Results are calculated after logit-log transformation of the dose-response curves and represent the means of at least three different experiments. rT_3, $3,3',5'$-triiodothyronine; T_2, diiodothyronine; T_1, iodothyronine; T_0, thyronine; S, sulfate.

crossreactivity by T_3 but much less interference by T_4 and T_4 sulfate (T_4S). On the other hand, T_4S was a potent competitor for binding of T_3S to antiserum #8193 while T_3 was much less reactive. Low to absent crossreactivity was observed with all other iodothyronine analogs whether sulfated or not. Less differences were seen between the specificities of T_3 binding to the two antisera (Fig. 2 and Table 1). T_3S was more effective in competing

with T_3 for antiserum #8190 compared with #8193. However, crossreactivity remained below 1% as was the case with all analogs tested, including T_4.

DISCUSSION

This study represents the first report of a RIA for the measurement of an iodothyronine conjugate. Previous studies had already demonstrated that it is feasible to measure sulfate conjugates of steroids by RIA. An established method for the determination of plasma dehydroepiandrosterone sulfate makes use of the high crossreactivity of the conjugate with antiserum produced against the nonconjugated steroid (10). However, recent reports have also described the production of specific antibodies to sulfate conjugates, e.g. estradiol-3-sulfate, that do not crossreact with the free steroid (11).

Although we immunized rabbits against a conjugate prepared by coupling pure T_3S to albumin, the antisera produced contain distinct types of antibody, the most abundant of which specifically binds T_3. The reason for this is unknown but it is possible that part of the sulfate conjugate is hydrolysed during immunogen preparation or in vivo. Extensive hydrolysis has been observed during coupling of phenolic steroid sulfates to protein using the mixed anhydride method but not with carbodiimide (12), the coupling reagent also used by us. The crossreactivity of T_3S with the binding of T_3 tracer to the antisera was of similar magnitude as that observed with antibodies raised against free T_3 as the antigen (7). It is not excluded that part of this

crossreactivity by T_3S is due to slight contamination with free T_3.

Of greater interest to our studies is the other class of antibodies that show preference for T_3S as the ligand. Especially T_3S binding to antiserum #8193 is little affected by T_3, although this favorable property may in part result from the sequestration of T_3 by the high-capacity T_3-specific antibodies. Notwithstanding the low crossreactivity of T_4 in the T_3S RIA, it may still create a significant problem for the measurement of T_3S in samples with high T_4 levels such as plasma. The similar effects of T_4 and T_3 in the T_3S RIA with antiserum #8193 suggest that in this case the steric hindrance of the extra iodine in T_4 is counter-balanced by the favorable influence of the dissociated hydroxyl group. The latter introduces a negative charge in the same region of the molecule as the sulfate group.

In conclusion, the present study demonstrates the feasibility of the development of a RIA for T_3S. Preliminary studies have shown accumulation of immunoreactive T_3S in incubations of T_3 with isolated rat hepatocytes if subsequent deiodination of the conjugate is inhibited (13). T_3S immunoreactivity has also been detected in plasma of PTU-treated rats (14). Studies are now in progress in our laboratory to adapt this RIA for the measurement of T_3S in human plasma.

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CHAPTER V

METABOLISM OF TRIIODOTHYRONINE IN RAT HEPATOCYTES

ABSTRACT

The metabolism of 3,3′,5-triiodothyronine (T_3) by isolated rat hepatocytes was analysed by Sephadex LH-20 chromatography, high performance liquid chromatography and by radioimmunoassays for T_3 sulfate (T_3S) and 3,3′-diiodothyronine (3,3′-T_2). Type I iodothyronine deiodinase activity was inhibited with propylthiouracil (PTU) and phenol sulfotranferase activity by SO_4^{2-} depletion or with competitive substrates or inhibitors. Under normal conditions, labeled T_3 glucuronide (T_3G) and I^- were the main products of [3′-^{125}I]T_3 metabolism. Iodide production was decreased by a) inhibition (PTU) or saturation ($\geqslant$100 nM T_3) of type I deiodinase or b) phenolsulfotransferase, accompanied by the accumulation of a) T_3S and 3,3′-T_2S or b) 3,3′-T_2 and 3,3′-T_2G independent of PTU. 3,3′-T_2 and its conjugates were only produced at $\leqslant$10 nM T_3. Thus, T_3 is metabolized in rat liver cells by 3 quantitatively important pathways: glucuronidation, sulfation and direct inner ring deiodination (IRD). Whereas T_3G is not further metabolized in the cultures, T_3S is rapidly deiodinated by the type I enzyme. As confirmed by incubations with isolated rat liver microsomes, direct IRD of T_3 is mediated by a low-Km, PTU-insensitive, type III-like iodothyronine deiodinase, and production of 3,3′-T_2 is only observed if its rapid sulfation is prevented.

INTRODUCTION

In euthyroid subjects thyroxine (T_4) is the predominant secretory product of the thyroid. T_4 itself has virtually no thyromimetic activity but it serves as a precursor of the bioactive thyroid hormone 3,3′,5-triiodothyronine (T_3). Approximately 20 % of plasma T_3 is secreted by the thyroid and 80% is derived from peripheral deiodination of T_4 (1). This process involves the outer ring deiodination (ORD) of T_4 by the so-called type I iodothyronine deiodinase in many extrathyroidal tissues, predominantly liver and kidney (2,3). In rats two other iodothyronine deiodinases ,type II and III, have been described which differ in several aspects from the type I enzyme. While the type I enzyme is capable of both ORD and inner ring deiodination (IRD), the type II enzyme catalyses only the ORD and the type III enzyme only the IRD of iodothyronines (for a review, see refs. 2,3). Thus, there are two routes for peripheral T_3 production, namely type I and II ORD of T_4. Likewise, there are two T_3-deiodinating pathways, i.e. type I and III IRD to 3,3′-diiodotyronine (3,3′-T_2).

The metabolic kinetics of many iodothyronines have been studied

extensively in vivo both in man and rat (1,4). However, the use of these plasma sampling techniques do not allow to estimate the actual contribution of the different tissues to the total body metabolism of thyroid hormone. Moreover, the rapid succession of intracellular processes may remain undetected. Therefore, detailed studies of thyroid hormone metabolism in isolated tissues may contribute to a better understanding of the integral process.

Previously, we reported on the metabolism of both 3,3'-T_2 and T_3 in monolayers of isolated rat hepatocytes (5,6). Whereas deiodination and conjugation with either glucuronic acid or sulfate have been generally considered as independent metabolic pathways, our studies revealed that sulfation is a rate-limiting step preceding the hepatic deiodination of these compounds (5,6). In contrast to 3,3'-T_2, which is mainly metabolized by sulfation and subsequent deiodination, T_3 metabolism also involves conjugation with glucuronic acid. T_3 sulfate (T_3S) is metabolized in rapid succession by IRD and ORD with 3,3'-T_2 sulfate (3,3'-T_2S) as a transient intermediate (5,7).

In this paper we describe the quantative contributions of the different pathways contributing to the metabolism of T_3 in rat hepatocytes as well as the effects of several compounds that interfere with sulfation and glucuronidation.

MATERIALS AND METHODS

The materials used are essentially the same as described previously (8). 3,3'-T_2 and T_3 were obtained from Henning GmbH, Berlin, Federal Republic of Germany. Carrier-free [3'-^{125}I]T_3 with a specific activity of 3300 Ci/g was prepared in our laboratory by radioiodination of 3,3'-T_2 using the chloramine-T method. [3'-^{125}I]T_3S and unlabeled T_3S were prepared by reaction of labeled or unlabeled T_3 with chlorosulfonic acid and purified by Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography (9). Standard T_3S was also a gift from Henning. Salicylamide (SAM), 2,6-dichloro-4-nitrophenol (DCNP) and pentachlorophenol (PCP) were purchased from Riedel-de Haen AG, Hannover, Federal Republic of Germany. 6-Propyl-2-thiouracil (PTU) and galactosamine (GAM) were from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest quality commercially available.

Hepatocytes

Male Wistar rats were used (180-250 g BW). After isolation of the

hepatocytes by collagenase perfusion (10), the cells were suspended in culture medium (10^6 cells/2 ml). This culture medium was composed of Ham's F_{10}, 10.6 mM PIPES, 11.2 mM BES, 8.9 mM Hepes (pH7.4), 2 mM $CaCl_2$, 12 mU/l insulin, 10 U/ml penicillin, 10 ug/ml streptomycin sulfate and 10% fetal calf serum. Monolayers of hepatocytes were obtained by plating 2 ml of the cellsuspension into uncoated 3.5-cm wells of plastic 6-well dishes (Nunc, Roskilde, Denmark). The plates were kept for 4 h at 37 C in a culture stove under atmospheric conditions. Cell viability, tested before each experiment by trypan blue exclusion, exceeded 85%.

Incubation procedures

All experiments were performed in triplicate under atmospheric conditions at 37 C. The dishes were placed on a slightly-angled, slowly-rotating plate. Hepatocytes were preincubated for 30 min with Dulbecco's balanced salt solution containing 2 mM glutamine, 1 mM vitamin C and either 1 mM $MgSO_4$ without SAM or 1 mM $MgCl_2$ with 100 uM SAM (8). Incubations were performed for 180 min in Dulbecco's medium with 1 mM $MgSO_4$ (SO_4^{2-}-containing incubations) or 1 mM $MgCl_2$ (SO_4^{2-}-free incubations) in the presence or absence of 0.1% bovine serum albumin (BSA). Sulfation was inhibited with 10-1,000 uM SAM, 1-10 uM DCNP or 1 uM PCP, deiodination with 100 uM PTU and glucuronidation with 0.5-5 mM GAM (8,11-13). Substrate levels were 1-1,000 nM T_3 with or without 0.1 uCi [^{125}I]T_3.

Analysis of incubation medium

Incubation media with labeled T_3 were analysed by Sephadex LH-20 column chromatography. For this purpose 0.5 ml medium was acidified with an equal volume of 1 M HCl. This mixture was applied to small Sephadex LH-20 columns (bed volume 0.75 ml) equilibrated in 0.1 M HCl. The columns were eluted with 1 ml fractions of 0.1 M HCl (2x), 0.1 M sodium acetate (pH 4) (8x) , H_2O (5x) and 0.1 M NaOH-ethanol (1:1, vol/vol) (3x).

After incubation with unlabeled T_3, media were analysed by specific radioimmunoassays (RIA) for T_3, 3,3'-T_2 (14) and T_3S content (15). The T_3S RIA was recently described by us. In short, antibodies against T_3S were obtained by immunizing rabbits with T_3S coupled to BSA using carbodiimide. Antiserum #8193 was selected for use in the T_3S RIA because of a high titer of T_3S antibodies (final dilution 1: 50,000), a high sensitivity to T_3S (<2.5 fmol/tube), and a low crossreactivity by T_3 (0.4 %). Aliquots of 50-100 ul incubation medium were assayed in duplicate in mixtures with approximately 25,000 cpm [^{125}I]T_3S (~8 fmol) and T_3S antiserum in a final

volume of 1 ml RIA buffer (0.06 M barbital, 0.15 M HCl, 0.1 % BSA, pH 8.6). After 48-72 h at 4 C, antibody-bound radioactivity was precipitated by addition of goat anti-rabbit IgG antiserum and polyethylene glycol 6000. After 1 h at 4 C the tubes were centrifuged, the supernatants were aspirated and the pellets were counted for radioactivity.

High performance liquid chromatography (HPLC)

The various 3,3'-T_2 and T_3 conjugates and nonconjugated iodothyronines were analysed by reversed-phase HPLC. This system consists of a 10x0.3-cm CP^{tm}Spher C18 column (Chrompack, Middelburg, The Netherlands), model 6000 A and 510 solvent pumps (Waters, Milford, MA, USA) and a model 680 automated gradient controller. Flow was 0.8 ml/min and absorbance was measured at 254 nm by a Model 440 fixed wavelength detector (Waters).

Separation of 3,3'-T_2 and T_3 was accomplished by isocratic elution with a 50:50 (vol/vol) mixture of methanol and 0.02 M ammonium acetate (pH 4). By employing a non-linear gradient of acetonitrile in 0.02 M ammonium acetate (pH 4) (15-40% in 30 min using program 7) 3,3'-T_2, T_3 and their sulfate and glucuronide conjugates were separated in a single run.

Sample preparation for the HPLC analysis of nonconjugated iodothyronines was done by Sephadex LH-20 chromatography of medium as described above. After the H_2O fractions, columns were rinsed with a mixture of 0.1 M ammonia and ethanol (1:1, vol/vol) and the iodothyronines were eluted with 1.25 ml 0.1 M ammonia in ethanol. The solvent was evaporated at 50 C under a stream of N_2. For the simultaneous analysis of free and conjugated 3,3'-T_2 and T_3 on HPLC, precleaning of samples on Sephadex LH-20 was modified as follows. After application of acidified medium, columns were washed with 2x1 ml 0.1 M HCl and with 0.75 ml H_2O. All retained radioactivity was eluted subsequently with 1.25 ml 0.1 M ammonia in ethanol. The solvent was evaporated as described above.

The elution position of reference compounds was determined using synthetic T_3, 3,3'-T_2, T_3S, 3,3'-T_2S (9) and biosynthetic T_3 glucuronide (T_3G) and 3,3'-T_2G (16).

Analysis of cell content

The incubation medium was removed after 180 min and cells were extracted immediately with 1 ml 0.1 M NaOH. After centrifugation, 0.5 ml supernatant was chromatographed on Sephadex LH-20 as described for medium, or cellular T_3, 3,3'-T_2 and T_3S content were determined by RIA.

Miscellaneous

Deiodination of [^{125}I]T_3 and [^{125}I]T_3G (isolated from hepatocyte cultures; ref. 16) was also studied using rat liver microsomes (14). In short, [^{125}I]T_3 (100,000 cpm) and 0-1,000 nM T_3 were incubated for 1 h at 37 C with 0.5 mg/ml microsomal protein in 0.2 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA and 10 mM dithiotreitol (DTT) in the absence or presence of 1 mM PTU and, if applicable, 1 uM rT_3. Incubations with [^{125}I]T_3G (20,000 cpm) were performed for 15-30 min with 0.1 mg/ml microsomal protein in phosphate-EDTA buffer and 5 mM DTT. Reactions were stopped with 800 ul 0.1 or 1 M HCl and the mixtures were chromatographed on Sephadex LH-20 as described above to isolate the iodide, conjugate and iodothyronine fractions. The latter fractions were further analysed by HPLC for the quantitation of the production of 3,3'-T_2G and 3,3'-T_2, respectively (see above).

The effect of phenol sulfotransferase inhibitors on the type I deiodinase was tested in similar experiments using [^{125}I]rT_3 as the substrate (8). The effect of these inhibitors on cellular ATP content was measured as described previously (8).

