ENTEROHEPATIC CIRCULATION OF TRIIODOTHYRONINE

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ENTEROHEPATISCHE KRINGLOOP VAN TRIJODOTHYRONINE

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Voor Herma,
In herinnering aan mijn vader

CONTENTS

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BAT	brown adipose tissue
BHDB	butyl 4-hydroxy-3,5-diiodobenzoate
BW	body weight
CNS	central nervous system
CV	conventional
DCNP	2,6-dichloro-4-nitrophenol
EHC	enterohepatic circulation
E-SH	enzyme with free sulfhydryl group
E-SI	enzyme-sulfenyl iodide
G	glucuronide
GF	germ-free
ID	intestine-decontaminated
ig	intragastric
im	intramuscular
ip	intraperitoneal
IRD	inner ring deiodination
iv	intravenous
NTI	non-thyroidal illness
ORD	outer ring deiodination
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PST	phenol sulfotransferase
PTU	6-propyl-2-thiouracil
RIA	radioimmunoassay
rT_3	3,3',5'-triiodothyronine (reverse T_3)
S	sulfate
sc	subcutaneous
T_1	monoiodothyronine
T_2	diiodothyronine
T_3	3,3',5-triiodothyronine
T_4	3,3',5,5'-tetraiodothyronine (thyroxine)
TA_3	3,3',5-triiodothyroacetic acid
TA_4	3,3',5,5'-tetraiodothyroacetic acid
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone (thyrotropin)
UDP	uridine diphosphate
UDPGT	UDP-glucuronyltransferase

I. PERIPHERAL METABOLISM OF IODOTHYRONINES

1. INTRODUCTION

The major factor regulating thyroid function is thyroid stimulating hormone (TSH), a glycoprotein released by the thyrotropic cells of the pituitary gland. Determinants of the TSH secretion rate are inhibition by thyroxine (T_4), 3, 3', 5-triiodothyronine (T_3), dopamine, glucocorticoids and somatostatin, and stimulation by TSH-releasing hormone (TRH), (nor)adrenaline and perhaps neurotensin [156]. In the control of thyroid hormone bioavailability, an important role is also played by iodothyronine transport into cells, enzymatic deiodination and conjugation.

Several groups have studied the enterohepatic metabolic pathways of iodothyronines. It has been generally accepted that these pathways have no more than a passive function in the elimination of the hormone. However, if enterohepatic circulation (EHC) of iodothyronines occurs, the intestinal tract may constitute an important pool of exchangeable hormone. Until recently, only few and inconclusive data existed concerning this EHC.

In our studies we have attempted to document the possible existence of an EHC of thyroid hormone and the role it may play in regulating overall hormone metabolism and excretion in the rat. Especially, we wanted to assess the importance of the intestinal microflora for this process.

We have studied the biliary clearance of T_3 and its conjugates, the hydrolysis of iodothyronine conjugates by intestinal bacteria and intestinal contents, and the metabolism of T_3 and its conjugates in conventional (CV) and intestine-decontaminated (ID) rats.

It is the purpose of this thesis to discuss the role of the EHC of iodothyronines in thyroid hormone metabolism, with special emphasis on the results of my own studies of this subject, described in detail in the appendix papers.

2. METABOLIC PATHWAYS OF IODOTHYRONINES

The thyroid gland of healthy humans secretes on average 115 nmol T_4, about 9 nmol T_3 and 2 nmol 3, 3', 5'-triiodothyronine (reverse T_3, rT_3) per day per 70 kg body weight (BW). T_4 has little intrinsic bioactivity and rT_3 lacks any bioactivity. T_3 can be considered as the only active thyroid hormone that regulates metabolic processes and energy consumption in different tissues [40, 70, 157, 158].

In principle, iodothyronines may undergo four metabolic processes (Fig. 1.). These are: deiodination (section 2.1.), conjugation (section 2.2.), ether-link cleavage (section 2.3.), and oxidative deamination and decarboxylation (section 2.4.).

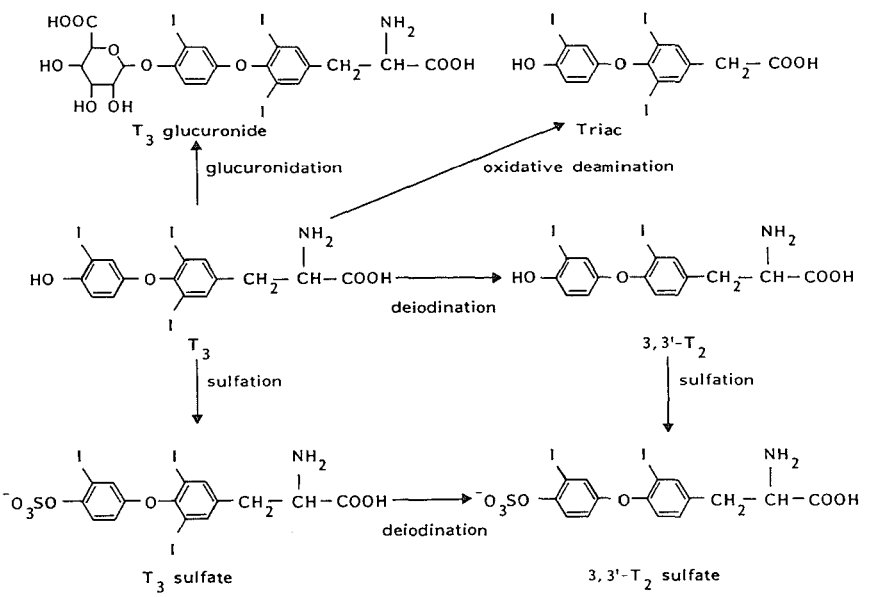

Figure 1. Pathways of T_3 metabolism.

2.1. DEIODINATION

Extensive reviews of this subject have been published recently [40, 70, 93, 157-159]. Two distinct deiodination reactions are recognized. The process through which T_4 is converted to T_3 is called outer ring deiodination (ORD) or 5'-deiodination, while rT_3 is formed by inner ring deiodination (IRD) or 5-deiodination of T_4. Further IRD of T_3 or ORD of rT_3 results in the formation of 3, 3'-diodothyronine (3, 3'-T_2). ORD of T_3 and IRD of rT_3, thus forming 3, 5-diiodothyronine (3, 5-T_2) and 3', 5'-diiodothyronine (3', 5'-T_2), respectively, are considered minor reactions (Fig. 2.). As T_3 is the most important iodothyronine, 5'-deiodination can be regarded as an activating pathway and 5-deiodination as an inactivating pathway. It is the interplay between these two reactions which determines the biological effect of T_4. In healthy humans, roughly 80% of circulating T_3 and 95% of rT_3 are derived from conversion of T_4 in peripheral tissues. The plasma appearance rates of T_3 and rT_3 each amount to 40-50 nmol per day.

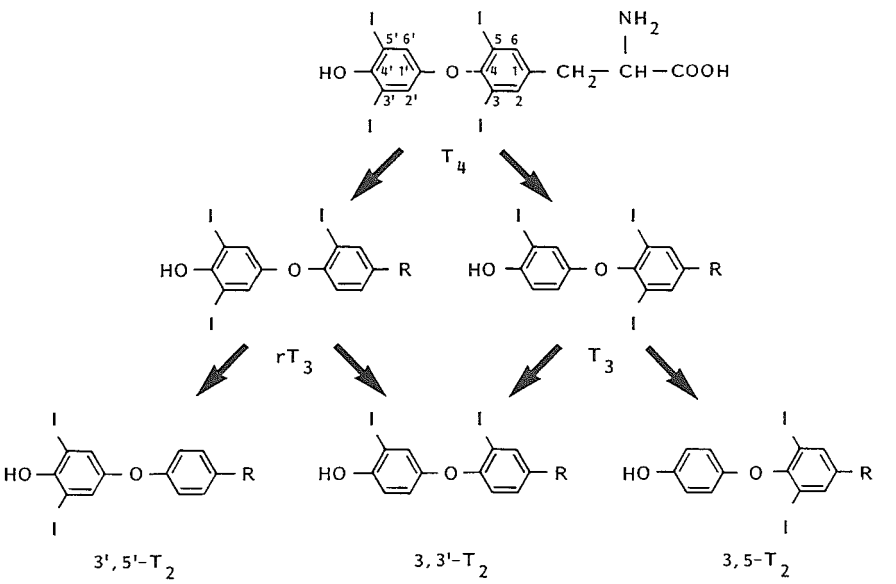

Figure 2. Sequential inner ring (↓) or outer ring (↘) deiodination of T_4

Until now, three different iodothyronine deiodinases have been identified. Type I iodothyronine deiodinase is a non-selective enzyme capable of both

ORD and IRD. High activities have been found in the liver, kidney and thyroid. In the rat liver it is an integral membrane protein located in the endoplasmic reticulum, but in the rat kidney it is associated with the plasma membrane. Simple thiols can serve as cofactor. At the active site of the enzyme, a sulfhydryl (E-SH) group of an essential cysteine residue acts as an acceptor for the iodonium (I^+) ion from the substrate. This results in the formation of an enzyme-sulfenyl iodide (E-SI) intermediate. The cycle is completed when E-SI is reduced to E-SH with cofactor. 6-Propyl-2-thiouracil (PTU) uncompetitively inactivates the enzyme by reacting with the E-SI intermediate forming an enzyme-PTU mixed disulfide (Fig. 3.). The preferred substrate for the type I deiodinase is rT_3. Its ORD to 3, 3'-T_2 is at least 500-fold more efficient than the deiodination of T_4 or T_3. Roughly equal fractions of T_4 are converted by ORD to T_3 and by IRD to rT_3, but the latter is rapidly further degraded to 3, 3'-T_2. IRD of T_3 to 3, 3'-T_2 is a relatively slow process. Type I enzyme activity is decreased in the hypothyroid and increased in the hyperthyroid state.

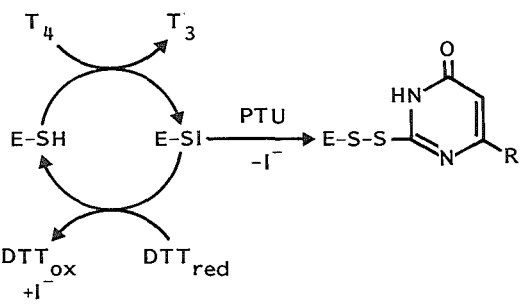

Figure 3. Mechanism of enzymatic deiodination by type I iodothyronine deiodinase. The outer ring deiodination of T_4 is shown as an example.

Facilitation of type I deiodination has been observed after sulfation of 3, 3'-T_2, T_3 and T_4 in incubations with isolated rat hepatocytes and microsomes. Evidence for the facilitated deiodination of T_3 sulfate (T_3S) also has been obtained *in vivo* in rats (see also sections 2.2., 4.1.2. and Appendix paper 1). IRD of T_4 is stimulated 200-fold by sulfation, but in contrast to free T_4, ORD of T_4 sulfate (T_4S) does not occur. Deiodination of rT_3 is not enhanced after sulfation, presumably because it is already an optimal substrate in the non-sulfated form. IRD of T_3S is 40 times as effective as that of T_3 and ORD of 3, 3'-T2 is accelerated 50-fold by sulfation.

The type II enzyme is a true ORDase, converting T_4 to T_3 and rT_3 to 3, 3'-T_2. Its activity has been localized in the central nervous system (CNS), especially in the neurons of the cerebral cortex and cerebellum, in the pituitary, especially in the anterior lobe, in brown adipose tissue (BAT) and in the placenta. Simple thiols also serve as cofactor, but higher concentrations are required as compared with the type I enzyme. The enzyme is not inhibited by micromolar concentrations of PTU. T_4 is preferred over rT_3 as substrate. A large increase in type II enzyme activity has been observed in the CNS, BAT and pituitary of hypothyroid rats.

The type III enzyme is a true IRDase, catalyzing the production of rT_3 from T_4 and producing 3, 3'-T_2 from T_3. It has been detected in rat CNS, especially in glial cells in cerebral cortex, in human and rat placenta, in rat skin, in monkey hepatocarcinoma cells, in fetal rat liver and in chicken embryo heart and liver cells. Recently, evidence has been provided for the existence of a type III-like enzyme in adult rat liver [38]. In common with the type I and II enzymes, the enzyme is located in the microsomal fraction of the tissues and is activated by thiols. Like the type II deiodinase, the type III enzyme is not inhibited by sub-millimolar concentrations of PTU. T_3 is preferred over T_4 as substrate. Unlike the type I deiodinase, this enzyme is unable to deiodinate sulfated iodothyronines. Similar to the type I deiodinase, the activity of this enzyme is decreased in hypothyroidism and increased in hyperthyroidism.

2.2. CONJUGATION

Conjugation is a phase II reaction, which transforms lipophilic endogenous compounds and xenobiotics into hydrophilic derivatives, thus facilitating their excretion in the bile and urine [34, 95].

Glucuronidation is performed by the UDP-glucuronyltransferases (UDPGT; EC 2.4.1.17.), a group of enzymes located in the endoplasmic reticulum of predominantly liver, kidney and intestine, but also in other tissues. UDP-glucuronic acid is used as the cofactor for conjugation of hydroxyl and other functional groups of different classes of compounds [34]. The identity of the UDPGT(s) and the substrate preference of these enzyme(s) for glucuronidation of the iodothyronines has not yet been established.

The phenol sulfotransferases (PST; EC 2.8.2.1.) have been identified in the cytosolic fraction of especially liver but also kidney, small intestine and brain, and catalyze the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a phenolic acceptor molecule [95]. 3'-T_1, 3-T_1 and 3, 3'-T_2 are good substrates for the partially purified phenol sulfotransferases of rat liver, whereas lower rates of sulfation of T_3 and a negligible sulfation of rT_3 and T_4 have been found [135]. Recently, T_3 sulfotransferase

activity has been localized in the cytosolic fraction of human liver homogenates [165]. In incubations of T_3 with cultured rat hepatocytes, T_3 glucuronide (T_3G) is a stable conjugate, which accumulates in the culture medium, while in the absence of inhibitors of the type I deiodinase, T_3S and 3, 3'-T_2 sulfate (3, 3'-T_2S) are rapidly deiodinated (see also section 2.1. and refs. 38, 157-159).

T_4 glucuronide (T_4G), rT_3 glucuronide (rT_3G), T_3G, 3, 3'-T_2 glucuronide (3, 3'-T_2G), T_3S and 3, 3'-T_2S have been determined in the bile of several animal species and humans (see also sections 4.1.1. and 4.1.2.). The biliary excretion of iodothyronine conjugates in rats with a genetic UDPGT deficiency (Gunn rats) will be discussed in section 4.1.4. Normally, rats do not excrete conjugated iodothyronines in the urine [14, 29, 30]. However, predominantly 3, 3'-T_2S, T_3S and minor amounts of T_3G are excreted in the urine in normal dogs [14, 47, 52]. Hepatectomized rats and dogs excrete T_4G in the urine after intravenous (iv) administration of radiolabeled T_4, while also 3, 3'-T_2S, T_3S, T_3G and rT_3G have been detected in the urine of these dogs [14, 45-48, 52]. This implicates that iodothyronines can be conjugated in the extrahepatic tissues of these animals. Faber et al. have detected conjugates of 3, 3'-T_2, 3', 5'-T_2, T_3, rT_3 and T_4 in the urine of hypothyroid and hyperthyroid humans. These investigators have incubated the urine with commercially available β-glucuronidase and arylsulfatase. Sensitive RIA's have been used to establish the amounts of iodothyronines released after the enzymatic hydrolysis. However, the sensitivity and specificity of the methods has not been checked with reference compounds [43]. In a variety of studies, iodide has been identified as the predominant product in human urine [29, 30, 151].

Iodothyronine glucuronides have not been identified in systemic venous blood of rats (see also Appendix paper 6 and refs. 30, 131, 132). However, after ligation of the common bile duct, T_4G and T_3G have been detected in the plasma of rats and dogs [23, 49, 109-111]. Recently, we have identified high concentrations of T_3G in the serum of rats with an autosomal recessive defect that causes conjugated hyperbilirubinemia (TR^-rats; ref. 28, see also section 4.1.4.). Also, T_4G has been determined in the plasma of patients with obstructive biliary disease [152]. Minor amounts of T_3S have been detected in the plasma of normal rats, dogs and humans [14, 39, 52], whereas significant amounts of T_3S have been found in the plasma of rats and dogs after ligation of the bile duct or after hepatectomy [14, 23, 52, 109-111, 113]. Increased amounts of T_3S have also been found in the plasma of rats and humans treated with the type I deiodinase inhibitors PTU or iopanoic acid [37, 39] and in the plasma of hypothyroid rats or hypothyroid humans treated with T_3 [23, 44, 117, 122]. The PTU-induced increase in plasma T_3S can be explained by its diminished clearance in the bile. However, in rats treated with PTU, T_4S has been found in the bile but not in the plasma. This may be explained by the

slower metabolic clearance of T_4 and the smaller proportion of this compound which is conjugated with sulfate [131].

It can be concluded that, under normal conditions, glucuronidated iodothyronines are not found in the blood and urine but are excreted in the bile. Only small amounts of T_3S appear in the plasma of rats, dogs and humans, and insignificant amounts of the iodothyronine sulfates and glucuronides are excreted in the urine.

2.3. ETHER LINK CLEAVAGE

In disease states associated with an enhanced leucocyte phagocytic capacity, such as infectious illnesses, ether link cleavage (ELC) is the major pathway of iodothyronine metabolism in these cells. This process is associated with the "respiratory burst" and involves an increased oxygen (O_2) uptake, enzymatic superoxide (O_2^-) and H_2O_2 production, subsequently causing the breakdown of iodothyronines. ELC is dormant in the resting leucocyte. The quantitative importance of ELC in health or in various non-infectious diseases has still to be established [16, 70].

2.4. OXIDATIVE DEAMINATION AND DECARBOXYLATION

Oxidative deamination, which involves the conversion of the alanine side chain of iodothyronines to the acetic acid derivative, has been observed in mammalian liver, kidney and brain homogenates *in vitro* [16, 70, 129, 130]. In humans it accounts for the daily turnover of perhaps 2% of T_4 and up to 14% of T_3, resulting in daily production rates of 1.6 nmol 3, 3', 5, 5'-tetraiodothyroacetic acid (TA_4) and 8 nmol 3, 3', 5-triiodothyroacetic acid (TA_3), respectively [16, 60]. TA_3 glucuronide (TA_3G) is the major TA_3 metabolite in the bile of rats and dogs [53, 55, 56, 129, 130]. TA_3 sulfate (TA_3S) is the best known substrate for IRD by the type I deiodinase [129, 130]. In hypothyroid rats and in rats treated with the type I deiodinase inhibitor PTU, significantly increased plasma levels of TA_3S have been found. Also, a proportional increase in biliary-excreted TA_3S has been observed after treatment of rats with PTU [121, 123, 130]. Until now, very little information exists on the occurrence and effects of decarboxylated iodothyronines or iodothyronamines [53, 70].