Data analysis

In each experiment I^- production was corrected for the amount of I^- recovered from control incubations (<2%). Production of 3,3'-T_2 and T_3S were corrected for crossreactivity of T_3 in the 3,3'-T_2 (<0.1%) and T_3S (0.4%) RIA. Results are given as means $\pm$ SEM. Statistical analysis of the data was performed with a one way classification, first performing an analysis of variance followed by comparisons among class means (17).

RESULTS

Sephadex LH-20 chromatography

In Fig. 1 are shown the chromatographic patterns of I^-, T_3G, T_3S and T_3 on Sephadex LH-20. The stepwise elution procedure resulted in the clear separation of these compounds with minimal overlap between the different fractions. More than 97.5 % of I^- eluted in fractions 1-4, >98% of T_3G in fractions 5-12 (sodium acetate), >98% of T_3S in the H_2O fractions 13-16 and >98% of T_3 in fractions 17-19 (NaOH-ethanol). Not shown are the chromatographic profiles of 3,3'-T_2G, 3,3'-T_2S and 3,3'-T_2. More than 98% of 3,3'-T_2G activity eluted in fractions 4-8. Approximately 80% of 3,3'-T_2S activity was found in fractions 6-12 and 15% in fractions 13-16 . 3,3'-T_2 had the same elution profile as T_3.

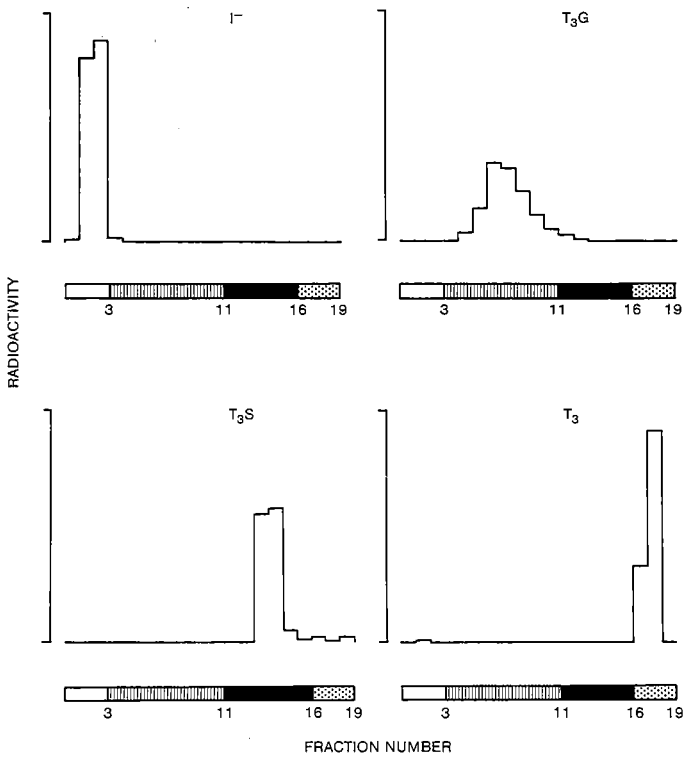

Figure 1
Sephadex LH-20 chromatography of I^-, T_3G, T_3S and T_3. Each ^{125}I-labeled compound was applied separately in 1 ml 0.5 N HCl to a small Sephadex LH-20 column with a bed volume of 0.75 ml which was equilibrated in 0.1 N HCl. Subsequently, columns were eluted with 1 ml fractions of 0.1 N HCl (2x), 0.1 M sodium acetate pH 4 (8x), H_2O (5x), and finally ethanol-0.1 N NaOH (1:1 vol/vol) (3x). Each 1-ml fraction was counted for radioactivity.

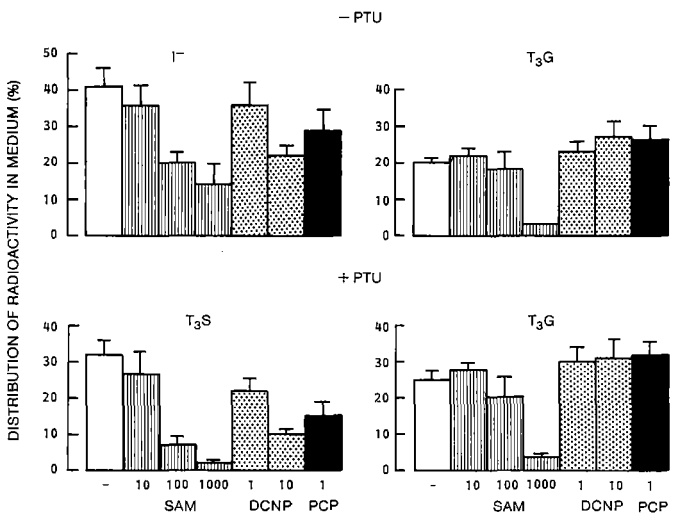

Figure 2
Effects of SAM, DCNP and PCP on T_3 metabolism. 10 nM T_3 with 0.1 uCi [^{125}I]T_3 were incubated for 180 min with 10^6 hepatocytes in 2 ml SO_4^{2-}-containing Dulbecco medium (n = 5) with or without 10-1,000 uM SAM, 1-10 uM DCNP and 1 uM PCP (upper panels). Parallel incubations were performed in the presence of 100 uM PTU (bottom panels). Incubations were performed in the absence of BSA. Medium was analyzed by Sephadex LH-20 chromatography. I^- production in incubations without PTU and T_3S production in incubations with PTU was significantly reduced by any concentration of inhibitors tested ($p < 0.001$).

Effects of SAM, DCNP and PCP on T_3 metabolism

Initially, incubations were performed in SO_4^{2-}-containing medium without BSA at a substrate concentration of 10 nM [^{125}I]T_3. Under these conditions I^- and T_3G were the main metabolites in incubations without PTU. Inhibition of type I deiodination with PTU did not affect T_3G, but induced a large increase in T_3S. To elucidate the importance of sulfation for I^- production, the effects of the competitive sulfotransferase inhibitors SAM, DCNP and PCP were investigated (Fig. 2). These inhibitors produced a dose-dependent diminution of I^- production in the absence of PTU and that of T_3S production in the presence of PTU. At the various inhibitor levels, percentage I^- production in incubations without PTU (Y) was significantly correlated with the percentage T_3S formation in incubations with PTU (X) according to the equation $Y = 0.83X + 14.8$ ($r = 0.79$; $p<0.001$). This indicates that the effects of these inhibitors on medium I^- are somewhat less than their effects on medium T_3S. For example, at 100 uM SAM I^- production without PTU was inhibited by 50%, whereas T_3S production with PTU was inhibited by 73%. Only at the highest concentration (1,000 uM) SAM also affected the T_3 glucuronidation rate, while DCNP and PCP had no such effect at the concentrations tested.

After the 3 h incubation period, cellular ATP levels were little affected by the sulfotransferase inhibitors. Expressed as percentage of controls, ATP levels amounted to 88$\pm$ 12% at 1,000 uM SAM, 81$\pm$ 6% at 10 uM DCNP and 109$\pm$ 9% at 1 uM PCP.

Effect of SO_4^{2-} concentration on the metabolism of T_3 (Fig. 3)

In SO_4^{2-}-deplete cultures , T_3 clearance was low and T_3G was the main metabolite found in the medium, independent of the presence of PTU. Addition of 0.1- 1 mM SO_4^{2-} induced an increase in T_3 sulfation (see incubations with PTU), in I^- production (without PTU) and in T_3 disappearance rate. Once again, I^- production in incubations without PTU (Y) and T_3S generation in incubations with PTU (X) showed a close correlation: $Y = 0.97X + 2.8$ ($r = 0.995$, $p<0.001$). T_3G production was constant over this range of SO_4^{2-} concentrations.

Effect of substrate concentration on the metabolism of T_3 (Fig. 4)

Initially, incubations were performed in the absence of BSA. In SO_4^{2-}-containing incubations without PTU, I^- was the main metabolite at low substrate concentrations, i.e. 47.1% of medium radioactivity at 1 nM and 40.7% at 10 nM. At higher T_3 concentrations, I^- production gradually

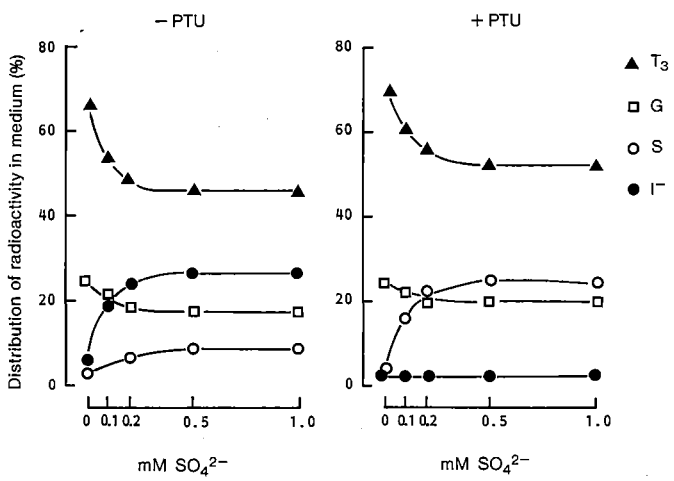

Figure 3
Effects of SO_4^{2-} concentration on the metabolism of T_3. Monolayers of 10^6 hepatocytes were preincubated for 30 min in SO_4^{2-}-free Dulbecco's medium plus 100 uM SAM. Subsequently, incubations were performed for 180 min with 10 nM [^{125}I]T_3 in 2 ml Dulbecco's medium containing 0-1 mM $MgSO_4$ and 0.1% BSA. Parallel incubations were performed in the presence of 100 uM PTU. Medium was analyzed by Sephadex LH-20 chromatography. Data are taken from one out of three closely agreeing experiments. (● I^-, □ T_3G, ○ T_3S and ▲ T_3).

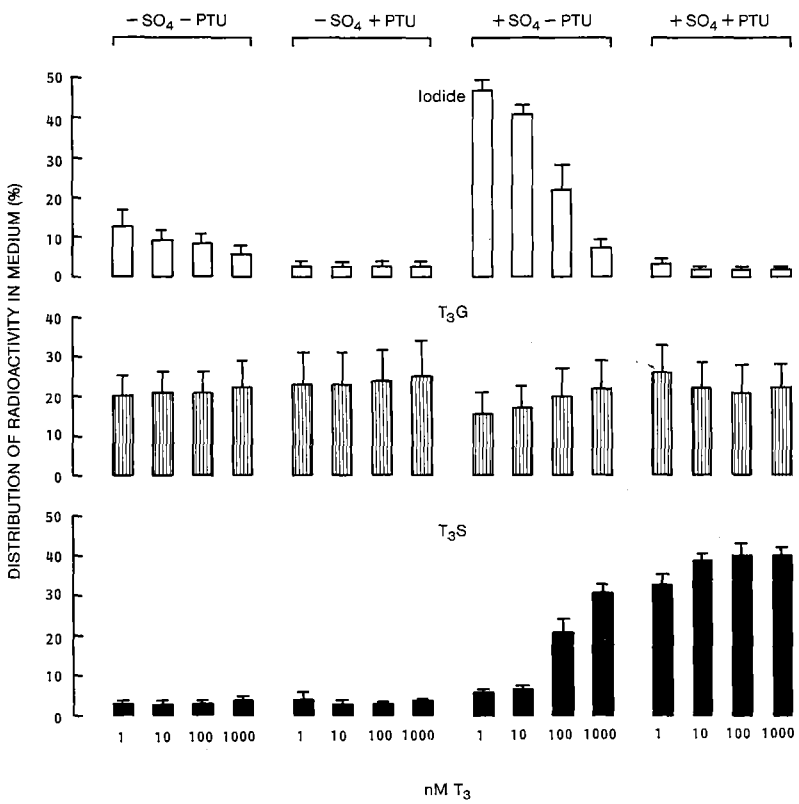

Figure 4
Effects of substrate concentration, SO_4^{2-} and PTU on the metabolism of T_3. Monolayers of 10^6 hepatocytes were preincubated for 30 min in SO_4^{2-}-deplete Dulbecco's medium plus 100 uM SAM or SO_4^{2-}-replete medium. After removal of the medium, parallel incubations were performed for 180 min in 2 ml SO_4^{2-}-deplete or SO_4^{2-}-replete Dulbecco's medium with or without 100 uM PTU in the absence of BSA (n = 4). Substrate concentration was 1-1,000 nM [^{125}I]T_3. Medium was analyzed by Sephadex LH-20 chromatography. Completion of medium radioactivity to 100% represents the proportion of unaltered T_3.

decreased to ultimately 7.4% at 1,000 nM T_3. In incubations without SO_4^{2-}, I^- production was lowered but not completely nullified compared with incubations with SO_4^{2-}, varying from 12.9% at 1 nM to 5.9% at 1,000 nM T_3. In incubations with PTU, generation of I^- was always less than 4.6%, independent of the presence of SO_4^{2-}.

In SO_4^{2-}-containing incubations without PTU, little (<8%) T_3S accumulated in the medium at 1-10 nM T_3. At higher T_3 concentrations (100-1,000 nM) or in incubations with PTU, T_3S accumulated to 20.9-39.9%. In incubations without SO_4^{2-} <3% of radioactivity in the medium eluted in the H_2O fractions.

Despite the 10^3-fold rise in T_3 concentrations radioactivity in the T_3G peak was a fairly constant fraction of total radioactivity in the medium.

HPLC analysis of products (Figs. 5 & 6)

HPLC analysis of incubations especially with 1 nM T_3 in the experiments described in Fig. 4 demonstrated the presence of 3,3'-T_2 and its conjugates. Furthermore, in incubations without BSA, a considerable amount of T_3 and the various metabolites accumulated intracellularly. We therefore made a detailed investigation of all possible metabolites produced in incubations of 1 and 10 nM T_3 with hepatocytes in the presence of 0.1% BSA. The results of these studies are presented in Fig. 5 which depicts the quantitation of iodide, conjugates and iodothyronines on Sephadex LH-20. The conjugate and iodothyronine fractions were subdivided into the different components as determined by HPLC (Fig. 6).

When hepatocytes were incubated with SO_4^{2-}, I^- production amounted to 48.5% at 1 nM and 29.3% at 10 nM T_3. In SO_4^{2-}-deplete incubations I^- production was still considerable, i.e. 20.7% at 1 nM and 8.9% at 10 nM T_3. This was not due to slight SO_4^{2-} contamination in these incubations as no sulfate conjugates were detected by HPLC even in the presence of PTU (Fig. 6). Addition of PTU to the incubations always decreased I^- production to less than 5.5%, independent of SO_4^{2-}.