3. IODOTHYRONINE TRANSPORT INTO CELLS

In hepatocytes, human hepatocarcinoma cells, pituitary tumor cells, muscle cells and fibroblasts, the uptake of T_4, rT_3 and T_3 occurs, at least in part, through a high-affinity, limited-capacity transport system. This is an active process, which is dependent on temperature and cellular ATP concentrations [85]. It requires an intact Na^+-gradient across the cell membrane as evidenced by the inhibitory effects of the Na^+, K^+-ATPase inhibitor ouabain [85].

It has been demonstrated *in vitro* that the uptake of T_4 and rT_3 into isolated rat hepatocytes occurs through a different system than that of T_3. However, T_4 is a competitive inhibitor of the uptake of T_3 and vice versa. Also, structural thyroid hormone analogues such as radiocontrast agents and amiodarone, and a plasma factor of patients with non-thyroidal illness (NTI) have been shown to inhibit the uptake system.

Indirect evidence exists for the active transport of T_3 from cytoplasm into the nucleus [85]. These gradients may have major implications for the regulation of the thyroid hormone bioactivity.

4. EXCRETION OF IODOTHYRONINES AND THEIR CONJUGATES IN THE INTESTINAL TRACT

4.1. EXCRETION IN THE BILE

In 1919 Kendall observed the excretion of 43% of iodine in the bile within 50 h after iv administration of 200 mg T_4 in dogs [82]. In rats, the biliary excretion of iodine after iv administration of T_4 was first reported by Krayer et al. in 1928 [84]. Subsequent studies in humans, dogs, rats and other animals have been carried out to elucidate the nature of this iodine. The following variations in experimental design make comparison of the results from these studies difficult:

1. Almost all studies have been done with bile-diverted animals. However, interruption of the EHC of bile acids leads to a reduced bile flow [17, 33, 83, 87, 105, 138, 139, 163].
2. Animals were either unanesthetized and kept in restraining cages or anesthetized with substances such as pentobarbital, droperidol or diethylether. Anesthesia may markedly influence the findings obtained in such studies. Ether depresses glucuronidation through inhibition of liver UDP-glucose dehydrogenase, which results in a dramatic reduction of UDP-glucuronic acid [161]. Apart from a marked fall in plasma T_4, also inhibition of glucuronidation and a 60% increase in bile flow have been reported after pentobarbital [86]. On the other hand, restraint of the animals induces stress and results in an increase of plasma glucocorticoids and adrenaline. These substances may reduce hepatic and renal T_3 production through a reduction of type I deiodinase activity [13, 18, 92].
3. Biliary excretion has been studied after iv, intraperitoneal (ip), intramuscular (im), or subcutaneous (sc) injections of radiolabeled iodothyronines or their conjugates. As will be discussed, the amount of radioactivity excreted as a function of time varies depending on the route of administration.
4. Findings obtained after a single injection of hormone may not be representative for the physiological (endogenous) situation. Especially the administration of supraphysiological or even pharmacological doses of labeled compounds with a low specific activity may exceed the capacity of the excretory systems.

5. Large variations in conjugation patterns and biliary excretion exist between species and even between different strains of a single species [105, 139].
6. Some workers have studied the biliary excretion in hypothyroid or hyperthyroid animals. Hypothyroidism in rats causes a cholestatic condition with a 50% decrease in bile flow, while hepatic conjugation capacity towards bilirubin is enhanced. Hyperthyroidism may result in an 1.3-1.4-fold increase in bile flow in rats, while the bilirubin UDPGT-activity towards bilirubin is lowered [153, 154].

4.1.1. BILIARY CLEARANCE OF T_4

In 1952 Taurog et al. were the first to identify T_4G in the bile of rats, an observation that has been subsequently confirmed by others [144-146]. With low doses (<100 μg T_4 per rat) the biliary excretion of non-conjugated T_4 decreases and that of T_4G increases as a function of time after injection of T_4 [144-146]. More time has passed before the radioactivity appears in the bile after sc injection than after iv administration of radiolabeled T_4 [144-146]. Apart from T_4G and non-conjugated T_4, small amounts of T_3G, rT_3G, T_4S and iodide have been detected in the bile, while thyroidectomized rats also excrete T_3S in the bile [11, 14, 30, 31, 111-114, 131, 142]. Rapid excretion of radioactivity in the bile occurs after administration of high doses of T_4 (>100 μg - 20 mg per rat). Also, a greater part of this radioactivity is non-conjugated T_4, and it has been suggested that this is caused by saturation of the conjugation pathways [89, 98, 144-146].

After treatment of rats with PTU, increased amounts of T_4S and T_4G are excreted in the bile [131]. Also, a decreased biliary T_3G/rT_3G ratio has been found after PTU or in fasting-induced hypothyroidism, which in the latter case has been prevented by replacement doses of T_4 [131, 142]. As PTU inhibits the type I deiodinase, T_4S is not deiodinated in the liver and will be excreted in the bile. PTU inhibits the clearance of T_4 which results in the increased excretion of T_4G. The PTU-induced reduction in the excretion of T_3G is explained by the inhibition of the conversion of T_4 to T_3 by the type I deiodinase. Similarly, the type I deiodination of rT_3 is reduced, resulting in the increased biliary excretion of rT_3G. The findings in non-substituted, fasted rats are consistent with a decrease in type I deiodinase activity due to fasting-induced hypothyroidism, while fasting per se may be associated with a diminished tissue uptake of iodothyronines [81, 142, 148]. These findings are in compatible with results obtained by Flock and Bollman, who have studied the biliary clearance of T_4 in rats treated with thiouracil and butyl 4-hydroxy-3, 5-diiodobenzoate (BHDB), which is another inhibitor of type I deiodinase [14, 51, 54-56]

Biliary excretion of T_4G and T_4 has also been observed after administration of radiolabeled T_4 in cats, sheep, horses, monkeys, rabbits and fish [24, 25, 35, 75, 100, 103, 146]. Within 24 h after iv administration of small doses of radiolabeled T_4, dogs have excreted 19% of radioactivity in the bile, whereas in rats this amounts to 24%. In the bile of dogs 22% of the radioactivity is excreted as T_4G, but in rats this amounts to 43% [14, 49, 52, 146]. Only qualitative data exist on the biliary clearance of T_4 in humans. T_4 and T_4G have been detected in human bile, but T_4G has not been detected in the bile of patients with viral hepatitis, suggesting an impaired hepatic glucuronidation of T_4 [9, 91, 96, 152].

4.1.2. BILIARY CLEARANCE OF T_3

In 1951 Roche et al. first observed the biliary excretion of T_3G and T_3S after iv or sc administration of radiolabeled T_3 to thyroidectomized rats [23, 110-113, 115-117, 119, 122, 123]. After administration of radiolabeled T_3 to euthyroid, bile-diverted rats, mainly T_3G and small amounts of T_3S, 3, 3'-T_2G, 3, 3'-T_2S, non-conjugated T_3 and iodide are excreted in the bile [14, 30, 31, 48, 52]. As with T_4, biliary excretion of radioactivity increases after administration of higher doses of T_3, with a relative decrease in the amount of T_3G and an increase in non-conjugated T_3 [23]. Treatment with PTU results in a dramatic increase in the biliary excretion of T_3S and 3, 3'-T_2S, whereas the excretion of T_3G is unaffected (see also Appendix paper 1). 3, 3'-T_2S may arise from sulfation of 3, 3'-T_2 produced by PTU-insensitive type III-like deiodination of T_3. This activity has recently been identified in incubations with rat hepatocytes and in microsomal fractions [38, 159]. Furthermore, the type I deiodinase may not have been completely inhibited by the doses of PTU administered, so that some 3, 3'-T_2S may still be produced being type I IRD of T_3S. The biliary T_3 clearance is not influenced by treatment of the rats with 2, 6-dichloro-4-nitrophenol (DCNP), but the increased biliary excretion of T_3S and 3, 3'-T_2S in PTU-treated rats is inhibited. This may implicate that, in the rat, iodothyronines are sulfated by DCNP-sensitive PSTs (see also Appendix paper 1 and section 2.2.). In other conditions that are associated with an impaired type I deiodinase activity, such as after thyroidectomy or after treatment with BHDB, also a significantly increased excretion of T_3S in the bile has been observed [14, 23, 55, 56, 70, 110-113, 115-117, 119, 122, 123].

Within 24 h after the iv administration of radiolabeled T_3, dogs excrete 16-29% radioactivity in the bile, while 33% is excreted in rats. In dogs, about 24% of this radioactivity is T_3G, whereas in rats this amounts to 45%. In the bile of these dogs, also 3, 3'-T_2S, T_3S and small amounts of T_3 and iodide have been detected [14, 25, 47, 52].

Only preliminary and qualitative data exist on the biliary clearance of T_3 in humans. After iv injection of physiologic amounts of radiolabeled T_3 in hypothyroid humans, the bile contains predominantly T_3G and some T_3S and T_3 [44].

4.1.3. BILIARY CLEARANCE OF IODOTHYRONINE CONJUGATES

After iv injection of radiolabeled T_4G or T_3G, normal rats with a bile canula have excreted up to 72% and 88%, respectively, of radioactivity in the bile in 60 min [7, 8, 71, 132]. The rapid clearance of T_3G is unaffected by PTU, which supports the view that T_3G is resistant to deiodination. It also explains why normally T_3G can not be detected in the plasma (see also section 2.2. and ref. 132). After iv administration of radiolabeled T_3S, normal rats have excreted <20% of the radioactivity in the bile, whereas PTU-treated rats have excreted up to 75% of the radioactivity within 4 h. Apart from T_3S, no other conjugated or non-conjugated iodothyronines have been detected in the bile of these rats [128, 132]. Also, thyroidectomized, bile-diverted rats have excreted 42% of radioactivity in the bile within 24 h after iv administration of radiolabeled T_3S and 66% after administration of T_3. Up to 5 h after injection of T_3S, the main radioactive compound in the bile is T_3S, but at 24 h 50% of biliary radioactivity consists of T_3G. This implicates that in these bile-diverted rats some T_3S must have been hydrolyzed to T_3, which is successively glucuronidated, suggesting that T_3S may have been desulfated outside the intestine [23, 118, 120, 124].