Total conjugates in SO_4^{2-}-replete cultures with PTU varied from 52.4-46.0% and without PTU from 19.2-26.1% at 1 and 10 nM T_3, respectively. Whereas T_3G production was fairly constant in these incubations, T_3S production increased from 4-5% in the absence to 17.5% in the presence of PTU. Beside these T_3-conjugates, HPLC also demonstrated the accumulation of 3,3'-T_2S in incubations with SO_4^{2-} and PTU. At 1 nM T_3, contribution of

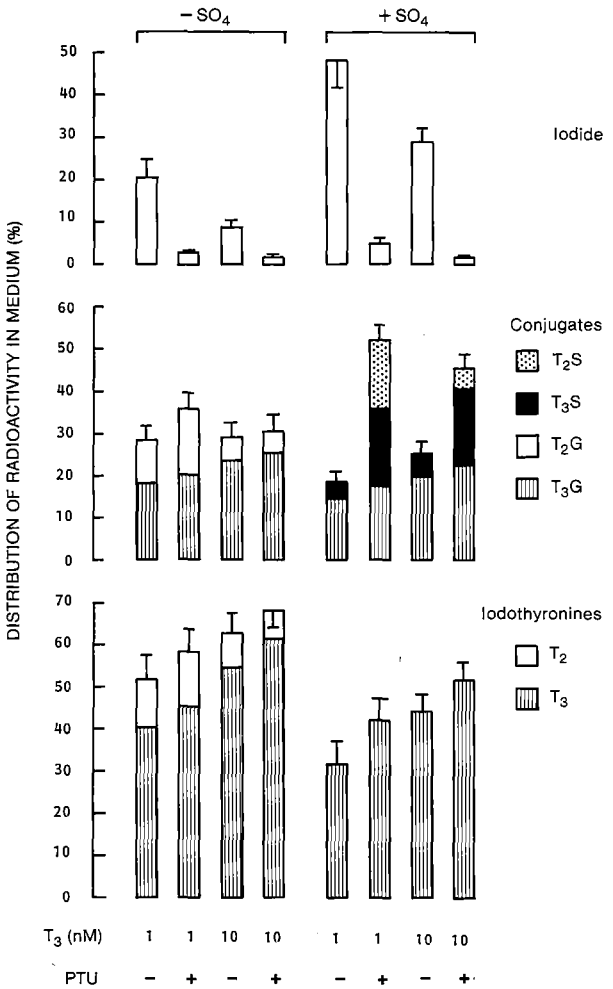

Figure 5
Effects of SO_4^{2-} and PTU on the metabolism of T_3 at low substrate levels. Monolayers of 10^6 hepatocytes were preincubated and incubated in the presence of 0.1% BSA (n = 14), essentially as described in the legend of Fig. 4. Substrate level was 1-10 nM [^{125}I]T_3. Medium was analyzed by Sephadex LH-20 chromatography. The conjugate and iodothyronine fractions were subdivided into the different components on the basis of HPLC analysis of 6-7 experiments.

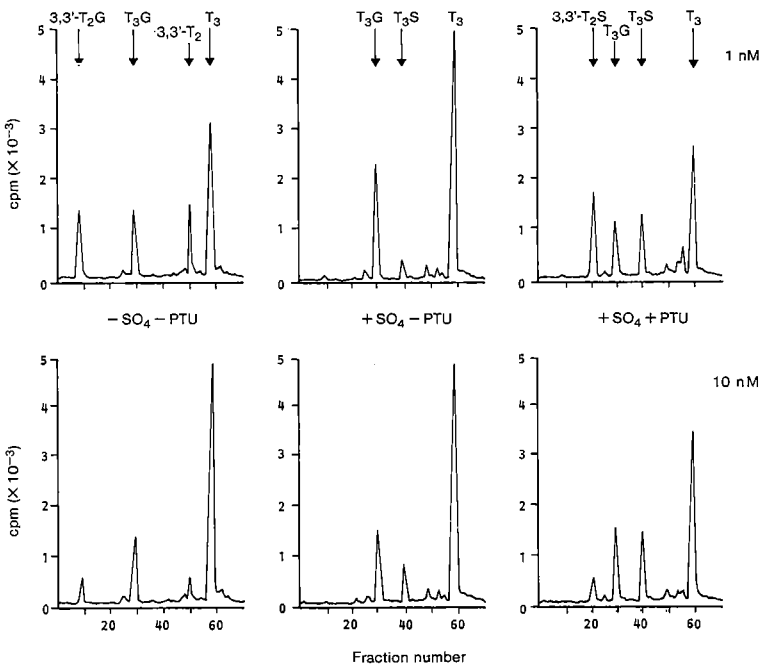

Figure 6
Identification and quantitation of metabolites of T_3 incubations by HPLC analysis. Medium of incubations with 1 nM (upper panel) or 10 nM T_3 (lower panel) as described in the legend of Fig. 5 was chromatographed on Sephades LH-20 with slight modification (see Methods). All radioactivity, except I^-, was collected in 1.25 ml 0.1 M ammonia in ethanol and evaporated at 50°C under a stream of N_2. Free and conjugated 3,3'-T_2 and T_3 were separated in a single run on HPLC using a non-linear gradient of 15-40% acetonitrile in 0.02 M ammonium acetate (pH 4). Fractions of 0.4 ml were collected and counted for radioactivity. The elution position of reference compounds was determined using synthetic T_3, 3,3'-T_2, T_3S and 3,3'-T_2S and biosynthetic T_3G and 3,3'-T_2G.

3,3′-T_2S to total conjugates was 33%, at 10 nM only 11%, and at higher substrate levels 3,3′-T_2S was undetectable (not shown).

Total conjugates in incubations without SO_4^{2-} or PTU were higher than in the corresponding incubations with SO_4^{2-}. Addition of PTU to incubations without SO_4^{2-} had little effect on total conjugate formation, in particular on T_3G production. However, HPLC analysis also demonstrated the presence of 3,3′-T_2G. At 1 and 10 nM T_3, contribution of 3,3′-T_2G to total conjugates was 40% and 18%, respectively, independent of PTU.

In these experiments, the percentage remaining T_3 was slightly higher in incubations without SO_4^{2-} than with SO_4^{2-} ($p<0.001$) and always higher at the 10 nM than at the 1 nM substrate level ($p<0.001$). In incubations without SO_4^{2-}, HPLC analysis showed that the iodothyronine fraction of the Sephadex LH-20 chromatography also contained significant amounts of 3,3′-T_2, the production of which was hardly influenced by addition of PTU. At 1 and 10 nM T_3, contribution of 3,3′-T_2 to the iodothyronine fraction was 22% and 12%, respectively. At higher T_3 concentrations or in SO_4^{2-}-replete incubations no 3,3′-T_2 or 3,3′-T_2G could be demonstrated by HPLC analysis.

Cell-associated radioactivity amounted from 20% of total radioactivity in SO_4^{2-}-containing incubations to 25-30% in SO_4^{2-}- deplete cultures. Addition of PTU led to a slight (25%) increase in cellular radioactivity. However, independent of the different circumstances, >80% of total T_3G and T_3S and >65% of total T_3 radioactivity was found in the incubation medium.

In view of the presence of 3,3′-T_2 (and 3,3′-T_2G) in incubations without SO_4^{2-} independent of PTU and the presence of 3,3′-T_2S in incubations with SO_4^{2-} plus PTU, we hypothesized the involvement of a PTU-insensitive, type III-like deiodinase. We therefore performed incubations in which the type I enzyme was completely inhibited by preincubation of hepatocytes with PTU and 1 uM rT_3. Otherwise, the incubations were done as described above in the presence of PTU at a substrate concentration of 1 nM T_3. As demonstrated by HPLC (not shown), 3,3′-T_2G and 3,3′-T_2 production in SO_4^{2-}-deplete cultures and 3,3′-T_2S production in SO_4^{2-}-containing cultures were not inhibited by complete inactivation of type I deiodinase. The latter was confirmed in parallel incubation with radioactive rT_3 (not shown).

RIA of medium 3,3′-T_2, T_3 and T_3S

Fig. 7 shows the effects of SO_4^{2-} and PTU on T_3 metabolism as measured by RIA. In SO_4^{2-}-deplete cultures a readily saturable 3,3′-T_2 production

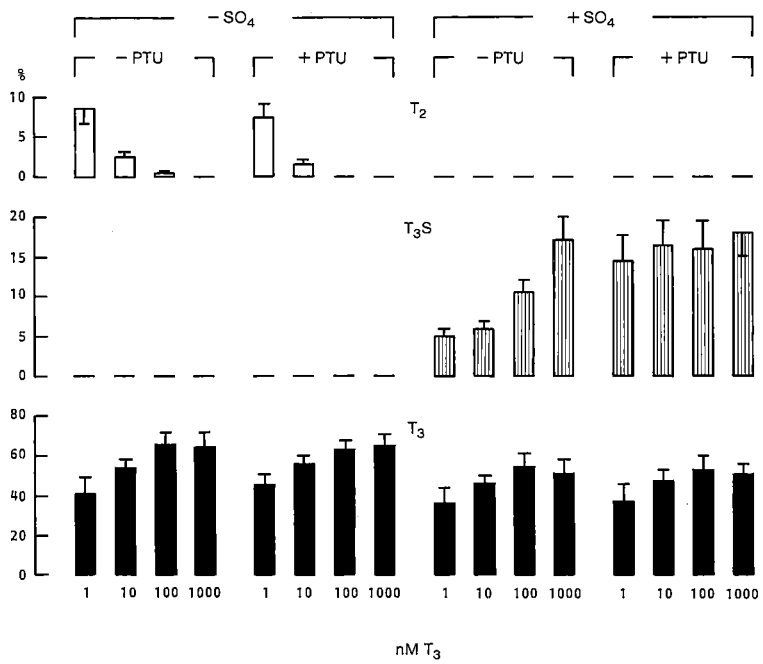

Figure 7
RIA of medium 3,3'-T_2, T_3 and T_3S content. Monolayers of 10^6 hepatocytes were preincubated and incubated in the presence of BSA as described in the legend of Fig. 4 (n = 8-16). Substrate concentration was 1-1000 nM unlabeled T_3. In SO_4^{2-}-deplete incubations no T_3S and in SO_4^{2-}-replete incubations no 3,3'-T_2 could be measured.

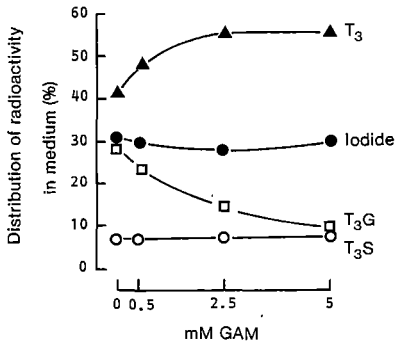

Figure 8
Effect of GAM on the metabolism of T_3. Monolayers of 10^6 hepatocytes were preincubated and incubated in SO_4^{2-}-replete Dulbecco medium in the presence of 0.1% BSA. Parallel incubations were performed with 0-5 mM GAM at a substrate level of 1 nM [^{125}I]T_3. Medium was analyzed by Sephadex LH-20 chromatography. Results are taken from a representative experiment reproduced twice.

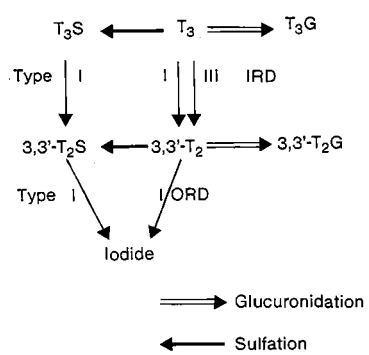

Figure 9
Metabolic pathways of T_3 in rat hepatocytes.

was observed, ranging from 8.7- 0.04% at 1- 1,000 nM T_3. Production of 3,3'-T_2 was not inhibited by PTU at 1 nM T_3 and only partially at 10 nM. In SO_4^{2-}-containing incubations without PTU only 5-6% of the substrate was recovered as immunoreactive T_3S at 1-10 nM T_3. Addition of PTU or incubation at higher substrate concentrations (100-1,000 nM) induced the accumulation of T_3S up to 17%. 3,3'-T_2 was not detectable by RIA in SO_4^{2-}-replete cultures and T_3S was not detectable in SO_4^{2-}-deplete cultures.

As shown by T_3 RIA, in all SO_4^{2-}-containing incubations less T_3 remained after 180 min than in the corresponding SO_4^{2-}-deplete cultures ($p<0.001$). Addition of PTU did not greatly influence the percentage remaining T_3.

RIA measurements were also performed in cell extracts. Again, a fairly constant medium:cell ratio was observed for the different compounds irrespective of the different circumstances: 71-83% of total 3,3'-T_2, >92% of total T_3S and 74-85% of total T_3 was found in the medium.

Effect of GAM on the metabolism of T_3 (Fig. 8)

The effects of GAM, an inhibitor of UDP-glucuronyl transferase (11,12), on the metabolism of T_3 were investigated. Addition of 0.5-5 mM GAM in the incubation medium led to a stepwise reduction in medium T_3G content by 65%. T_3 clearance was inhibited with 25%, but I^- production was not influenced. Cellular ATP levels were only slightly reduced by GAM, i.e. 8% at 2.5 mM and 19% at 5 mM.

The metabolism of T_3G was further investigated in incubations of [^{125}I]T_3G with microsomes as described in Materials and Methods, or with hepatocytes. Under these conditions, which resulted in extensive deiodination of T_3S (ref. 7 and Eelkman Rooda, S.J., and T.J. Visser, unpublished observations), I^- and 3,3'-T_2G production from T_3G were undetectable.

Conversion of T_3 to 3,3'-T_2 by rat liver microsomes (Table 1)

At the lowest substrate concentration (<0.1 nM), 5.8% of [^{125}I]T_3 was converted to 3,3'-T_2. The percent production of 3,3'-T_2 progressively decreased with increasing substrate concentration to a plateau of 2.3% at 100-1,000 nM T_3. Inhibition of 3,3'-T_2 production by PTU was minimal at <0.1-1 nM T_3, partial at 10-100 nM T_3 and complete at 1,000 nM T_3.

Coincubation of microsomes with PTU and rT_3 resulted in the complete inactivation of the type I deiodinase as demonstrated in experiments with ^{125}I-rT_3 as substrate (not shown). This led, once again, to the complete

inhibition of 3,3′-T_2 production at the highest T_3 concentration, while the fraction of T_3 converted to 3,3′-T_2 at each lower substrate concentration was also diminished by ~2%.

Table 1

CONVERSION OF T_3 TO 3,3′-T_2 BY RAT LIVER MICROSOMES

	% 3,3′-T_2		
nM T_3	Control (n=6)	PTU (n=5)	PTU+rT_3 (n=3)
0	6.1±0.1	5.3±0.1	4.0±0.1
1	5.0±0.1	4.4±0.1	3.0±0.1
10	3.2±0.1	2.1±0.1	1.8± 0
100	2.4±0.1	0.9±0.3	0.7±0.3
1000	2.2±0.2	0.1±0.1	0

Rat liver micosomes were incubated for 1 h at 37C with [^{125}I]T_3 plus 0-1,000 nM T_3 in 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA and 5 mM DTT in the presence or absence of PTU or rT_3. The reactions were stopped by addition of 0.1 M HCl and analysed by Sephadex LH-20 as described in Materials and Methods.