These results emphasize that the role of glucuronidation of iodothyronines is to facilitate their excretion with the bile. Sulfated iodothyronines are preferred substrates for the type I deiodinase in hepatocytes. Intracellular accumulation of T_3S in hepatocytes, after treatment with PTU or BHDB and in hypothyroidism, leads to an significant increase in the canalicular excretion of T_3S into the bile and the sinusoidal excretion of T_3S into the plasma. However, plasma T_3S may also originate from extrahepatic sources (section 2.2.).

4.1.4. BILIARY CLEARANCE OF IODOTHYRONINES IN GUNN AND TR$^-$ RATS

A rat strain with an autosomal recessive deficiency of UDPGT activity towards bilirubin was first described by Gunn in 1938 [62]. This Gunn rat is an animal model of the human Criggler Najjar syndrome [78]. Heterozygotes possess normal enzyme activities towards bilirubin. Deficient transferase activities have also been reported for other substrates than bilirubin.

Flock et al. have found that within 24 h after iv injection of labeled T_3 Gunn rats have excreted 19% of radioactivity in the bile, whereas normal rats have excreted 33%. This decrease in biliary excretion of radioactivity is compensated for by an increase in the urinary excretion from 36% in normals to 49% in Gunn rats. Only 44% of biliary radioactivity in these Gunn rats is in the form of glucuronides, whereas in normal rats this amounts to 65%. However, the iodothyronine sulfate fraction amounts to 30% of biliary radioactivity in the Gunn rats and 12% in normals. After the iv administration of labeled T_4, the biliary excretion of radioactivity has decreased from 24% within 24 h in normal rats to 11% in Gunn rats. A compensatory increase in the excretion of radioactivity in the urine from 28% to 34% has been observed. Similary, the proportion of iodothyronine glucuronides in the bile has decreased from 68% in normals to 29% in Gunn rats. The iodothyronine sulfate fraction in the bile has increased from 6% to 24%, respectively [14, 50, 56]. In Gunn rats, the biliary clearance of iv injected T_4G, like that of bilirubin glucuronide, is not significantly different from normal rats [7].

Mutant TR^- rats have a normal functioning hepatobiliary excretion pathway for bile acids and a defective pathway for the excretion of other anions, such as bilirubin and sulfobromophthalein-glutathione. This defect was first described by Jansen et al. in 1985. The TR^- rats resemble human Dubin-Johnson syndrome in many respects [76-78]. Preliminary results in these rats have shown a strongly impaired biliary T_3G excretion and a dramatic accumulation of T_3G in serum. In PTU-treated TR^- rats the biliary excretion of T_3S seems only partially affected by the defect [28].

4.2. DIRECT MESENTERIAL SECRETION OF IODOTHYRONINES

After iv administration of tracer T_4 to bile-diverted rats or sheep, up to 35% of radioactivity is still excreted in the feces. It has been concluded that this is due to direct secretion of iodothyronines from blood to the gastrointestinal lumen [3, 4, 59, 75, 80, 99]. From equilibrium studies using constant sc infusions of tracer T_3 or T_4 into intact CV rats, DiStefano et al. have calculated that the "mesenterial arterial fluxes of T_3 and T_4 to the gut" are 1.0 and 2.5 times the "biliary fluxes of these hormones to the gut", respectively [30, 31]. This will implicate a significant mesenterial secretion of T_3 and T_4. However, direct experimental evidence for such an excretory pathway is lacking. The reduction in fecal excretion of radioactivity by 80-90% after iv injection of radiolabeled T_4 in rats, after ligation of the bile duct, suggests that direct mesenterial secretion plays an minor role [4, 99].

4.3. FECAL EXCRETION OF IODOTHYRONINES AND THEIR CONJUGATES

From the foregoing it follows that the fecal excretion of iodothyronines and their metabolites is the sum of the biliary and mesenterial excretions minus the amount which is reabsorbed. Normally, only non-conjugated iodothyronines are excreted with the feces in rats, dogs, cats, sheep and humans [29-31, 94, 151]. As will be shown in the next chapters, intestinal decontamination of rats by means of antibiotics results in the fecal excretion of iodothyronine conjugates. The important role of anaerobic intestinal bacteria for the deconjugation of these glucuronides and sulfates will be discussed.

5. ABSORPTION OF IODOTHYRONINES AND THEIR CONJUGATES IN THE INTESTINAL TRACT

In connection with the oral replacement therapy for hypothyroidism, extensive absorption studies have been carried out with iodothyronines in animals and humans.

In rats, the absorption of T_4 differs throughout the intestinal tract, being the lowest in the stomach and the highest in the colon [1, 19-21, 26, 68, 90]. DiStefano et al. have infused tracer T_3 and T_4 separately at constant rates into the duodenum of intact rats. After 3 days, steady-state conditions have been established. They have calculated that up to 42% of infused T_3 and 49% of infused T_4 have been absorbed into the systemic circulation from the 4th to the 7th day of infusion. Concentrations of T_3 and T_4 under steady-state conditions are higher in portal blood than in systemic arterial blood [30, 31]. Such a difference, which will be in support of a significant EHC, has not been documented for other routes of administration. In the ileum and colon, a higher absorption of T_4 has been found in the absence of intestinal contents [19, 20]. Also, higher absorption rates have been reported for the colon, but not for the ileum, of germ-free (GF) rats [19, 20]. Therefore, it was suggested that intestinal contents such as proteins, fibers and bacteria interfere with the absorption of iodothyronines by binding these compounds [19, 20, 68].

Absorption of oral T_4 and T_3 in humans amounts to 40-88% and 69-94%, respectively. These values are equal or only slightly elevated in hypothyroidism or hyperthyroidism [68, 151]. The variance in absorption rates can be explained by differences in bioavailability due to different vehicles and adherence to intestinal contents of these compounds [68]. Also, certain drugs such as colestipol, cholestyramine and activated charcoal, and dietary substances such as liver residue, walnuts, soybean infant formula and cottonseed meal interfere with the enteral absorption of T_4 [12, 68, 102, 104, 127]. Thyroxine-substituted hypothyroid patients with a short bowel, after jejuno-ileal bypass surgery for obesity, or patients with enteral diseases may require higher replacement doses [5, 68, 141, 147, 150]. It has to be determined if absorption of T_4 and T_3 is a saturable process.

Animals and humans poorly absorb conjugated compounds [34, 95]. The absorption of radioactivity within 2 h after the installation of labeled T_4G into the duodenum of CV rats was 15%, whereas after T_4 this is 41% [26]. Feces of thyroidectomized rats collected up to 3 days after intraduodenal administra-

tion of labeled T_3S contain 73% of label, whereas after T_3 this amounts to 58%. It has been concluded that these rats must have absorbed at least 27% and 42% of label, respectively [23, 120, 124]. Relative to T_3, a reduced absorption of radioactivity in the isolated small intestine, but an equal absorption in the large intestine has been found after the administration of labeled T_3G [27]. As the intestinal contents have not been removed, the results may be explained by hydrolysis of the conjugates in the colon, with subsequent absorption of the released aglycon. In CV rats, conjugated iodothyronines have been detected in the small intestine, but not in the large intestine [30, 31]. As will be discussed in the next chapter, the intestinal tract is colonized with anaerobic, iodothyronine conjugate-hydrolyzing bacteria. Relative to the distal tract, significantly lower numbers of these bacteria have been found proximal of the coecum, implying a reduced hydrolyzing capacity [64, 73, 125, 133]. In ID rats, the absorption of radioactivity within 26 h after intragastric (ig) administration of radiolabeled T_3G or T_3S is reduced 4.9 and 2.8 times, respectively, whereas after T_3 it is 1.5 times higher than in CV rats (see also Appendix paper 6). The number of anaerobic bacteria in the intestinal tract of these rats is virtually eliminated (see also Appendix papers 5 and 6). Since the absorption of radioactivity after ig administration of radiolabeled T_3 in ID rats is increased compared to CV rats, a nonspecific interference of the antibiotics with the absorption of radioactivity can be excluded. Plasma radioactivity in all the different groups of rats is composed of T_3 and iodide only (see also Appendix paper 6). Intestinal hydrolysis is a prerequisite for the absorption of iodothyronine conjugates. The importance of the bacterial glucuronidases and sulfatases for the EHC of thyroid hormone will be further discussed in chapters 6 and 7.

From studies with everted gut sacks it has been concluded that T_3 and T_4 are conjugated in mucosal cells and that the conjugates subsequently appear in the serosal fluid [69, 143]. However, since conjugated iodothyronines have not been retrieved from mesenteric venous or portal blood, these experiments seem to have little implications *in vivo* [30, 31]. Despite suggestions to this effect, lymphatic transport of enterally absorbed iodothyronines has not yet been documented.

6. HYDROLYSIS OF IODOTHYRONINE CONJUGATES IN THE INTESTINAL TRACT

β-Glucuronidase (EC 3.2.1.21.) occurs in several tissues of animals, in bacteria and in plants [34, 160]. In the gastrointestinal tract, this activity is present in the bile, intestinal juices, salivary secretions, mucosal cells and intestinal bacteria [34, 125, 160]. The enzyme promotes the liberation and consequently the reabsorption of aglycons from several glucuronides of endogenous and foreign compounds, which are excreted in the bile [34, 160].

During incubation of T_3G with 20 times diluted suspensions of human or rat feces under anaerobic conditions, approximately 80% of the conjugate is hydrolyzed in 1 h (see also Appendix paper 5). In 5 times diluted suspensions of feces from GF rats, or from rats which have been decontaminated by 5 days of oral administration of antibiotics (ID rats, see also chapter 7), only 10-15% of T_3G is hydrolyzed in 24 h (see also Appendix paper 5). It has been suggested that this residual hydrolysis is caused by glucuronidase activity, released by the intestinal mucosa (see also Appendix paper 5 and refs. 41, 125).

β-Glucuronidase activity has been associated *in vitro* and *in vivo* with facultatively and obligately anaerobic bacteria belonging to the genera *Streptococcus*, *Peptostreptococcus*, *Corynebacterium*, *Proprionibacterium*, *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Catenabacterium*, *Lactobacillus* and *Escherichia* [58, 67]. In contrast to intestinal epithelial β-glucuronidase, which has a pH optimum of 6-7, bacterial β-glucuronidase has a pH optimum of 4-5 [125]. Higher numbers of facultatively and obligately anaerobic bacteria occur in the distal intestinal tract as compared with the proximal part and here the intestinal β-glucuronidase activity is predominantly of microbial origin [64, 125, 133, 134]. We have isolated potent T_3G-hydrolyzing, anaerobic bacteria from the human fecal flora, belonging to the genus *Eubacterium* (see also Appendix paper 4).

The arylsulfatases (EC 3.1.6.1.) catalyze the hydrolysis of arylsulfates and occur widely distributed in animals, bacteria and plants [32, 95, 101, 126]. Since the enzymes are absent in intestinal cells, intestinal juices, salivary secretions and the bile, no hydrolysis of arylsulfates has been found in the intestinal tract of GF and ID rats [6, 41, 42, 73, 108].

Closon et al. have observed 80% hydrolysis of T_3S after incubation for 38 h at 37°C with fecal suspensions of CV rats [22]. We have observed up to 91%

hydrolysis of 3'-T_1 sulfate (3'-T_1S), 3, 3'-T_2S, T_3S, rT_3 sulfate (rT_3S), or T_4S in incubations for 1 h at 37°C with 20 times diluted suspensions of human or CV rat feces (see also Appendix paper 5). In agreement with the experiments of Closon et al., no hydrolysis of the iodothyronine sulfates has been observed during incubation with suspensions of human or rat feces which have been preheated for 20 min at 80°C (see also Appendix paper 5 and refs. 22, 23). 5 times diluted fecal suspensions from GF and ID rats do not hydrolyze the sulfates during incubation for 24 h at 37°C (see also Appendix paper 5).

Bacterial arylsulfatases have been associated *in vitro* and *in vivo* with the genera *Enterobacter*, *Klebsiella*, *Serratia*, *Proteus*, *Salmonella*, *Alcaligenes*, *Pseudomonas* and *Mycobacterium* [32]. Closon et al. have found that iodothyronine sulfatase activities are associated with *Bacillus faecalis* and to a minor extent with *Salmonella paratyphi* B [22, 23]. We have found that these activities originate from bacteria belonging to the genera *Lactobacillus*, *Eubacterium* or *Lachnospira*, and *Peptostreptococcus* (see also Appendix papers 2-3).

Extensive and rapid hydrolysis of T_3G and iodothyronine sulfates thus occurs in incubations with diluted fecal suspensions from CV rats and humans as well as with facultatively and obligately anaerobic intestinal bacteria. As already mentioned, this may form a prerequisite for the EHC of thyroid hormone.

7. ENTEROHEPATIC CIRCULATION OF IODOTHYRONINES

Compounds, which are excreted in the bile, come in contact with the huge absorptive surface of the intestine, where they may be absorbed into the portal blood and return to the liver. This process is called "enterohepatic circulation" (EHC). It implies that biliary excretion, unlike clearance with the urine, does not necessarily result in the elimination of compounds from the body. EHC has been demonstrated for many endogenous compounds such as bile acids, bilirubin and steroid hormones, as well as for many drugs and food additives [6, 17, 33, 42, 61, 87, 149, 163]. The intestinal bacteria are an essential part of the EHC of these compounds. In most cases the microflora convert highly polar biliary metabolites into more lipid soluble forms suitable for reabsorption. However, some metabolic reactions performed by the gut flora, such as ester hydrolysis, may produce more polar metabolites and thereby increase elimination in the feces. An enterohepatic circulation can delay the elimination of a drug and, thus, prolong its action. Interspecies and interindividual differences in an enterohepatic circulation may arise from differences in hepatic metabolism and excretion of the compound into the bile, but also in the nature and metabolic capacity of the gut flora [106].

Indirect evidence has been obtained for the EHC of T_3 and T_4 in rats, dogs, cats, sheep, fish and humans. Albert and Keating have studied the fecal excretion of radioactivity after the installation of bile containing radiolabeled T_4 into the duodenum of CV rats or rats with ligated bile ducts. In 48 h about 68% and 34% of radioactivity, respectively, has been recovered from the feces of these rats. It can be concluded that in this time at least 34% of total radioactivity, or half of the fecal radioactivity in CV rats has been absorbed and has circulated through the liver and biliary tract [2, 3]. When bile, which has been obtained from rats injected with radiolabeled T_4, is introduced in the duodenum of these rats, estimations of this circulation of radioactivity vary from at least 10% in 68 h to 37% in 48 h. However, the composition of the donor bile in these experiments is not known [2, 15, 146]. In case of a significant direct mesenterial secretion of T_3 and T_4, the magnitude of the EHC will be underestimated, if this is calculated from the difference between the biliary and fecal clearance (section 4.2.).

In chapter 6 we already hinted at the role of the intestinal microflora in the EHC of iodothyronines through the hydrolysis of conjugates excreted in the bile. In rats, the intestinal tract can be almost completely freed from bacteria by adding antibiotics to the drinking water. After this treatment, these ID rats are very similar to GF rats [63]. The upper intestinal tract of rats mainly contains conjugated T_3 and T_4, but in the colon predominantly non-conjugated iodothyronines have been found [30, 31]. We have not detected conjugated iodothyronines in the feces of CV rats within 70 h after iv or ig administration of radioactive T_3, T_3G, or (after pretreatment with PTU) T_3S (see also Appendix paper 6 and ref. 132). However, in the 70 h period after the iv injection of radiolabeled T_3 to ID rats, the feces contain 30% T_3, 11.5% T_3G and 11% T_3S. Over this period, these rats have excreted 1.6 times more radioactivity in the feces than CV rats (see also Appendix paper 6). Also, within 13 h after the iv administration of radiolabeled T_3G to ID rats, fecal radioactivity consists of 30% T_3 and 67% T_3G, whereas after iv administration of T_3S to PTU-treated ID rats, 95% of fecal radioactivity consists of T_3S. Plasma radioactivity reappears in CV rats after clearance of the injected labeled conjugates, but not in ID rats. This radioactivity is greater after T_3G than after T_3S administration [132].

Therefore, in the intestinal tract of ID rats, only small amounts of glucuronides are hydrolyzed by the mucosal β-glucuronidase, whereas arylsulfatase activity in the gut wall is apparently of no importance. Consequently, the EHC of iodothyronines in these animals is reduced. Our results are not only conclusive for a significant EHC of iodothyronines in CV rats but also for the important role of the intestinal microflora in this process.

Only a few studies have been made of the EHC of iodothyronines in dogs, cats, sheep and fish. From the results it may be concluded that the EHC in these animals is less extensive than in rats [24, 36, 68, 75]. Myant has administered donor bile containing radiolabeled T_4G in the upper intestinal tract of humans. Within 6-7 days about 0.2 - 7% of the radioactivity has appeared in their bile, which has been collected by means of a T-tube positioned in the common bile duct, and 70% has appeared in the feces. A maximum absorption of 30% of the radioactivity excreted in the bile in this time has been suggested [97].

As discussed already, interference with the enteral absorption of T_3 and T_4 by certain drugs, diets, diseases or in conditions with a short bowel, results in interruption of the EHC with successive increased fecal loss (see also chapter 5 and ref. 150). Colestipol and cholestyramine presumably can bind T_4 in the gut and, hence, interfere with the EHC of the hormone [12, 164]. Also increased fecal losses of T_4 and iodine have been reported in protein losing enteropathy, ulcerative colitis and Crohns disease [66, 79]. Rats, which have been treated with soy flour diets, excrete more radioactivity with the feces

after iv injection of radiolabeled T_4 than normal rats [10, 136]. Also, overt thyroid dysfunction, resulting in goiter or hypothyroidism, has occurred when children or neonates, with presumably subliminal defects in thyroid hormone synthesis or utilisation, have been fed with a soy flour diet [65, 74, 88, 107, 137, 155, 162]. Patients with pancreatic steatorrhea excrete 2.4 times more organic iodine with the feces than normals, and this can not be reversed by treatment with pancreatic enzymes [72]. Decreased serum T_3 levels after jejuno-ileal bypass surgery for obesity may result from a reduced EHC of thyroid hormone [140]. However, in adults with adequate thyroid function and iodine intake, no overt hypothyroidism ensues during interference with the EHC of iodothyronines, presumably because of a compensatory increase in the thyroidal production of T_4 and/or extrathyroidal production of T_3.

8. GENERAL DISCUSSION

From chapter 2 it follows that deiodination and conjugation are major pathways of thyroid hormone metabolism *in vitro* and *in vivo*, and from section 4.1. it follows that T_4 and T_3 predominantly appear as glucuronides in the bile of CV rats. In vitro, the deiodination of the different iodothyronines by the type I deiodinase in hepatocytes, except that of rT_3, is greatly facilitated after sulfation, but ORD of T_4S (in contrast to IRD) is inhibited. In CV rats, minor amounts of iodothyronine sulfates are excreted in the bile, but the biliary excretion of T_4S and T_3S significantly increases after inhibition of type I deiodinase activity.