DISCUSSION

It has been known for some time that T_3 is metabolized in rats primarily by conjugation and deiodination. Morreale de Escobar and Escobar del Rey reported on the metabolism of T_3 in thyroidectomized rats, substituted with T_3 (18). After isotopic equilibration with ^{125}I-labeled T_3, approximately equal proportions of radioactivity appeared in the urine and feces. Administration of PTU led to a decrease in urinary I^- excretion with a concomitant increase in fecal radioactivity. The identity of the excretory products was not investigated in this study.

Already in 1960 Flock et al. observed that glucuronidation and sulfation are important pathways in the metabolism of T_3 and T_4 (19,20). After administration of labeled T_3, T_3G was the predominant excretory product in the bile of normal rats. Excretion of especially T_3S was increased by treatment of the rats with butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) (19,20), a known inhibitor of the type I deiodinase (2,3).

In contrast to rats, normal dogs injected with ^{125}I-T_3 also excrete, in addition to T_3G, significant amounts of T_3S and 3,3′-T_2S into the bile and the urine (19). In hepatectomized dogs urinary I^- and T_3G excretion

diminished while that of T_3S and $3,3'-T_2S$ increased. Nephrectomy, however, led to a decrease in the production of the latter conjugates (19). This suggested that in dogs the liver is an important site for deiodination and for the glucuronidation of T_3, while the kidneys and perhaps other organs may be more important for the sulfation of iodothyronines.

Roche and coworkers identified T_3S as the main T_3 metabolite in the bile and plasma of thyroidectomized rats (21,22), a finding which can now be ascribed to the impaired type I deiodinase activity in hypothyroidism (2,3). Recently, we have used HPLC to identify T_3 metabolites in the bile and plasma of rats (23,24). Within 4 h after injection of $^{125}I-T_3$ to normal rats, 22.4% of the dose was excreted in the bile, mainly in the form of T_3G. In PTU-treated rats the biliary excretion amounted to 36% of the dose after 4 h, due to an increased output of T_3S and $3,3'-T_2S$ (23). PTU-treatment also induced a 4-5 fold increase in plasma T_3S after $^{125}I-T_3$ injection (24). Although DCNP by itself had little effect on the biliary clearance of T_3, it greatly diminished the PTU-induced excretion of T_3S and $3,3'-T_2S$ (23). This indicates that, in vivo, sulfation of T_3 in rats is mediated predominantly by a DCNP-sensitive phenol sulfotransferase.

There is now overwhelming evidence that the type I deiodinase catalyses both the IRD and ORD of iodothyronines (2,3). Thus, the same enzyme generates T_3 from T_4 as well as catalyses the degradation of this hormone. Another important aspect of type I deiodination is the fact that sulfate conjugation of the 4'-hydroxyl group of T_3 greatly facilitates this reaction (7). This is reflected in the 30-fold increase in the V_{max} of the IRD of T_3S compared with IRD of nonconjugated T_3. Sulfation has also been shown to accelerate the IRD of T_4 (25) and the ORD of $3,3'-T_2$ (5). Previous studies have demonstrated that deiodination of T_3 and $3,3'-T_2$ in rat hepatocytes greatly depends on the prior sulfation of these compounds (5). It was the purpose of the present study to determine into more detail the contribution of the various pathways to the metabolism of T_3 in rat liver cell cultures. Based on findings from the literature and data presented in this paper the hepatic metabolism of T_3 in rats can be summarized as shown in Fig. 9 (p 82).

In rat liver, T_3 is metabolized via three, quantitatively important pathways which will be described consecutively. The first metabolic pathway is glucuronidation. In vivo, the product T_3G is probably excreted by hepatocytes directly into the bile (see above). The undetectable levels

of T_3G in plasma suggest that it is exported specifically through the canalicular cell membrane of the hepatocyte, perhaps involving the same transport system as described for the excretion of other conjugates and organic anions (26). Glucuronidation has a high capacity in view of our in vitro experiments, which show that the fraction of T_3 which is glucuronidated is constant over a wide range of substrate concentrations (Fig. 4). Furthermore, T_3G is not liable to further metabolism, e.g. deiodination, as no $^{125}I^-$ or 3,3'-T_2G were demonstrated after incubation of T_3G with liver microsomes or hepatocytes.

The second metabolic pathway for the metabolism of T_3 in hepatocytes is sulfation. This process also has a high capacity, as it is not saturated even at 1,000 nM T_3 (Figs. 4 & 7). As mentioned above, the product T_3S is a good substrate for IRD by the type I deiodinase. This deiodinative process is readily saturated at added T_3 concentrations >10 nM (through intracellular T_3S production) and is inhibited by PTU, resulting in the accumulation of T_3S (Fig. 4). At the low endogenous concentrations of type I deiodinase substrates, the enzyme is probably not saturated to a significant extent in vivo and deiodination of T_3S will not be limited, explaining the low levels of T_3S in bile and plasma in normal rats (19,23,24). However, biliary excretion and plasma levels of T_3S increase when the type I enzyme activity is impaired in the hypothyroid state and by PTU or BHDB treatment (2,20-24).

The third pathway for T_3 metabolism in rat liver cells is direct IRD to 3,3'-T_2. The latter was only observed at low (<10 nM) T_3 concentrations in SO_4^{2-}-deplete cultures, independent of the presence of PTU (Figs. 5 & 7). The 3,3'-T_2 produced by IRD of T_3 accumulates in these cultures because the main pathway for the metabolism of 3,3'-T_2 (i.e. sulfation) is excluded. Similar findings have been obtained in studies of the production of 3,3'-T_2 from rT_3 in this system (8). Direct IRD of T_3 to 3,3'-T_2 is apparently due to the presence of a PTU-insensitive, type III-like iodothyronine deiodinase. This was concluded from experiments in which type I deiodinase activity was completely inactivated by incubation of hepatocytes (see Results) or the microsomal fraction (Table 1) with rT_3 and PTU. It is known that rT_3 is the preferred substrate for the type I enzyme and that PTU is an uncompetitive inhibitor which reacts with an enzyme intermediate generated during catalysis (2,3). The degree of PTU inhibition, therefore, depends on both quality and quantity of the

substrate. T_3 itself is a poor substrate for the type I deiodinase with a high K_m of ~10 uM (14), and at T_3 concentrations <1 uM PTU inhibition is only partial. However, complete inactivation of the type I enzyme is achieved with PTU in the presence of 1 uM rT_3, independent of the T_3 concentration (2,3). Under these conditions production of 3,3'-T_2 from low T_3 concentrations persists (e.g. Table 1), compatible with the involvement of a type III-like deiodinase (see below).

Although 3,3'-T_2 production was only observed in SO_4^{2-}-deplete hepatocytes, direct IRD of T_3 also occurs under normal (i.e. SO_4^{2-}-replete) conditions. In SO_4^{2-}-containing incubations in which the type I enzyme was completely inhibited (i.e. by PTU and rT_3), 3,3'-T_2S production was still observed. As under these conditions type I IRD of T_3S is prevented and evidence exists that type III IRD of T_3S does not occur (27,28), the only remaining route for this 3,3'-T_2S production is direct IRD of T_3 and successive sulfation of 3,3'-T_2.

In SO_4^{2-}-deplete cultures production of 3,3'-T_2G in addition to 3,3'-T_2 was observed at low T_3 concentrations. As T_3G is no substrate for IRD by the type I enzyme, 3,3'-T_2G is only formed by glucuronidation of 3,3'-T_2. Virtually no 3,3'-T_2G is observed in rat bile (23), consistent with our in vitro observations that glucuronidation of 3,3'-T_2 is much slower than its sulfation (8).

The production of radioiodide in incubations of outer ring-labeled T_3 with hepatocytes may be derived from different routes (Fig. 9). Roughly equal amounts of I^- are produced via the pathways $T_3 \longrightarrow T_3S \longrightarrow 3,3'\text{-}T_2S$ and $T_3 \longrightarrow 3,3'\text{-}T_2 \longrightarrow 3,3'\text{-}T_2S$. This is concluded from the similar quantities of T_3S and 3,3'-T_2S that accumulate in SO_4^{2-}-replete cultures at 1 nM T_3 in the presence of PTU (Fig. 5). At higher substrate concentrations levels the contribution via the pathway $T_3 \longrightarrow 3,3'\text{-}T_2 \longrightarrow 3,3'\text{-}T_2S$ decreases due to saturation of the first reaction (Fig. 5). In SO_4^{2-}-deplete cultures I^- is mainly produced by PTU-sensitive (type I) ORD of 3,3'-T_2 (Figs. 4 & 5). Principally, two other I^- producing pathways are possible, namely type I ORD of T_3 or T_3S. However, no 3,5-T_2 could be measured by specific RIA in SO_4^{2-}-deplete cultures (Visser, T.J., and S.J.Eelkman Rooda, unpublished observations). Furthermore, experiments with microsomes strongly suggest that T_3S primarily undergoes IRD and succesive ORD of the product 3,3'-T_2S (7).

As described above, we obtained evidence for the presence of a type

III-like enzyme in our hepatocyte cultures. So far, the type III enzyme, which is PTU insensitive and catalyses only the IRD of iodothyronines, has been localized in rat brain (29), skin (30) and placenta (31). A similar enzyme has been described in less-differentiated liver tissues and cells, i.e. monkey hepatocarcinoma cells (27,28) and in fetal rat (32) and chicken liver (33), but to our knowledge not in adult liver. One explanation for our finding is the possible induction of the enzyme under our culture conditions. Direct IRD of T_3 in the hepatocytes appeared more variable than the other metabolic pathways. However, the results in Table I clearly show for the first time that the type III enzyme is present in the microsomal fraction of adult rat liver. The reason that no type III-like activity has been described previously in liver microsomes is probably due to the low V_{max} and K_m values of this enzyme for T_3. Therefore, the contribution of this enzyme to the 3,3'-T_2 production at the high T_3 concentrations generally used in studies of the type I IRD of T_3 is also low. Further studies are needed to characterize this type III-like deiodinase of rat liver.

The principles of the various methods used in this paper have been described previously (8). Firstly, a 30 min preincubation of hepatocytes in Dulbecco medium without SO_4^{2-} or possible SO_4^{2-} precursors such as methionine and cysteine in the presence of 100 uM SAM, provides an easy and reliable method to deplete cells of SO_4^{2-} (8). Since SAM is extensively sulfated in liver cells, it is added to the preincubations to further deplete cellular SO_4^{2-} stores. This action of SAM in the preincubation does not interfere with T_3 metabolism in the incubation period, as shown by the restoration of T_3 metabolism after readdition of SO_4^{2-} to SO_4^{2-}-deplete cultures (8). Secondly, at T_3 concentrations >10 nM Sephadex LH-20 chromatography provides a rapid means for the study of the T_3 metabolism, with results in close agreement with HPLC analysis and RIA measurements. For analysis of incubations with <10 nM T_3 Sephadex LH-20 chromatography is less suitable, due to accumulation of 3,3'-T_2 and its conjugates. Therefore, these incubations were analyzed by HPLC and specific RIA's. Thirdly, analysis of the medium reflects intracellular events and can thus be used for quantitation of the T_3 metabolism. This is concluded from the findings that, especially in the presence of BSA, the various products are observed predominantly in the medium while, furthermore, their distribution over medium and cells is independent of the different circumstances.

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CHAPTER VI

INCREASED PLASMA T_3 SULFATE IN RATS WITH INHIBITED TYPE I IODOTHYRONINE DEIODINASE ACTIVITY AS MEASURED BY RADIOIMMUNOASSAY

ABSTRACT

In contrast to the glucuronide conjugate, T_3 sulfate (T_3S) undergoes rapid deiodinative degradation in the liver and accumulates in rats and in rat hepatocyte cultures if type I iodothyronine deiodinase activity is inhibited. We here report the radioimmunoassay (RIA) of plasma T_3S in rats treated with the antithyroid drugs propylthiouracil (PTU) or methimazole (MMI), of which only PTU inhibits type I deiodinase. Male Wistar rats were treated acutely by ip injection with 1 mg PTU or MMI per 100 g body wt and subsequently for 4 days by twice-daily injections with these drugs together with 0.5 ug T_4 or 0.25 ug T_3 per 100 g body wt. Blood was obtained 4 h after the last injection and plasma T_4, rT_3, T_3 and T_3S were determined by RIA and compared with pretreatment values. Serum concentrations (mean $\pm$ SEM nmol/l) in untreated rats were: T_4 51 $\pm$ 1; T_3 1.37 $\pm$ 0.03; T_3S 0.09 $\pm$ 0.01; rT_3 0.03 $\pm$ 0.002. Serum T_3 was decreased and T_3S and rT_3 were increased by acute PTU treatment (T_3 1.16 $\pm$ 0.05, $p<0.01$; T_3S 0.33 $\pm$ 0.04, $p<0.001$; rT_3 0.27 $\pm$ 0.02, $p<0.001$) but unaffected by acute MMI treatment (T_3 1.37 $\pm$ 0.05; T_3S 0.09 $\pm$ 0.01; rT_3 0.02 $\pm$ 0.003). In T_4-treated rats, serum T_3 was decreased and T_4, T_3S and rT_3 were increased by PTU vs MMI (T_4 86 $\pm$ 5 vs 58 $\pm$ 4, $p<0.001$; T_3 0.51 $\pm$ 0.07 vs 0.88 $\pm$ 0.06, $p<0.001$; T_3S 0.38 $\pm$ 0.03 vs 0.12 $\pm$ 0.01, $p< 0.001$; rT_3 0.86 $\pm$ 0.19 vs 0.08 $\pm$ 0.01, $p<0.005$). In T_3-substituted rats T_3S was increased by PTU vs MMI (1.09 $\pm$ 0.13 vs 0.25 $\pm$ 0.03, $p<0.001$). The T_3S/T_3 ratio in the PTU-treated, T_3-replaced rats (0.60 $\pm$ 0.09) was in agreement with that determined by HPLC of serum radioactivity in animals that in addition to this treatment also received ~10 uCi [^{125}I]T_3 with the last 2 injections (0.92 $\pm$ 0.13). In conclusion, this investigation demonstrates the feasibility of the measurement of serum T_3S by RIA. Our findings confirm previous observations with radioactive isotopes, suggesting that sulfation is an important pathway for the metabolism of T_3 in rats. Analogous to rT_3, the accumulation of T_3S in PTU-treated rats indicates that this conjugate is metabolized predominantly by type I deiodination.

INTRODUCTION

Although thyroxine (T_4) is the predominant secretory product of the thyroid, it is thought to have little intrinsic biological activity (1). Outer ring deiodination (ORD) of T_4 yields the bioactive hormone 3,3',5-triiodothyronine (T_3). Complete loss of bioactivity results from the inner ring deiodination (IRD) of T_4 with the formation of 3,3',5'-

triiodothyronine (rT_3). Peripheral conversion of T_4 is the major source for circulating T_3 (80%) and rT_3 (95%) in normal humans and rats (1). For this purpose many tissues contain iodothyronine deiodinases (called type I, II and III), enzymes that act not only on T_4 but also on its metabolites (2,3). The type I deiodinase, located in liver and kidney, is a nonselective enzyme capable of both ORD and IRD, while the type II and III enzymes specifically act on either the outer or the inner ring (2,3). A further distinction is the potent inhibition of type I deiodinase by propylthiouracil (PTU), which drug does not influence type II or III activity. In euthyroid rats, peripheral production of plasma T_3 is effected primarily by type I ORD of T_4 and to a minor extent through the type II deiodinase (4). Besides conjugation, T_3 is metabolized by type I and III IRD to 3,3'-diiodothyronine (3,3'-T_2).