The excretion rate of metabolites from endogenous T_4 in rats can be calculated using data on the biliary excretion of iv injected radiolabeled T_4 under steady-state conditions (ref. 131 experiment I) and by considering the thyroidal secretion of approximately 10 nmol/day/kg BW T_4. The results show an average excretion of 3.5 nmol/day/kg BW T_4G and 1.5 nmol/day/kg BW T_3G. Note that in these calculations the contribution of the thyroidal T_3 secretion to the biliary excretion of T_3G is neglected. Almost similar results can be obtained by using data from Földes et al., who have determined total and free iodothyronines in rat bile using sensitive RIA's before and after β-glucuronidase and arylsulfatase treatment [57]. With the use of the same techniques, the biliary excretion of iodothyronines and their metabolites has also been determined in post-cholecystectomy patients [91]. The daily biliary excretion of T_4 and its conjugates in these patients amounts to 10 nmol and that of T_3 and its conjugates 1.3 nmol, or 10% and 5% of the total metabolic clearance of T_4 and T_3, respectively. However, in these patients reduced serum levels of T_3 and increased serum levels of rT_3 have been found, which may be indicative of a disturbed thyroid hormone economy in NTI [91].

The biliary-excreted iodothyronine glucuronides and sulfates are subject to hydrolysis by intestinal β-glucuronidases and arylsulfatases, respectively, allowing for reabsorption of liberated iodothyronines (see also chapter 6). However, as glucuronides are the predominant forms in which T_4 and T_3 normally appear in the bile and reabsorption of T_3 from intestinal T_3G seems more effective than from T_3S (see also chapter 5 and Appendix paper 6), it is likely that the enterohepatic circulation of thyroid hormone primarily proceeds through biliary excretion of T_4G and T_3G. The major purpose of sulfation appears to be the induction of inactivation of T_4 and T_3 through

IRD; the released iodine may be reutilized for thyroid hormone synthesis.

In a few studies the biliary and fecal clearance rates of endogenous T_4, representing the proportions of the plasma T_4 clearance accounted for by the biliary and fecal excretion of T_4 metabolites, have been determined. Myant has estimated a mean fecal clearance of 19.5 ml/day in rats. The biliary clearance determined in bile duct-diverted rats amounts to 44.6 ml/day. From the difference between the fecal clearance and the biliary clearance it can be concluded that there is a significant EHC of thyroid hormone [99]. However, Galton et al. have found that the fecal clearance of endogenous T_4 was similar or even higher than the biliary clearance in ether-anesthetized rats [59]. As already mentioned in section 4.1. the use of ether anesthesia may have markedly influenced these results.

A significant direct mesenterial secretion of iodothyronines, as already mentioned in section 4.2., may result in an underestimation of the EHC of iodothyronines, if this is calculated from the difference between the biliary and fecal clearance.

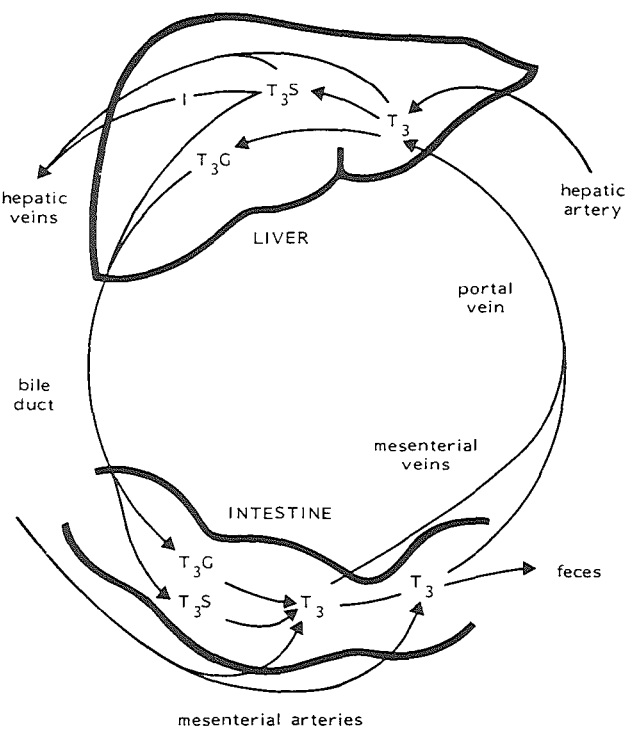

Figure 4. Enterohepatic circulation of T_3.

In Fig. 4. a schematic representation of the EHC of T_3 in rats is produced. Only a few studies have considered the EHC of iodothyronines in humans. Conjugation of iodothyronines as well as biliary excretion of iodothyronines and their conjugates have been demonstrated in humans. Also, iodothyronine conjugates are hydrolyzed by major strains of facultatively and obligately anaerobic bacteria of the human intestinal microflora (see also chapter 6). Therefore, in the human body all the conditions for an EHC of thyroid hormones are present, the extent of which still has to be determined.

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II. APPENDIX PAPERS

Appendix paper 1

Effects of Inhibition of Type I Iodothyronine Deiodinase and Phenol Sulfotransferase on the Biliary Clearance of Triiodothyronine in Rats*

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ABSTRACT. Recent studies using isolated rat hepatocytes have indicated that the bioactive form of thyroid hormone, T_3, is metabolized in liver predominantly by conjugation with glucuronic acid or sulfate. In contrast to T_3 itself and the stable glucuronide, T_3 sulfate is rapidly degraded by successive deiodination of the tyrosyl and phenolic rings. In the present study we have investigated the biliary excretion of T_3 metabolites in male Wistar rats under pentobarbital anesthesia. The animals were injected iv with 1) saline, 2) the deiodinase inhibitor propylthiouracil (PTU; 1 mg/100 g BW), 3) the phenol sulfotransferase inhibitor dichloronitrophenol (2.6 μmol/100 g BW), or 4) a combination of both drugs. After 15 min, 10 μCi [^{125}I]T_3 were administered iv, and bile was collected for 30-min periods until 4 h after tracer injection. Secretory products were analyzed by HPLC. In control animals, 22.4% of the dose was excreted in bile mainly in the form of T_3 glucuronide. In PTU-treated rats biliary excretion was increased to 36.0% of the dose ($P < .001$) due to a dramatic increase in the sulfates of T_3 and 3,3′-diiodothyronine. Dichloronitrophenol by itself had no effect on the biliary clearance of T_3, but greatly inhibited PTU-induced excretion of sulfates. These results strongly suggest that sulfation and subsequent deiodination is an important pathway of T_3 metabolism *in vivo*.

THE PRINCIPAL secretory product of the follicular cells of the thyroid gland, T_4, is thought to have little intrinsic biological activity (1). It serves as a precursor for the actual hormone T_3, which involves enzymatic deiodination of the phenolic ring of T_4 (1). If, on the other hand, the tyrosyl ring of T_4 is deiodinated, rT_3 is produced with complete loss of thyromimetic activity (1). Various tissues contain deiodinases that act not only on T_4 but also on its metabolites (2). Prominent among these is the liver (type I) deiodinase which is the major source of peripheral T_3 production from T_4 (3). Surprisingly, the type I deiodinase is most active in phenolic ring deiodination of rT_3 to 3,3′-diiodothyronine (T_2), while it also converts T_4 to rT_3 and T_3 to T_2 by tyrosyl ring deiodination (2). The nonspecific nature of the type I deiodinase contrasts with the high specificity of the type II and type III deiodinases in other tissues, which only act on either the phenolic or the tyrosyl ring. A further distinction is the potent inhibition of type I deiodinase by the antithyroid drug 6-propyl-2-thiouracil (PTU) in contrast to the lack of PTU effect on the other types of enzyme (2).

Since the biological effect of T_4 is determined by the position of the iodine that is removed, it is logical that the enzymatic mechanisms of deiodination have received wide attention. However, conjugation of the phenolic hydroxyl group with glucuronic acid or sulfate is at least as important as deiodination for the metabolic clearance of thyroid hormone in rats (4). Bollman and Flock (4) have also demonstrated extensive conjugation of iodothyronines in dogs, but less is known about this pathway in humans. Recent studies have shown that T_3 is metabolized in isolated rat hepatocytes predominantly by sulfation and glucuronidation (5). While T_3 glucuronide is a stable end product, T_3 sulfate is prone to rapid deiodination by the type I enzyme, much more so than T_3 itself (6). Only if deiodination is inhibited, for instance with PTU, is significant accumulation of T_3 sulfate observed in these cultures (5). By analysis of the biliary clearance of T_3 in rats we now present evidence that successive sulfation and deiodination is also an important pathway for the metabolism of this hormone *in vivo*.

Materials and Methods

Male Wistar rats, weighing 250–350 g, were anesthetized by ip injection of pentobarbital (5 mg/100 g BW), and the abdomen

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was opened by midline incision. The common bile duct was cannulated with Silastic tubing (id, 0.3 mm; od, 0.6 mm; Dow-Corning, Midland, MI), and the abdomen was closed with stitches. All subsequent iv injections were given in the penile vein, using 1 ml 0.01 N NaOH in saline as the vehicle. Control rats received vehicle, and the treatment groups received PTU (1 mg/100 g BW), 2,6-dichloro-4-nitrophenol (2.6 μmol/100 g BW), or a combination of both drugs. The doses used have been shown to produce near-maximum inhibition of hepatic deiodinase (3) and phenol sulfotransferase (7) activities. After 15 min, 10 μCi [3′-^{125} I]T_3 were administered to each rat, but similar results were obtained if the period between PTU and tracer injection was extended to 150 min. During the experiments rats were placed under infrared lamps to maintain body temperature. Together with the T_3 injection and 2 h thereafter they were given sc injections with 3 ml saline to prevent dehydration and additional pentobarbital if appropriate. Bile was collected for 30-min periods until 4 h after T_3 administration, at which time rats were killed by cervical dislocation, and blood was obtained by heart puncture. The volume and radioactivity of the bile and serum samples were determined, which were then stored at −20 C until further analysis. In each experimental session five rats were studied in parallel, one of each experimental group as well as a sham-operated control without bile cannule.