We have recently investigated the metabolism of T_3 in primary cultures of rat liver cells (Chapter V, ref. 5) and in intact rats (6,7). In hepatocytes, T_3 is metabolized via three quantitatively important pathways, i.e. glucuronidation, sulfation and direct IRD. Both in vitro (at <10 nM T_3 concentrations) and in vivo, radioiodide and labeled T_3G were the main metabolites of outer ring ^{125}I-labeled T_3 (5-7). While T_3 glucuronide (T_3G) is a stable conjugate that is rapidly excreted into the bile, normally little T_3 sulfate (T_3S) was observed in vitro and in vivo due to rapid deiodination of T_3S by the type I enzyme. Inhibition of the deiodinase with PTU induced the accumulation of T_3S and 3,3'-T_2 sulfate (3,3'-T_2S) in the hepatocyte cultures (5) as well as in bile and plasma of the intact animal (6,7). These results strongly suggested that successive sulfation and deiodination is an important pathway in the hepatic metabolism of T_3.

We have developed a specific radioimmunoassay (RIA) for T_3S (8) and we now report its use in the measurement of plasma T_3S levels in rats treated with PTU or methimazole (MMI).

METHODS

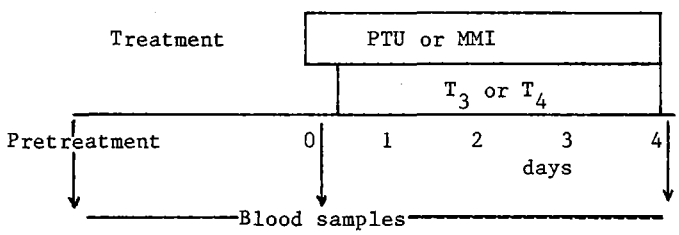
Materials

3,5-Diiodothyronine (3,5-T_2), T_3, rT_3 and T_4 were obtained from Henning Berlin GmbH (Berlin, FRG). [3'-^{125}I]T_3 was obtained from Amersham (Amersham, UK; >1200 uCi/ug) or synthesized by radioiodination of 3,5-T_2 in our laboratory (3300 uCi/ug) using standard methods. [3'-^{125}I]T_3S and non-radioactive T_3S were prepared by reaction of labeled or unlabeled T_3 with chlorosulfonic acid and purified by Sephadex LH-20 (Pharmacia Fine

Chemicals, Uppsala, Sweden) chromatography as previously described (9). Standard T_3S was also a gift from Dr. H. Rokos (Henning). PTU was purchased from Sigma Chemical Co. (St Louis, MO) and MMI (2-mercapto-1-methyl-imidazole) from Janssen Chimica (Beerse, Belgium). All other reagents were of analytical grade.

Experimental procedures

Male Wistar rats, 200-250 g body wt, were treated acutely (day 0) by ip injection with 1 mg PTU or MMI in 250 ul 0.1 N NaOH in saline per 100 g body wt. Subsequently the rats were injected ip twice daily with 1 mg PTU or MMI together with 0.5 ug T_4 or 0.25 ug T_3 in 250 ul 0.1 N NaOH in saline per 100 g body wt for 4 days. Blood samples (2 ml) were taken under ether anesthesia from the tail vein or by orbital puncture before treatment and 4 h after the initial PTU or MMI injection on day 0. The animals were bled by heart puncture 4 h after the last injection on day 4 (Scheme 1). In addition, 5 rats were treated with PTU and T_3 as described above, but with the last 2 injections they also received ~10 uCi [^{125}I]T_3.



Scheme 1

Analysis of samples

RIA

Serum was kept at -20 C until assayed for T_4, T_3, T_3S and rT_3 content by specific RIA. RIA of plasma T_4, T_3 and rT_3 was performed using standards prepared in thyroid hormone-free human or rat serum with identical results. The T_3S RIA was recently described by us (8) and adapted for serum T_3S measurements. Although the antiserum (#8193) used in this assay is fairly specific, the low crossreactivities with T_3 (0.4%) and, more importantly because of its higher plasma levels, T_4 (0.6%) are not negligible and necessitate the isolation of the serum T_3S fraction as follows. To 0.25-0.5 ml serum was added 3 volumes of a HCl-ethanol solution to obtain final concentrations of 0.5 M HCl and 10% ethanol. The mixtures were applied to

small Sephadex LH-20 columns (0.75-3 ml bed volume), which were eluted successively with 1-2 ml 0.1 M HCl (2x), H_2O (2x), 20% ethanol in water (7x) and ethanol-0.1 M NaOH (1:1, vol/vol) (3x). Appropriate 20% ethanol in water fractions (usually 3) were pooled and freeze-dried. Alternatively, the pooled T_3S fractions were concentrated on a second Sephadex LH-20 column and transferred to a small volume of 0.1 M ammonia in ethanol as described (Chapter V), followed by evaporation of the solvent at 50 C under a stream of N_2. Residues were dissolved in 0.5-1 ml 0.01 M NaOH or RIA buffer and assayed for T_3S content. T_3S fractions were identified by chromatography of parallel serum samples spiked with [^{125}I]T_3S. Recovery of the tracer over the entire procedure amounted to 76 $\pm$ 2% (mean $\pm$ SEM, n=19).

Recovery of 500 pM T_3S added to pool serum was 106 $\pm$ 6% (n=8). Addition of T_3 (10 nM) and T_4 (1 uM) increased serum T_3S immunoreactivity with <50 pM (n=8). The within-assay CV was found to be 7.4% (n=8). The between-assay CV calculated on the basis of repeated determinations on the same samples in separate assays was 12.0%.

Radioactive products

Serum samples from rats treated with [^{125}I]T_3 were analyzed as follows. Plasma $^{125}I^-$ was measured as trichloroacetic acid (TCA)-soluble radioactivity. For this purpose, 50-100 ul serum was mixed with 10% (wt/vol) ice-cold TCA to a final volume of 500 ul. After 10 min at 0 C, mixtures were centrifuged and radioactivity was determined in the supernatant. For identification of T_3 metabolites by HPLC, iodothyronines and conjugates were separated on Sephadex LH-20 or they were obtained collectively by solid-phase extraction (SPE). Fractionation of serum was done by stepwise Sephadex LH-20 chromatography similar to the method described above, modified such that conjugates were eluted with H_2O and iodothyronines with 0.1 M ammonia in ethanol. SPE of serum was performed as follows. Serum (0.5 ml) 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., Philipsburg, NJ). Columns were washed successively with 2xl ml of each 0.1 M NaOH, H_2O, 0.1 M HCl and H_2O, before both conjugates and iodothyronines were collected in 1 ml methanol. Reversed-phase HPLC was done using a 10x0.3-cm Chromspher C_{18} analytical column (Chrompack BV, Middelburg, The Netherlands) as described previously (6). Elution was performed with a 25 min gradient of 16-40% acetonitrile in 0.02 M ammonium acetate (pH 4). Flow was 0.8 ml/min

and fractions of 0.5 min were collected.

Data analysis

The T_3S RIA was corrected for recovery of [^{125}I]T_3S added to pool serum run in triplicate parallel with experimental samples in each chromatography session. Data are presented as mean ± SEM. Student's t-test was used to determine the significance of the difference between serum levels in untreated rats versus (vs) acute PTU or MMI treatment, or between those in the chronically PTU- vs MMI-treated rats.

RESULTS

Fig. 1 shows the results of the serum T_4, T_3, rT_3 and T_3S measurements as well as the T_3/T_4, rT_3/T_4 and T_3S/T_3 ratios in rats before and 4 h after a single MMI or PTU injection. Control values were: T_4 51 ± 1, T_3 1.37 ± 0.03, T_3S 0.09 ± 0.01 and rT_3 0.03 ± 0.002 nmol/l. Serum T_4 was not affected while serum T_3 was decreased by 15% and serum rT_3 and T_3S were increased 9- and 3.7-fold, respectively, by acute PTU treatment. No changes were observed after a single MMI-injection. Control values for the different ratios were: $T_3/T_4(x10^2)$ 2.8 ± 0.1, $rT_3/T_4(x10^2)$ 0.6 ± 0.05, and T_3S/T_3 0.07 ± 0.005. Acute PTU treatment decreased the T_3/T_4 ratio by 25% and increased the rT_3/T_4 and T_3S/T_3 ratios 8- and 4.7-fold, respectively. Acute MMI treatment did not produce significant changes in any of these parameters.

Figure 2 shows the results of the longer-term treatment of rats with PTU or MMI in combination with replacement doses of T_4. Treatment of T_4-substituted rats with PTU resulted in 48% higher T_4, 42% lower T_3, 11-fold higher rT_3 and 3.2-fold higher T_3S (0.38 ± 0.03 vs 0.12 ± 0.01 nmol/l) levels compared with corresponding values in MMI-treated rats. As a result, PTU induced a 63% decrease in the T_3/T_4 ratio, a 6-fold increase in the rT_3/T_4 ratio and a 6.5-fold increase in the T_3S/T_3 ratio (0.91 ± 0.16 vs 0.14 ± 0.01).

Figure 3 shows the effects of prolonged PTU or MMI administration in T_3-treated rats. Treatment with T_3 for 4 days lowered serum T_4 levels to 5-8 nmol/l in both groups of rats (not shown), and serum T_3 levels were not significantly different after PTU (1.87 ± 0.15 nmol/l) or MMI (2.22 ± 0.16 nmol/l). Compared with MMI, PTU-treatment caused a 4-fold increase in the serum T_3S levels (from 0.25 ± 0.03 to 1.09 ± 0.13 nmol/l) and a 5-fold increase in the T_3S/T_3 ratio (from 0.12 ± 0.02 to 0.60 ± 0.09).

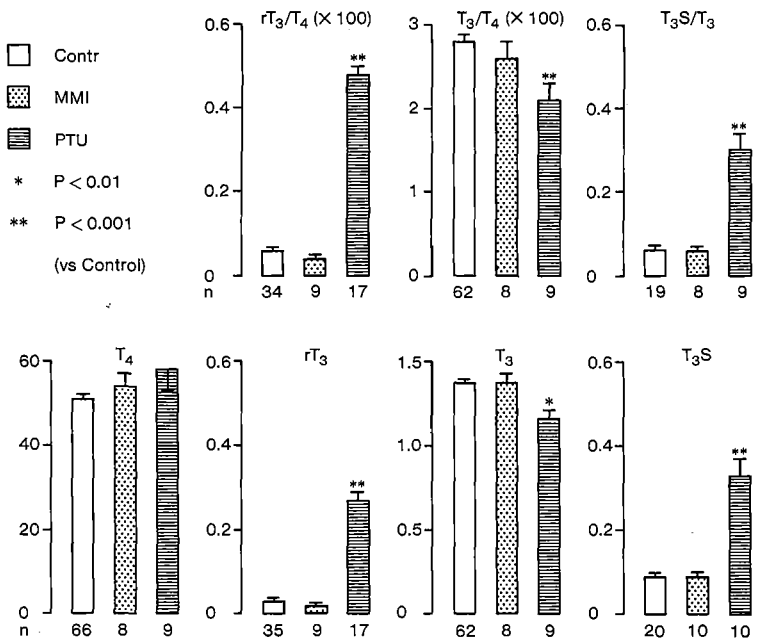


Figure 1
Serum T_4, rT_3, T_3 and T_3S (nmol/l) and the rT_3/T_4, T_3/T_4 and T_3S/T_3 ratios in rats before and 4 h after acute treatment with 1 mg MMI or PTU per 100 g body wt. Results are given as mean ± SEM and the significance of the effects of PTU and MMI was estimated using Student's t-test: * $p < 0.01$, ** $p < 0.001$

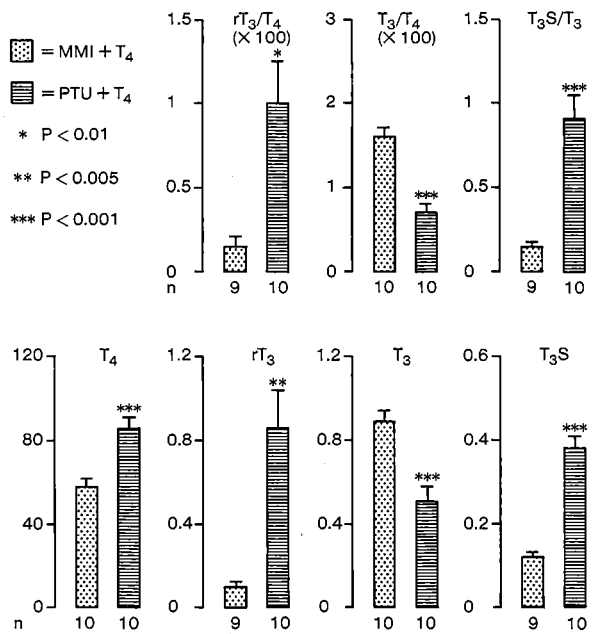


Figure 2
Serum T_4, rT_3, T_3 and T_3S (nmol/l) and the rT_3/T_4, T_3/T_4 and T_3S/T_3 ratios in rats treated with twice daily injections with 1 mg PTU or MMI and 0.5 ug T_4 per 100 g body wt. Results are given as mean ± SEM and the significance of the differences between PTU and MMI treatment was estimated using Student's t-test: * $p < 0.01$, ** $p < 0.005$, *** $p < 0.001$

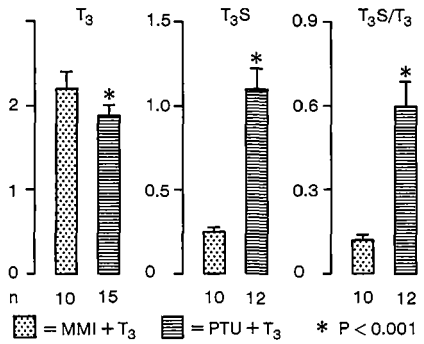


Figure 3
Serum T_3 and T_3S (nmol/l) and the T_3S/T_3 ratio in rats treated with twice-daily injections with 1 mg MMI or PTU and 0.25 ug T_3 per 100 g body wt. Results are given as mean ± SEM and the significance of the differences between PTU and MMI treatment was estimated using Student's t-test: * $p < 0.001$

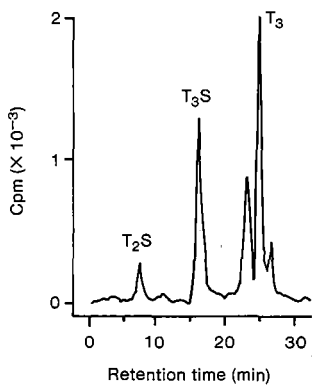


Figure 4
HPLC analysis of a solid-phase extract of serum from a rat treated with twice-daily injection of 1 mg PTU and 0.25 ug T_3 per 100 g body wt. On the last 2 occasions the rat also received 10 uCi [^{125}I]T_3 (for details, see methods). The elution position of reference compounds was determined using synthetic 3,3'-T_2S, T_3S and T_3.