Reverse phase HPLC was done on a 10 × 0.3-cm CPSpher C_{18} column (Chrompack, Middelburg, The Netherlands) with a 15–40% gradient of acetonitrile in 0.02 M ammonium acetate, pH 4 (8). For this purpose the nonlinear gradient no. 7 was selected, which was programmed by a model 680 automated gradient controller and delivered by a combination of model 6000A and 510 solvent pumps (Waters, Milford, MA). Samples consisted of 25 μl bile mixed with an equal volume of mobile phase. The gradient was started at the time of injection and was completed in 30 min, followed by a 15-min isocratic elution with the 40:60 acetonitrile-ammonium acetate mixture. Solvent flow was 0.8 ml/min, and 0.4-ml fractions were collected and counted for radioactivity. Recovery of applied radioactivity was, on the average, 97%.

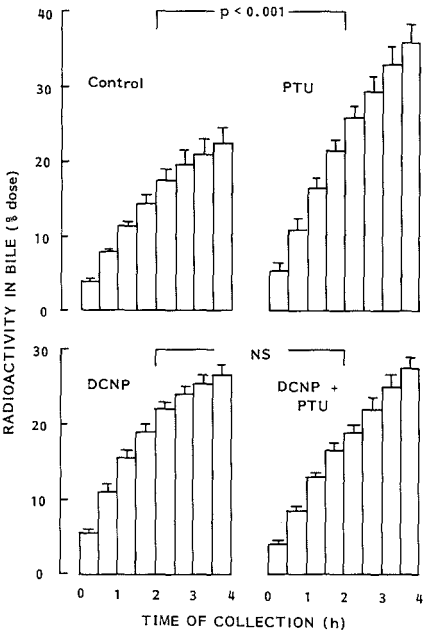

FIG. 1. Effects of the deiodinase inhibitor PTU and the phenol sulfotransferase inhibitor DCNP on the cumulative excretion of radioactivity in the bile of rats injected with labeled T_3. Data are the mean ± SE percent dose excreted in each group of five rats, and statistical significance of the PTU effect was tested by analysis of variance. The total volume of bile collected over the 4-h period was 4 ± 0.5 ml in the control group, 5 ± 1 ml in the PTU group, 5 ± 0.5 ml in the DCNP group, and 4 ± 0.5 ml in the DCNP plus PTU group (mean ± SE; n = 5).

Results

In control rats the cumulative excretion of radioactivity in the bile over the 4-h observation period amounted to 22.4 ± 2.1% (mean ± SE) of the administered T_3 dose (Fig. 1). On the average, 74% of biliary radioactivity was in the form of T_3 glucuronide, while 8% was excreted as T_3 sulfate and less than 1% as T_2 sulfate. The remainder consisted of small amounts of iodide, T_2 glucuronide, T_3, and T_2. The latter were not quantified as they overlapped with other minor peaks on HPLC (Figs. 2 and 3).

PTU treatment resulted in a significant 60% increase in the total radioactivity that was recovered after 4 h in the bile to 36.0 ± 2.7% of the injected dose. PTU did not affect the excretion of T_3 glucuronide if expressed as a percentage of the T_3 dose given. However, it induced a 5-fold increase in the excretion of T_3 sulfate and an even more dramatic increase in T_2 sulfate excretion. The sulfates now accounted for 24% and 14%, respectively, of the total radioactivity collected, while the contribution of T_3 glucuronide decreased from 74% to 44%. From 2 h after T_3 administration on, biliary excretion of sulfates exceeded that of T_3 glucuronide. As expected, the small amount of iodide in bile was further diminished by PTU.

Treatment of rats with dichloronitrophenol (DCNP) alone did not substantially affect the biliary clearance of T_3. Although Fig. 2 suggests that DCNP increased the excretion of radioactive material in the elution position of T_2, this was not a consistent finding in all DCNP-treated animals. Except for an increase in T_2 glucuronide and a more rapid excretion of T_3 glucuronide, the composition of the radioactivity in the bile was not changed by DCNP. After 4 h, 26.6 ± 1.3% of the injected radioactivity had appeared in bile. DCNP produced pronounced effects in rats that were also treated with PTU.

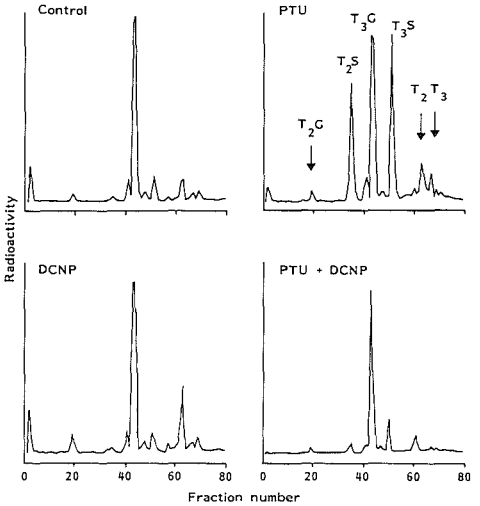

FIG. 2. Analysis of biliary T_3 excretion products by HPLC. The elution positions of reference compounds were determined using commercially available T_3 and T_2 (Henning GmbH, Berlin, West Germany), synthetic T_3 sulfate (T_3S) and T_2 sulfate (T_2S) (24), and biosynthetic T_3 glucuronide (T_3G) and T_2 glucuronide (T_2G) from incubations with isolated rat hepatocytes (8). The results are taken from a representative experiment, showing the analysis of bile collected 90–120 min after [^{125}I]T_3 injection into rats pretreated with saline, PTU, DCNP, or PTU plus DCNP.

The excretion of T_3 sulfate and T_2 sulfate in PTU-treated animals was inhibited by 58% and 61%, respectively, if they also received DCNP. There was only a small and insignificant increase in the excretion of T_3 glucuronide over that after PTU alone. Therefore, the PTU-stimulated biliary T_3 clearance was largely prevented by DCNP. In rats treated with a combination of the drugs, 27.4 ± 1.4% of the radioactivity was excreted in the bile within 4 h after the injection of labeled T_3.

Total plasma radioactivity 4 h after [^{125}I]T_3 administration was not different in control rats with or without bile cannula and was not affected by PTU or DCNP treatment. In the different groups, mean plasma radioactivity varied between 0.24–0.32% of the dose/ml (not shown).

Discussion

The three principal conclusions from this study are: 1) T_3 is predominantly excreted in the bile of normal rats as the glucuronide; 2) inhibition of type I iodothyronine deiodinase activity with PTU results in a selective increase in the excretion of sulfate conjugates; and 3)

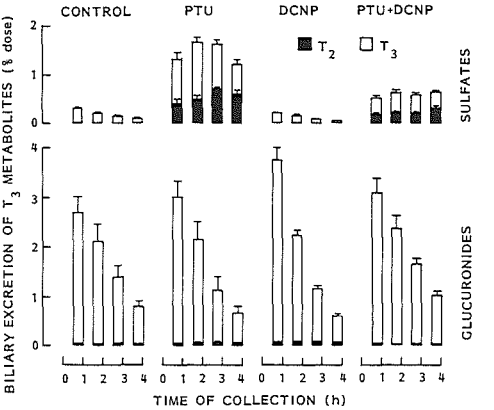

FIG. 3. Biliary excretion of T_3 and its metabolites as a function of time after injection of hormone. Results are the mean ± SE (n = 5) amounts of the glucuronides and sulfates of T_3 (□) and T_2 (■) in bile collected 30–60, 90–120, 150–180, and 210–240 min after T_3 administration. Bile was analyzed by HPLC, as shown in Fig. 2. Analysis of variance indicated that DCNP increased the excretion of T_3 glucuronide between 30 and 60 min ($P < 0.01$) and that of T_2 glucuronide in all periods tested ($P < 0.001$). Excretion of both T_3 sulfate and T_2 sulfate was increased by PTU ($P < 0.001$, *vs.* control), by PTU plus DCNP ($P < 0.001$, *vs.* control), and by PTU plus DCNP ($P < 0.001$, *vs.* DCNP) and was decreased by PTU plus DCNP ($P < 0.001$, *vs.* PTU).

sulfation of iodothyronines occurs largely by means of a DCNP-sensitive phenol sulfotransferase. These findings are reminiscent of early studies of the metabolism of thyroid hormone in rats. Morreale de Escobar and Escobar del Rey (9) observed that in rats equilibrated with ^{125}I-labeled T_3 or T_4, roughly equal proportions of radioactivity were eliminated with the feces or as radioiodide in the urine. The marked decrease in urinary output induced by PTU treatment was accompanied by an augmented loss via the fecal route before a change in plasma radioactivity could be discerned (9). Lang and Premachandra (10) also reported on the stimulatory effect of PTU on the biliary clearance of injected labeled T_4 in rats, again without apparent alterations in plasma radioactivity. These workers did not investigate the identity of the excretory products. However, such a study was undertaken by Flock and Bollman (11), who demonstrated that T_4 is excreted in rat bile largely as the glucuronide conjugate. After PTU administration, they noted an augmented biliary excretion of an acid-hydrolyzable T_4 conjugate, possibly T_4 sulfate (11). Similar results were obtained in a subsequent study using another type I deiodinase inhibitor, *i.e.* butyl 4-hydroxy-3,5-diiodobenzoate (12). In addition, evidence was presented that butyl 4-hydroxy-3,5-diiodobenzoate induced

a selective increase in the biliary excretion of T_3 sulfate as well as T_2 sulfate from injected T_3 (12), just as we found with PTU. It is also of interest that Roche and coworkers (13) noted the presence of T_3 sulfate in bile and plasma of thyroidectomized rats after injection with radioactive T_3. Hypothyroidism in rats is now known to be associated with impaired type I deiodinase activity (14).