Determination of the serum [^{125}I]T_3S/T_3 ratio

Serum radioactivity of the 5 rats treated with PTU and T_3 that also received [^{125}I]T_3 with the last 2 injections contained 25 ± 3% I^- (determined by TCA-precipitation), 36 ± 4% water-soluble conjugates and 40 ± 4% T_3, as determined by Sephadex LH-20 chromatography. The ratio of radioactive T_3S/T_3 estimated by HPLC was 0.92 ± 0.13 (n=5). In Fig. 4 a representative HPLC profile is shown of one of the rats with a T_3S/T_3 ratio of 0.76. The metabolites identified are 3,3'-T_2S, T_3S, T_3 and an additional conjugate which is probably the sulfate conjugate of 3,3'-diiodothyroacetic acid (M. Rutgers and T.J. Visser, unpublished observations).

DISCUSSION

Monodeiodination is the major pathway of T_4 metabolism, accounting for ~80% of its disposal in humans (1). Roughly equal proportions of T_4 are converted to T_3 and rT_3, although estimates of especially the total body rT_3 production vary considerably in the literature (1). In euthyroid rats, most plasma T_3 appears to be derived from the PTU-sensitive, type I ORD of T_4 in liver and kidney (4). PTU is also a strong inhibitor of the peripheral production of T_3 in humans (10-14). In contrast, PTU does not seem to affect the peripheral production of rT_3 but greatly inhibits the metabolic clearance of this compound (14). Although rT_3 is also produced in the liver by type I IRD of T_4, probably little of this metabolite is released into the circulation but instead is rapidly further degraded by type I ORD to 3,3'-T_2 (2). This explains that plasma rT_3 is mainly cleared by the liver and perhaps to some extent by the kidneys as substantiated by measurements of arterio-venous gradients of rT_3 across these organs in patients with mild liver failure (15). The source of plasma rT_3 has not been established but is probably derived from the type III IRD of T_4 perhaps in brain and skin (2).

Whereas type I deiodination is the major metabolic pathway of rT_3, the metabolism of T_3 is more complex. In extrahepatic tissues T_3 is deiodinated to 3,3'-T_2 especially by the type III IRDase while in rat liver two additional pathways have been detected, i.e. glucuronidation and sulfation (5). Whereas T_3G is a stable conjugate, T_3S is metabolized in rapid succession by IRD and ORD with 3,3'-T_2S as a transient intermediate. Due to its high susceptibility to type I deiodination (16), little T_3S is observed under normal conditions in rat serum or bile and in T_3 incubations with isolated rat hepatocytes (5-7). Only if type I deiodinase activity is

inhibited for instance with PTU, T_3S has been shown to accumulate in vivo and vitro (5-7).

The present experiments in rats were designed to investigate the usefulness of the RIA of plasma T_3S in the study of the peripheral metabolism of thyroid hormone. The properties of this RIA have been described in detail elsewhere (8). Although the cross-reactivity of T_4 and T_3 with the T_3S antibody are low, their relatively high serum concentrations necessitate that these iodothyronines are removed prior to the assay. This has been accomplished by isolation of the serum iodothyronine conjugate fraction in a simple, batch-wise precleaning step using Sephadex LH-20 as an adsorbent. This method is characterized by reproducible and high recoveries for T_3S. Using this procedure, interference with the estimation of plasma T_3S levels by even supra-physiological T_4 and T_3 concentrations was circumvented.

In this study we used PTU as a selective inhibitor of the type I deiodinase in liver and kidney. This drug has little or no effect on the type II and III deiodinases in other tissues (2). As a control, parallel groups were treated with MMI, another thyrostatic drug. Overwhelming evidence has been presented in the literature that MMI does not inhibit the peripheral deiodination of thyroid hormone (17), although a recent study has suggested that MMI may enhance tissue T_4 to T_3 conversion (18). No such action of MMI was evident in the present study. For instance, treatment of rats for 4 days with a replacement dose of T_4 in combination with MMI resulted in plasma T_4 levels 4 h after the last injection that were equal to those in untreated animals. The T_3/T_4 ratio in these animals was 40% lower than in the controls, which is largely accounted for by the cessation of thyroidal T_3 secretion.

Plasma T_3S levels were low in untreated rats and the T_3S/T_3 ratio in these animals (0.07) was not changed after a single injection with MMI (0.06) or after prolonged treatment with this drug in combination with T_4 (0.14) or T_3 (0.12). The effectiveness of PTU as an inhibitor of the type I deiodinase was demonstrated by the greater reduction in the plasma T_3/T_4 ratio compared with the parallel, MMI-treated rats. This was already evident 4 h after a single PTU injection but most impressive were the differences in the T_4-replaced animals, where the T_3/T_4 ratio was decreased by 63% in the PTU-treated rats compared with the corresponding MMI group. This figure is in excellent agreement with the findings reported by Silva

et al. (4), indicating that roughly 70% of peripheral T_3 production in euthyroid rats is accomplished by the PTU-sensitive (i.e. type I) deiodination of T_4. In the T_4-substituted rats, plasma T_4 levels were considerably higher in animals treated with PTU than in those receiving MMI, indicating that type I deiodination is an important, rate limiting step in the disposal of T_4. Plasma T_3S was increased 3.5-fold 4 h after acute PTU administration and was also greatly elevated by chronic treatment with this drug in the T_4-substituted rats despite the decrease of plasma T_3 in these animals. This seems to indicate that PTU exerts a greater inhibition on the metabolic clearance of T_3S than on the production of T_3 from T_4. This is explained by assuming that type I deiodination is virtually the only pathway for the metabolism of T_3S, while there exists an alternative, PTU-insensitive (type II) pathway for the peripheral production of T_3. The mean T_3S/T_3 ratio in the PTU-treated rats varied from 0.31 in the acutely treated rats to 0.60-0.91 after prolonged treatment of the animals substituted with T_3 or T_4. The magnitude of these T_3S/T_3 ratios was similar to that found previously 4 h after iv administration of $[^{125}I]T_3$ to rats treated with a single PTU dose. This was further substantiated in the present study by the results obtained with PTU-treated, T_3-substituted rats that also received $[^{125}I]T_3$. The $[^{125}I]T_3S/T_3$ ratio in these animals (0.92 ± 0.13) was not significantly different from the T_3S/T_3 ratio determined by RIA.

In the absence of accurate data on the MCR of T_3S and steady-state plasma levels of this conjugate under different conditions, it is not possible from the results in this paper to provide estimates of the quantitative contribution of sulfate conjugation to the metabolism of T_3 in rats. However, the in vitro studies with isolated rat hepatocytes (chapter V, ref. 5) as well as the analyses of T_3 metabolites in bile (7) suggest that sulfation is equally important as glucuronidation, accounting together for roughly half of the T_3 disposal.

Except, of course, for the T_3-replaced rats there appears to be a close correlation between the PTU-induced increase in plasma rT_3 and T_3S. The plasma rT_3 levels in the untreated rats (0.03 nmol/l) were close to the detection limit of our RIA and in agreement with the values reported by Kaplan et al. (19) but substantially lower than those published by most other investigators (20-23). The 8-fold increase in plasma rT_3 in response to acute as well as chronic PTU-treatment is also in excellent agreement

with data published by Kaplan et al. (19) and Heinen et al. (23). The parallel increase in plasma rT_3 and T_3S after PTU further underscores the importance of the type I deiodinase for the disposal of these compounds.

In conclusion, this paper demonstrates the feasibility of the RIA for plasma T_3S as a valuable tool in the study of the peripheral metabolism of thyroid hormone in rats. Analogous to rT_3, the accumulation of plasma T_3S in PTU-treated rats confirms previous findings with radioactive isotopes (6,7). showing that type I deiodination is the main pathway for the metabolism of T_3S. The high plasma T_3S levels encountered in PTU-treated rats suggests that indeed sulfation is an important step in the elimination of T_3. Preliminary results of the RIA of plasma T_3S in humans indicate that although levels are much lower than in rats, they are also increased by drugs that inhibit type I deiodinase activity (S.J. Eelkman Rooda and T.J. Visser, unpublished observations).

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CHAPTER VII

SERUM TRIIODOTHYRONINE SULFATE IN MAN MEASURED BY RADIOIMMUNOASSAY

ABSTRACT

In humans deiodination and, perhaps, glucuronidation are important pathways in thyroid hormone metabolism. In addition, sulfation plays an important role in T_4 and especially in T_3 metabolism in rats, but little is known about sulfate conjugation of thyroid hormone in humans. In the present study we used a specific T_3 sulfate (T_3S) RIA to address this question. Eight euthyroid volunteers were treated for 7 weeks with oral T_3 (1 ug/day/kg body wt). In the 5^{th} week they also received during 2 days propylthiouracil (PTU, 4x250 mg/day) and in the 7^{th} week during 3 days iopanoic acid (IOP, 1 g/day). Pretreatment values were (mean $\pm$ SEM nmol/l): T_4 92 $\pm$ 6; rT_3 0.24 $\pm$ 0.02; T_3 2.30 $\pm$ 0.10 and T_3S <0.1 (at or below the detection limit of the RIA). After 4 weeks of T_3-suppression these values were: T_4 39 $\pm$ 6; rT_3 0.11 $\pm$ 0.01; T_3 5.31 $\pm$ 0.39; T_3S 0.10 $\pm$ 0.01. After 2 days of PTU treatment, T_4 increased to 48 $\pm$ 7 ($p<0.01$), rT_3 to 0.20 $\pm$ 0.03 ($p<0.01$), T_3S to 0.13 $\pm$ 0.01 ($p<0.025$), but T_3 did not change (4.91 $\pm$ 0.35). The effect of IOP was more pronounced; after administration for 3 days it increased T_4 to 49 $\pm$ 8 ($p<0.001$), rT_3 to 0.48 $\pm$ 0.09 ($p<0.005$), T_3S to 0.29 $\pm$ 0.04 ($p<0.005$), and it decreased T_3 to 3.95 $\pm$ 0.25 ($p<0.005$). The T_3S/T_3 ratio was increased by PTU from 0.018 $\pm$ 0.003 to maximally 0.024 $\pm$ 0.004 ($p<0.025$) and by IOP to maximally 0.055 $\pm$ 0.007 ($p<0.005$). In conclusion: 1) serum T_3S is virtually undectable in euthyroid humans; 2) low but significant serum T_3S concentrations are detected in humans treated with T_3; 3) serum T_3S in T_3-treated subjects is increased by inhibition of type I deiodinase activity with PTU and especially IOP; and 4) in comparison with previous estimates of the serum T_3S/T_3 ratio in rats, the low ratios determined in humans may be explained by: a lesser importance of sulfation for the metabolism of T_3 in humans, and/or differences in kinetics of plasma T_3 and T_3S between humans and rats.

INTRODUCTION

In the rat, conjugation with glucuronic acid and sulfate are important pathways in the metabolism of thyroxine (T_4) and 3,3',5-triiodothyronine (T_3). The glucuronides of T_4 (T_4G) and T_3 (T_3G) are the main products in the bile of rats injected with radioactive T_4 or T_3 (1-3). Glucuronidation, in contrast to sulfation, does not seem to be an irreversible pathway for the elimination of these iodothyronines, since strains of obligately anaerobic bacteria in the intestine are capable of deconjugating these glucuronides (4-5). The liberated iodothyronines are, at least partly,

reabsorbed but the extent of such an enterohepatic cycle for thyroid hormone has not been established (6-8). However, the sulfate conjugates of T_4 (T_4S) and T_3 (T_3S) are much better substrates than the nonsulfated hormones for the type I iodothyronine deiodinase, which is located mainly in the liver and kidneys (9). This explains why little labeled T_3S was found in serum and bile of normal rats after iv injection of [3'-^{125}I]T_3. Treatment of rats with propylthiouracil (PTU) or iopanoic acid (IOP), which inhibit the type I deiodinase, strongly increased T_3S in serum and bile (10).

Little is known about the possible role of glucuronidation and sulfation in the metabolism of T_4 and T_3 in man. T_4G and T_3G have been detected in human bile (11) but the quantitative importance of this pathway has not been assessed. Recent findings suggest that successive sulfation and deiodination of T_3 may occur in humans. Young and coworkers have shown that in human liver T_3 is a substrate for both the thermostable and thermolabile forms of phenol sulfotransferase (12). Visser et al. have shown that in vitro the type I deiodinase of human liver is capable of inner ring deiodination (IRD) of T_3S, which reaction is much more efficient than the IRD of T_3 itself. PTU and IOP are also effective inhibitors of the human type I deiodinase (13). Finally, T_3S, although in a very small amount, has been identified after an iv injection of labeled T_3 in serum of euthyroid volunteers (14) and in serum and bile of a hypothyroid patient (15).

With the use of a recently developed T_3S RIA (16), we demonstrated that it is possible to measure serum T_3S levels in rats (17). In the present study we used this RIA to investigate the role of sulfation in the metabolism of T_3 in humans by measurement of plasma T_3S before and during treatment with PTU and IOP. As mentioned above, both drugs inhibit the type I deiodinase, but IOP also inhibits other (i.e. type II and III) iodothyronine deiodinases (18). As inhibition of the type I enzyme by these drugs does not only lead to a decreased breakdown of T_3S but also, through a diminished T_4 to T_3 conversion, to a reduced T_3 substrate supply for sulfation, the ultimate effect on plasma T_3S levels is unpredictable. Therefore, the present study was carried out in healthy human volunteers in whom the endogenous T_4 production was suppressed by oral T_3 administration.

SUBJECTS AND METHODS

Subjects

Eight euthyroid volunteers, 6 males and 2 females, who took no medication participated in the study. The mean age was 33 yrs (range 27-39), and informed consent was obtained from all subjects. During the entire study period subjects were treated with oral T_3 (Cytomel^R), 1 ug/day/kg body wt, divided in 3 doses taken at 7 am, 3 pm and 11 pm. In the 5^{th} week of the study, the subjects also received oral treatment with PTU (250 mg/6 h) for 2 days, and in the 7^{th} week they were given oral doses of IOP (Telepaque^R; 1 g daily) for 3 days. (Telepaque was obtained from Sterling Winthrop, courtesy of Dr. D. Glinoër). Blood samples were always taken at 1 PM, once a week during the first 4 weeks and in the 6^{th} week, and once a day during the 5^{th} and 7^{th} week in which PTU and IOP were given (for details, see also the legend to Fig.1).

RIA measurements

Serum was kept at -20 C until assay. TSH was measured with a sensitive immunoradiometric assay (RIA-gnost^R, Behring, Marburg, FRG). The free T_4 (FT_4) concentration was calculated by multiplying the FT_4-fraction (SPAC, BYK Mallinckrodt, Dietzenbach, FRG) and the total T_4 concentration. Total T_4 and T_3 were measured by established RIA procedures and total rT_3 as previously described (19). Iodothyronine standards were obtained from Henning Berlin GmbH (West Berlin, FRG). Standard T_3S was prepared in this laboratory (20) or was a gift from Dr. H. Rokos (Henning). [3'-^{125}I]T_3S was prepared in this laboratory (20). The T_3S RIA has been described recently (16) and subsequently modified for serum T_3S measurements (17). The small but significant cross-reactivities of T_4 and T_3 necessitate the isolation of the T_3S fraction from serum in a batch-wise procedure using Sephadex LH-20 as an adsorbant. T_3S was isolated from 0.5-1.0 ml serum samples with a recovery of 76 $\pm$ 2% of added ^{125}I-T_3S (17). Results were corrected for recovery as determined in pool serum run in triplicate parallel with experimental samples in each chromatography session. Residues were dissolved in 0.5-1 ml RIA buffer and 50-100 ul aliquots were assayed in duplicate for T_3S content. Recovery of 500 pmol/l T_3S added to pool serum was 106 $\pm$ 6% (n=8). Addition of T_3 (10 nmol/l) and T_4 (1 umol/l) increased serum T_3S immunoreactivity with <50 pmol/l. The within and between assay CV's were 7.4 and 12.0%, respectively (17).

Data analysis

All serum samples from the same subject were processed for T_3S RIA in a single series. Except for T_4 and TSH, all rT_3, T_3, T_3S and $3,3'-T_2$ samples were run in the same assay. Serum thyroid concentrations (nmol/l) and ratios thereof are given as mean ± SEM. Statistical analysis was done by Students t-test for paired data.

RESULTS

In Table I are given the serum thyroid hormone measurements before and after 3 or 4 weeks of T_3 suppression. Thyroid parameters of all subjects were in the normal range before treatment. TSH levels were suppressed (<0.10 mU/L) by T_3 from the first week onward during the entire study period. After 3 weeks on T_3, serum T_4 was already maximally decreased by 54%. A close correlation was observed between the decrease in T_4 and the decrease in FT_4 and rT_3 during the first 4 weeks. At the start of the study serum T_3S was <0.1 nmol/l, at or below the detection limit of the RIA, and increased to 0.10 ± 0.01 nmol/l after 4 weeks of T_3 treatment. Serum thyroid parameters in week 6 were identical to those in week 4.

Table I

Serum TSH (mU/l) and iodothyronine measurements (nmol/l) before and after 3 or 4 weeks of T_3 suppression

	Pretreatment values	After 3 weeks of T_3 suppression	After 4 weeks of T_3 suppression	Normal values
TSH	1.53 ± 0.17	0.02 ± 0.003	0.02 ± 0.004	0.20-4.90
T_4	92 ± 6	42 ± 5	39 ± 6	60-150
FT_4	11.2 ± 0.6	5.7 ± 0.7	5.0 ± 0.8	8.1-15.4
T_3	2.30 ± 0.10	4.55 ± 0.27	5.31 ± 0.39	1.30-3.10
rT_3	0.24 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.15-0.52
T_3S	<0.1	ND	0.10 ± 0.01	

Data are mean ± SEM. ND= not determined

In Fig. 1 are shown the effects of PTU and IOP-treatment on serum T_4, rT_3 and T_3 concentrations. PTU and IOP both slightly increased T_4, which effect was evident from the second day of drug treatment onward. Two days

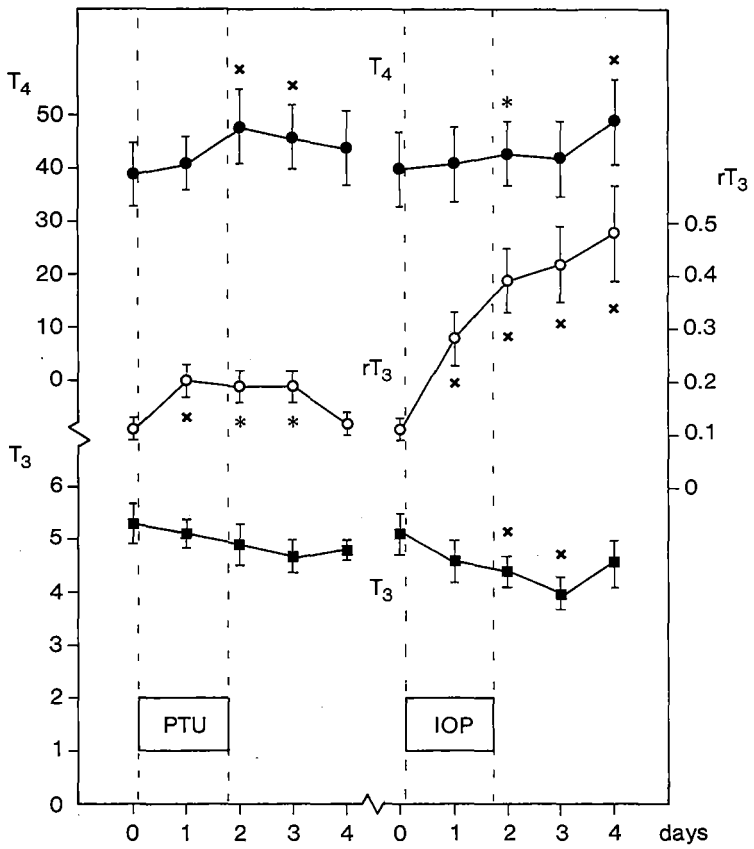

Figure 1
Effect of PTU (left) and IOP (right) on serum T_4, rT_3 and T_3 (nmol/l) during continuous T_3-suppression. Subjects were treated with 1 ug T_3/kg body wt per day divided over 3 oral doses at 7 am, 3 pm and 11 pm. In the 5th week, they also received oral doses of 250 mg PTU starting at 1 pm of day 0 and continuing at 6-h intervals until 7 am of day 2. In the 7th week IOP was given in oral doses of 1 g at 1 pm of day 0 and 7 am of days 1 and 2. Blood was obtained just before the first gift of the drugs on day 0 and at the same time (1 pm) of all subsequent days of week 5 and 7. Data are given as mean ± SEM. Serum measurements were compared to those on day 0 using Students paired t-test: $^{*}P < 0.025$ $^{+}P < 0.01$.

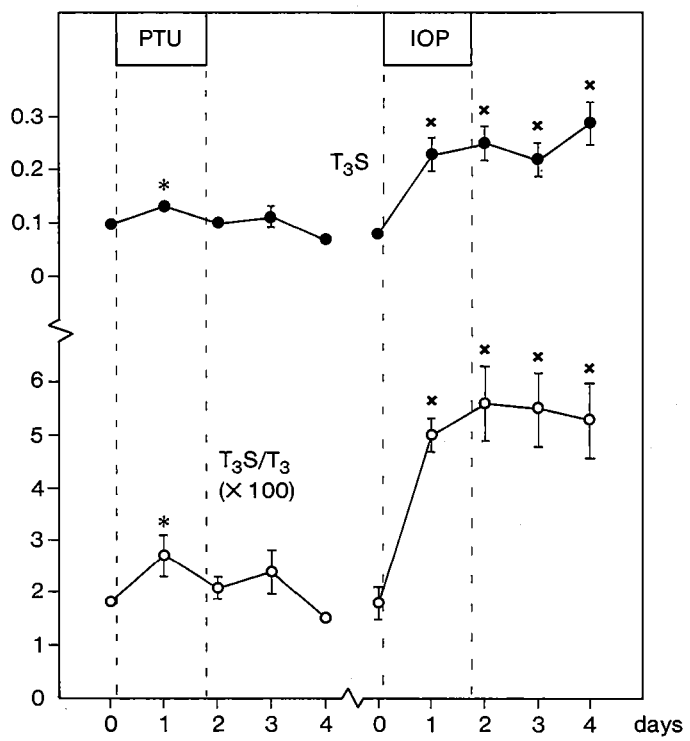

Figure 2
Effect of PTU (left) and IOP (right) on serum T_3S (nmol/l) and the T_3S/T_3 ratio during continuous T_3-suppression. Data are given as mean ± SEM. For further details, see the legend to Fig. 1.

after the last IOP dose T_4 was still 23% higher than before IOP. Reverse T_3 was increased to 180% by PTU, which effect was maintained until 1 day after the last PTU dose. IOP gradually increased rT_3, i.e. 2.5-fold after 1 day of treatment to 4.4-fold 2 days after the last IOP dose. Serum T_3 was decreased by PTU, although not significantly, and by IOP. Maximal decrements in serum T_3, i.e. 11 and 22%, occured 1 day after the last dose of PTU and IOP, respectively.

In Fig. 2 are depicted the effects of PTU and IOP treatment on serum T_3S levels and the serum T_3S/T_3 ratio. Again, the pretreatment values at the start of these 2 regimens were fairly similar. PTU and IOP increased T_3S maximally 1.4 and 3.2-fold, respectively. These maximal effects were reached after 1 day of PTU treatment and 2 days after the last IOP dose. The increase in the T_3S/T_3 ratio was more marked with IOP (maximal increase 3.2-fold) than with PTU (maximal increase 1.5-fold).

DISCUSSION

The thyroid predominantly secretes T_4 and a small amount of T_3, while thyroidal secretion of rT_3 is negligible. Normally, the major fraction of circulating T_3 (~80%) and rT_3 (>95%) is derived from peripheral conversion of T_4 (21). Most studies on the enzymatic mechanisms of thyroid hormone deiodination have been conducted in the rat. These studies have led to the recognition of three different iodothyronine deiodinases, termed type I, II and III (for a review, see ref. 9). The type I deiodinase is located in liver, kidney and thyroid, is capable of both outer- (ORD) and inner ring deiodination (IRD), and is inhibited by PTU. The type II enzyme, a selective outer ring deiodinase, is found, among others, in the central nervous system (CNS), pituitary gland and brown adipose tissue. The type III enzyme is found in the CNS and skin (22) and is only capable of IRD. The latter two enzymes are PTU-insensitive (9).

In euthyroid rats plasma T_3 production is predominantly derived from type I and to a minor extent type II ORD of T_4 (23). Although PTU-treatment lowers plasma T_3 levels in T_4-substituted subjects (24), it is presently unknown to what extent type I deiodination contributes to plasma T_3 production in humans. Plasma rT_3 is probably derived from type III IRD of T_4 (9), but the contribution of the different tissues with type III deiodinase activity has not been determined. Plasma rT_3 is mainly cleared by type I ORD to $3,3'-T_2$ especially in the liver but also in the kidneys (19). In humans, the liver does not seem important for the clearance of

plasma T_3 (25), but this probably occurs predominantly by type III IRD in extrahepatic tissues.

In euthyroid rats small amounts of labeled T_3S were found in serum and bile after iv injection of [^{125}I]T_3. When the type I enzyme was inhibited by PTU, levels of T_3S and 3,3'-T_2S in serum and bile were increased dramatically (10,26). These tracer findings were recently confirmed and extended in a study using a specific T_3S RIA (17). Serum T_3S levels were low (0.09 nmol/l) in euthyroid rats and increased 4 h after an ip injection with 1 mg PTU/100 g body wt (to 0.33 nmol/l) or after 4 days of treatment with PTU in combination with replacement doses of T_4 (to 0.34 nmol/l) or T_3 (to 1.09 nmol/l). Serum T_3S/T_3 ratios varied between 0.6-0.9 in these latter treatment regimens, indicating that in rats sulfation is an important pathway in the metabolism of T_3(17). Similar results were obtained when rats were treated with IOP (27).

The present report shows that in euthyroid humans serum T_3S values are at or below the detection limit of our RIA. During the first 4 weeks of T_3-suppression T_3S became detectable but remained low. Inhibition of the type I deiodinase by PTU and more particularly by IOP increased T_3S. Under the latter treatment, however, serum T_3S is still considerably lower than in rats with inhibited type I deiodinase activity. Also the serum T_3S/T_3 ratio in humans (maximally 0.055) is much lower than previously observed in rats. Several explanations are possible for this difference. Firstly, the potency of the drugs to inhibit type I deiodinase activity may differ between human and rats. Although in vitro the type I deiodinase of human liver shows the same or even higher sensitivity to PTU and IOP (13) than the enzyme from rat liver (9), possible differences in the bioavailability of these drugs may contribute to the relatively low response of serum T_3S to especially PTU in humans. The latter consideration is supported by the smaller effects of PTU on serum rT_3 in humans compared with rats (17). Secondly, sulfation may be a less important pathway of T_3 metabolism in humans than in rats. At present, there is little in vitro evidence to support this possibility but extension of the recent observation of T_3 sulfation by human liver sulfotransferases (12) may provide more information on this point. Thirdly, the lower serum T_3S concentrations in humans may be related to species-specific differences in plasma T_3 and T_3S kinetics. Although the half-life of plasma T_3 is much higher in humans (28) than in rats (23), preliminary evidence suggests that the half-lifes of plasma T_3S are less

different (10,29). This seems to indicate that differences between the rates of T_3 sufation between humans and rats are greater than those in plasma T_3S clearance. Finally, it is possible that after sulfation of T_3 in the human liver a greater proportion of T_3S is excreted into the bile rather than released into the circulation compared with the situation in the rat. Again, no evidence is available to support this possibility.

Only one previous attempt to measure serum T_3S levels in humans has been reported in abstract form (30). This method was based on the difference in T_3 RIA values before and after treatment of serum with arylsulfatase. In accordance with our results, serum T_3S levels in normal subjects were consistently <0.1 nmol/l. Increases were found after administration of propranolol (0.30 nmol/l), and after an acute oral T_4 load. In these conditions, T_3S was reported to represent 22 and 11% of circulating T_3, respectively. In patients with severe systemic illness 23-65% of serum T_3 was in the form of T_3S but absolute values were not mentioned.

The drug-induced increases in serum T_3S and rT_3 levels appeared to correlate very well. As mentioned above, the magnitude as well as the duration of the effects of IOP were greater than those of PTU with respect to both serum T_3S and rT_3. These findings are compatible with the view that type I deiodination is the main route for the metabolism of T_3S and rT_3 in vivo.

Finally, the decrease in serum T_3 by IOP is remarkable since the metabolism of T_3 by the type III deiodinase is also inhibited. This was substantiated by serum $3,3'-T_2$ measurements (19), which showed a significant decrease during IOP (from 0.082 ± 0.006 to 0.048 ± 0.003) but not during PTU. Displacement of T_3 from plasma transport proteins by IOP is unlikely since the free fraction of T_4 was not changed (not shown). Furthermore, thyroidal secretion of T_3 in the T_3-suppressed subjects is already negligible before IOP treatment. Therefore, inhibition of T_3 production from residual circulating T_4 is the most likely explanation for the IOP-induced decrease in serum T_3.

In conclusion, this paper demonstrates that T_3S is virtually undetectable in serum of euthyroid humans. Low but significant serum T_3S concentrations are detected in humans treated with suppression doses of T_3. Inhibition of the type I enzyme by PTU but more particularly by IOP induces a significant increase in serum T_3S levels. Compared with the earlier

reported serum T_3S/T_3 ratio in rats the much lower ratio in humans suggests that sulfation of T_3 is a less important pathway in humans than in rats, although other possible explanations have not been excluded.

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SUMMARY

The thyroid produces all circulating thyroxine (T_4), but plasma 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) are mainly derived from extrathyroidal conversion of this prohormone. T_3 is the only iodothyronine with significant thyromimetic bioactivity. The liver and kidneys are most important in the peripheral production of T_3. The exact sites of rT_3 production are presently not known, but the CNS and skin are thought to be important in this respect. Both T_3 and rT_3 are deiodinated to 3,3'-diiodothyronine (3,3'-T_2). These conversion reactions are catalyzed by iodothyronine deiodinases, of which at least 3 different types have been identified in the rat. These deiodinases, type I, II and III, differ in several aspects like substrate specificity, reaction kinetics and distribution among various tissues.

Chapter I provides a general introduction concerning thyroid hormone synthesis and factors which regulate thyroid hormone bioavailability.

In Chapter II the two conjugation pathways, glucuronidation and sulfation, are reviewed. Enzymes catalyzing these phase II detoxication reactions are ubiquitously present in mammals and serve the purpose to increase the water solubility of compounds to facilitate their excretion into bile and urine. They frequently compete for the same substrate. Glucuronidation appears to be a high-K_m, high-capacity process, whereas sulfation is a low-K_m, low-capacity reaction. In general, therefore, at low substrate concentrations sulfation prevails and at higher concentrations glucuronidation is predominant. The recent progress in the isolation and characterisation of the specific iodothyronine sulfotransferases in rat and man will stimulate further research in this area, whereas identification of the specific iodothyronine UDP-glucuronyltransferase(s) has not been reported.

In Chapter IIC available evidence concerning the importance of conjugation and deiodination for thyroid hormone metabolism is discussed. In the rat conjugation with glucuronic acid and sulfate are, next to deiodination, important metabolic pathways. Glucuronidation of T_4 and T_3 yields metabolites that are more water-soluble than the nonconjugated hormones, and the products T_4 glucuronide (T_4G) and T_3G are excreted into the bile. After biliary excretion, T_4G and T_3G are not lost in the feces but are hydrolyzed by β-glucuronidase-producing bacteria in the intestine. The liberated hormones are then, at least in part, reabsorbed (entero-

hepatic cycle). Whereas glucuronidation does not lead to the irreversible loss of thyroid hormone, sulfation does. This conjugation reaction is not an independent pathway of thyroid hormone metabolism but leads to the formation of derivatives that are better substrates for deiodination than the nonsulfated iodothyronines. The V_{max}/K_m ratios of the type I deiodinase for iodothyronine sulfates are 40-200 fold higher than those for the native hormones. Finally, the quantitative role of glucuronidation in T_4 and T_3 metabolism is deduced from studies in the Gunn rat, a species with an inherited deficiency of UDP-glucuronyltransferase. Little is known about the quantitative role of glucuronidation and sulfation in the metabolism of thyroid hormone in humans. T_4G and T_3G have been identified in human bile, and T_3S, although in a very small amount, has been detected in bile and serum of a hypothyroid patient after iv injection of labeled-T_3.

In Chapter III the role of the type I deiodinase in rT_3 metabolism in isolated rat hepatocytes was investigated. The results indicate that rT_3 is mainly metabolized by outer ring deiodination (ORD) to I^- and 3,3'-T_2. Normally, the product 3,3'-T_2 is not observed due to rapid sulfation and subsequent ORD of 3,3'-T_2S. Inhibition of sulfation does not affect rT_3 clearance, but the decrease in I^- formation from rT_3 is compensated by an increased recovery of 3,3'-T_2 up to 70% of rT_3 metabolized.

In Chapter IV a description of the development of a RIA for T_3S is given. Specific antibodies were raised by immunization of rabbits with a T_3S-bovine serum albumin conjugate. With one of the antisera (#8193) the studies described in Chapters V-VII were performed.

Chapter V contains a detailed investigation into the metabolism of T_3 in isolated rat hepatocytes. Three major pathways were identified: glucuronidation, sulfation and direct inner ring deiodination (IRD). Whereas T_3G is not further metabolized in these cultures, T_3S is rapidly deiodinated by the type I enzyme. Evidence is provided for the presence of a type III-like enzyme in rat liver which has not been recognized before. Direct IRD of T_3 by this enzyme is characterized by a low K_m and is not inhibited by PTU.

In Chapter VI the RIA for T_3S was used for determination of plasma T_3S in rats treated with PTU or methimazole (MMI). In contrast to the MMI treated rats, treatment with PTU alone or in combination with T_3 or T_4 increased plasma T_3S. The T_3S/T_3 ratio of 0.6-0.9 in the PTU-treated rats confirms previous observations with radioisotopes that sulfation is an important pathway in T_3 metabolism in rats.

In Chapter VII the role of sulfation in T_3 metabolism in man was investigated. Normally, serum T_3S was <0.1 nM, at or below the detection limit of the T_3S RIA. Inhibition of the type I enzyme by PTU and especially by IOP in combination with oral T_3 therapy led to a significant increase in plasma T_3S, although levels were much lower than in rats. Accordingly, the T_3S/T_3 ratio was lower in man than in rats. This suggests that sulfation is a less important route in T_3 metabolism in man than it is in rats, although the results may also be due to different rates of T_3 and T_3S turnover in rats and humans.

SAMENVATTING

Schildklierhormoon speelt een belangrijke rol in de stofwisseling en stimuleert de groei en ontwikkeling van het organisme. Het heeft o.a. invloed op het transport van aminozuren en electrolyten vanuit de extracellulaire ruimte naar de cel, en speelt een rol bij de synthese van eiwitten binnen de cel. Dit alles leidt tot veranderingen in celgrootte, celaantal en celfunktie.

Hoewel thyroxine (T_4) het belangrijkste door de schildklier geproduceerde hormoon is, worden bovenstaande effekten vrijwel uitsluitend gemedieerd door het biologisch aktieve 3,3',5-trijodothyronine (T_3). Van het in het plasma circulerende T_3 wordt slechts 20% door de schildklier geproduceerd, terwijl 80% afkomstig is van enzymatische dejodering van T_4 in de lever en nieren. Een derde jodothyronine dat in plasma aanwezig is, is het 3,3',5'-trijodothyronine (reverse T_3, rT_3). Deze stof heeft voorzover nu bekend geen invloed op bovengenoemde biologische processen. Ook plasma rT_3 is voor het overgrote deel afkomstig van enzymatische dejodering van T_4, waarschijnlijk in het centrale zenuwstelsel en de huid. Bij de rat zijn drie enzymen gevonden, die deze dejoderings-reakties katalyseren. Deze worden wel aangeduid met type I, II en III.

In hoofstuk I wordt een algemeen overzicht gegeven van de synthese van schildklierhormoon en van faktoren die een rol spelen bij de regulatie van schildklierhormoon op cellulair niveau.

In hoofdstuk II worden de twee conjugatie reakties, glucuronidering en sulfatering, besproken. Glucuronidering wordt gekatalyseerd door het enzym UDP-glucuronyltransferase. Phenolsulfotransferase is het enzym dat betrokken is bij de sulfatering. Beide conjugatie-reakties komen wijdverspreid voor, niet alleen bij de mens en andere dieren, maar ook in planten en bacteriën. Ze spelen een rol bij de detoxificatie van een groot aantal stoffen, zowel endogeen (bv. hormonen) als exogeen (bv. geneesmiddelen). Koppeling van deze stoffen met glucuronzuur of sulfaat maakt deze stoffen beter water oplosbaar, waarna ze uitgescheiden worden met de gal of urine.

In hoofstuk IIC wordt het belang van conjugatie en dejodering van schildklierhormoon besproken. In de rat en mens worden T_4 en T_3 geglucuronideerd tot de beter in water oplosbare stoffen T_4 glucuronide (T_4G) en T_3G. Deze worden in de gal uitgescheiden. In de darm worden deze stoffen echter weer gehydrolyseerd door glucuronidase-producerende bacteriën, waarna T_4 en T_3 worden gereabsorbeerd. Dank zij deze enterohepatische

kringloop wordt op een meer economische manier met deze hormonen omgesprongen dan wanner zij onveranderd het lichaam zouden verlaten met de faeces.

Sulfatering van schildklierhormoon is lange tijd een onopgemerkt proces geweest, omdat onder normale omstandigheden vrijwel geen sulfaten van schildklierhormoon aantoonbaar zijn. Nader onderzoek heeft uitgewezen dat deze conjugatie reaktie, althans bij ratten, wel degelijk voorkomt, maar dat bv. T_3 sulfaat (T_3S) na vorming snel gedejodeerd wordt. Sulfatering blijkt dus geen onafhankelijke route te zijn, maar nauw verbonden met dejodering. Bij de mens is weinig bekend over de rol van glucuronidering en met name sulfatering in het metabolisme van schildklierhormoon.

De vraagstelling, die ten grondslag ligt aan dit proefschrift, is in hoeverre sulfatering van belang is voor het metabolisme van het schildklierhormoon T_3 en de isomeer rT_3.

In hoofdstuk III is het metabolisme van rT_3 in geisoleerde rattehepatocyten onderzocht. Dejodering van rT_3 in de buitenring levert jodide (I^-) en 3,3'-dijodothyronine (3,3'-T_2) op. Dit laatste produkt wordt echter onder normale omstandigheden niet waargenomen, omdat 3,3'-T_2 snel gesulfateerd wordt en vervolgens door dejodering verder afgebroken. Wordt nu deze sulfatering van 3,3'-T_2 geremd, hetzij door sulfaat te onttrekken aan hepatocyt-culturen danwel remmers van deze reaktie toe te voegen, dan kan 3,3'-T_2 wel terug gevonden worden. Het metabolisme van rT_3 is echter niet afhankelijk van sulfaat.

Om het belang van sulfatering in het metabolisme van T_3 beter te kunnen bestuderen werd een radioimmunoassay (RIA) voor T_3S ontwikkeld. De methode die hiervoor gebruikt is wordt beschreven in hoofdstuk IV.

In hoofdstuk V is het metabolisme van T_3 in rattehepatocyten onderzocht. T_3 wordt op drie manieren gemetaboliseerd. Ten eerste wordt het geglucuronideerd, waarna T_3G in de gal wordt uitgescheiden. Ten tweede kan T_3 worden gesulfateerd tot T_3S, welk produkt snel verder afgebroken wordt door het type I enzym. Ten derde kan T_3 via direkte binnenring-dejodering worden gemetaboliseerd. Deze route was tot nu toe onbekend. Deze reaktie blijkt gekatalyseerd te worden door een enzym dat een tweetal eigenschappen gemeen heeft met het type III enzym (dat voorkomt in het centrale zenuwstelsel, huid en foetale lever), nl. 1) de reaktie wordt gekarakteriseerd door een lage K_m voor T_3 en 2) het enzym is ongevoelig voor het thyreostaticum

propylthiouracil (PTU). Nader onderzoek zal moeten uitwijzen of dit enzym gelijk is aan het type III enzym dan wel of het hier een nieuw enzym betreft.

In hoofdstuk VI wordt de T_3S RIA gebruikt om in ratteplasma T_3S te bepalen. Hiertoe werden ratten behandeld met de thyreostatica PTU of methimazol (MMI), waarvan alleen PTU ook het type I enzym remt. Behandeling met PTU alleen of in combinatie met T_4 of T_3, leidt tot een aanzienlijke stijging van het plasma T_3S, in tegenstelling tot de met MMI behandelde ratten. De plasma T_3S/T_3 ratio van 0.6-0.9 bevestigt eerdere studies met radioactief T_3 dat in ratten sulfatering een belangrijke route is in het metabolisme van T_3.

Tot slot wordt in hoofdstuk VII een eerste aanzet gegeven tot het onderzoek naar het belang van sulfatering in het metabolisme van T_3 bij de mens. Onder normale omstandigheden is T_3S in een lage concentratie aanwezig. Wanneer het type I enzym wordt geremd door PTU, maar meer in het bijzonder door IOP, wordt een aanzienlijke stijging van plasma T_3S gevonden. Desalniettemin zijn ook dan de waarden veel lager dan bij ratten. Ook de T_3S/T_3 ratio is veel lager bij de mens dan de rat, hetgeen suggereert dat sulfatering een minder belangrijke rol speelt in het metabolisme van T_3 bij de mens.

NAWOORD

Velen ben ik dank verschuldigd bij de tot stand koming van dit proefschrift.

Allereerst mijn promotor Theo Visser, die mij vanaf het begin op dit voor mij onbekende biochemische terrein begeleidde. Theo, jouw enthousiasme, ook in tijden met mindere resultaten, werkte zeer aanstekelijk. Daarnaast heb ik veel geleerd van de manier waarop jij ogenschijnlijk moeilijke problemen op eenvoudige manier wist op te lossen.

Marla van Loon voerde alle leverperfusies en een groot deel van de experimenten uit. Marla, je was altijd bereid om "nog even een assay in te zetten of een chromatografie te doen". Dit proefschrift is voor een belangrijk deel mede door jou tot stand gekomen.

Ellen Kaptein heeft de T_3S radioimmunoassay mede ontwikkeld en de vele T_3S bepalingen verricht.

Aan de discussies en experimentele samenwerking met Marja Rutgers denk ik met plezier terug.

Roel Docter was steeds behulpzaam met het geven van statistische adviezen. Zijn computerprogramma's waren van grote waarde bij het uitwerken en tekenen van de vele chromatografieën.

Joop van Buuren wijdde mij in in de geheimen van de "pc" en was altijd bereid eventuele problemen op te lossen. Alle medewerkers van het schildklierlab wil ik graag bedanken voor de gezelligheid en goede sfeer gedurende de afgelopen jaren.

De Audiovisuele Dienst verzorgde op uitstekende wijze de figuren.

Tot slot, Anky, jouw steun was in een woord geweldig.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 16 juni 1956 geboren in Delft. Hij deed in 1974 eindexamen Gymnasium β aan het Coornhert Gymnasium in Gouda. Na het behalen van de propaedeuse aan de Landbouw Hogeschool in Wageningen werd in 1975 begonnen met de studie geneeskunde aan de Medische Faculteit te Rotterdam. In 1980 werd het doctoraal examen en in 1983 het artsexamen afgelegd. In dat zelfde jaar werd begonnen met de opleiding tot internist op de afdeling inwendige geneeskunde III van het academisch ziekenhuis Dijkzigt te Rotterdam (hoofd: Prof.Dr. J.C. Birkenhäger). In deze periode werd een begin gemaakt met het onderzoek beschreven in dit proefschrift. Op 1 oktober 1987 werd hij ingeschreven in het specialisten-register, waarna hij tot 1 november 1988 werkzaam was op de afdeling inwendige geneeskunde III. Sinds 1 november is hij als internist verbonden aan het St. Elisabeth ziekenhuis te Amersfoort.