These previous observations as well as the present findings are explained by the recent recognition of the interplay between sulfation and type I deiodination of iodothyronines. Experiments with isolated rat hepatocytes have made it clear that sulfation strongly facilitates deiodination of initially the tyrosyl ring and subsequently the phenolic ring of T_3 (5). Direct tests of synthetic T_3 sulfate (6) and T_2 sulfate (5) as substrates for the type I deiodinase in rat liver microsomes have shown a 40- to 50-fold increase in deiodination efficiency compared with that of nonconjugated T_3 or T_2. Even greater stimulatory effects by sulfation have been observed for the tyrosyl ring deiodination of T_4, initiating complete deiodinative breakdown of the molecule (15). In contrast, sulfation appears to prohibit deiodination of the phenolic ring of T_4, making it impossible to generate bioactive T_3 by this pathway (15). Sulfation is, therefore, the first step in the irreversible inactivation of thyroid hormone which permits reutilization of the iodine by the thyroid gland. In contrast, biliary excretion of T_3 glucuronide may be a reversible pathway, as this conjugate is hydrolyzed by intestinal bacteria (16), enabling the reabsorption of free T_3. The extent of such an enterohepatic cycle for thyroid hormone, however, remains unsettled.

It is unclear which tissues are able to conjugate thyroid hormone and to what extent they contributed to the biliary excretion of conjugates in our experiments. Although substantial conjugation of T_3 with both glucuronic acid and sulfate occurs in isolated rat liver cells (5), extrahepatic conjugation is a prominent pathway for the elimination of certain xenobiotics and endogenous compounds (17). Indeed, extensive urinary excretion of iodothyronine conjugates has been observed in hepatectomized dogs (4). Since DCNP-sensitive phenol sulfotransferase is present in many tissues (18), the inhibitory effect of DCNP on the biliary excretion of the sulfate conjugates of T_3 and T_2 is not indicative of their site of production. The T_2 sulfate in the bile of PTU-treated rats may have been produced by tyrosyl ring deiodination of T_3 sulfate due to incomplete inhibition of the type I deiodinase. More likely, however, it arises from sulfation of T_2 produced by PTU-insensitive (type III) deiodination of the tyrosyl ring of T_3 itself in extrahepatic tissues.

Little is known about conjugation of thyroid hormone in humans other than the demonstration of the glucuronide as the predominant biliary excretory product of T_4 (19). It is also well known that fecal clearance of thyroid hormone is less important in man that it is in the rat, while the existence of an enterohepatic circulation remains controversial (20). However, recent findings suggest that the data reported here may be extrapolated to human physiology. Thus, a DCNP-sensitive phenol sulfotransferase has been identified in human liver which is quite active in the sulfation of T_3 (21). Furthermore, the type I deiodinase of human liver has been shown to deiodinate T_3 sulfate much faster than T_3 itself (22). The recent development of a sensitive RIA for T_3 sulfate (23) should enable us to disclose if successive sulfation and deiodination are indeed as important for the disposal of T_3 in humans as they appear to be in rats.

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Appendix paper 2

Hydrolysis of iodothyronine sulfates by sulfatase activity of anaerobic bacteria from the rat intestinal flora **

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1. SUMMARY

2 Obligately anaerobic bacteria isolated from rat cecal flora have previously been shown to possess sulfatase activity towards 3,3′-diiodothyronine sulfate [5]. These strains have now been tested for their ability to hydrolyze the sulfate conjugates of other iodothyronines, including the thyroid hormones thyroxine and 3,3′,5-triiodothyronine. In anaerobic incubations at 37°C with approximately 10^7 bacteria per ml, variable amounts of the conjugated substrates, ranging from 15–90%, were hydrolysed in 24 h. These results showed a potent iodothyronine sulfate hydrolysing capacity of rat intestinal microflora. The strains were characterized by carbohydrate fermentation tests. One strain belonged to the genus *Lactobacillus*, the other strain probably to *Eubacterium* or *Lachnospira*.

2. INTRODUCTION

Conjugation is used by the body as a mechanism of inactivation or detoxification of various substances. The conjugates are usually excreted in the bile. In the digestive tract the conjugated substances are reabsorbed less efficiently than their unconjugated forms [1]. Therefore, conjugation is an efficient means of fecal excretion. The possibility that the hydrolysis of conjugates by intestinal bacteria may play a role in establishing an enterohepatic cycle has been pointed out by several authors [2,3]. The thyroid hormone, thyroxine (T_4), and its metabolites are excreted in the bile partly in the free form, but mainly as sulfate or glucuronide conjugates [4].

We have recently demonstrated sulfatase activity towards the sulfate ester of the thyroid hormone metabolite, 3,3′-diiodothyronine (T_2) in 2 anaerobic bacterial strains, isolated from rate cecal contents [5]. Recently, a method has been developed to synthesize the sulfate esters of other iodothyronines, including the pro-hormone T_4, the biologically active hormone 3,3′-5triiodothyronine (T_3), and the inactive metabolites 3,3′,5′-triiodothyronine (rT_3) and 3′-iodothyronine (T_1) [6]. This has enabled us to extend our investigations to

** Parts of this study were presented at the 13th and 14th Annual Meetings of the European Thyroid Association, Madrid, 11–15 July 1983 [20] and Rotterdam, 3–7 September 1984 [21].

these conjugates. Simultaneously, a tentative identification was made of the 2 anaerobic bacterial strains (A and B) from our previous work [5].

3. MATERIALS AND METHODS

3.1. Synthesis of iodothyronine sulfates

The synthesis of the sulfates of T_1, T_3, rT_3 and T_4 (T_1S, T_3S, rT_3S and T_4S, respectively) was achieved according to a recently developed method [6]. In short, the procedure consists of the reaction of anhydrous [^{125}I]iodothyronine of varying specific activity for 12 h at 20°C with a mixture of *N,N*-dimethylformamide and chlorosulfonic acid (4/1, v/v) and subsequent purification by Sephadex LH-20 chromatography.

3.2. Isolation of strains A and B

Strains A and B were isolated from cecal contents of conventional rats as described previously [5]. The strains were cultured on Schaedler broth with 0.0002% resazurin solidified with 2% agar in anaerobic culture flasks or in medium in test-tubes with 0.3% agar.

Bacterial growth was estimated by the number of colony-forming units (cfu) on solid media of diluted subcultures as well as by measurement of absorption by the incubates at 420 nm. Anaerobic conditions during incubation (see also below) were verified by using resazurin in the media, as this indicator becomes pink at a redox potential above − 120 mV (pH 7.0).

3.3. Incubation of T_3S with strain A

Incubations of T_3S with strain A bacteria were performed under strictly anaerobic conditions in a mixture of 8 ml broth (steam sterilized at 120°C for 10 min) with 0.04% dithiothreitol, 1 ml of broth containing 5×10^8 bacteria and 1 ml [^{125}I]T_3S in water (sterilized by 0.2 μm pore filtration). The final concentrations of T_3S were 0.1, 1.0 and 5.0 μM, respectively. The incubations were carried out at 37°C in a test-tube with a paraffin seal, which allowed sequential anaerobic sampling of the culture medium. Samples of 1 ml were drawn at 0, 3, 6, 9, 12, 15 and 24 h and stored at − 20°C until further analysis. The samples were simultaneously extracted with 2 ml ethanol and centrifuged at 3000 × *g*. The supernatant was then evaporated at 50°C under a stream of N_2. The residue was dissolved in 1 ml 0.1M HCl and subsequently applied to a small (1 ml bed volume) Sephadex LH-20 column equilibrated in 0.1 M HCl. 3 compounds were isolated by successive elution with 0.1M HCl H_2O and ethanol/0.1M NaOH (1/1, v/v): I^-, resulting from nonspecific deiodination; T_3S; and free T_3, respectively. Product quantities were reflected by the radioactivity in the respective fractions. Control experiments without bacteria were conducted on every occasion (Fig 1).

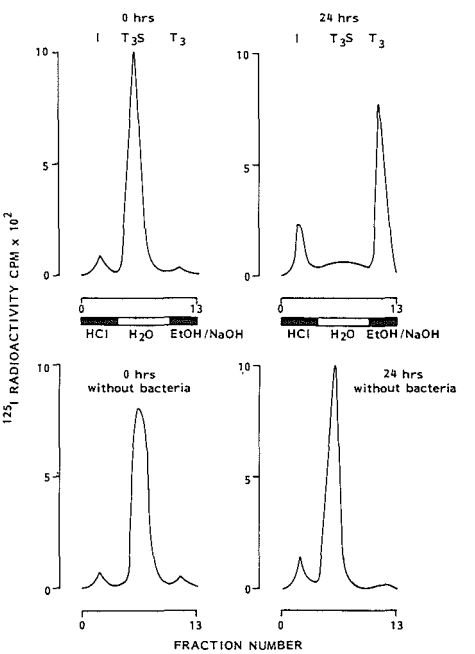

Fig. 1. Chromatography of culture medium containing 1.0 μM of the sulfate ester of 3,[3′-^{125}I],5-T_3 before and after 24 h incubation with strain A or without bacteria.

3.4. Incubations of different iodothyronine sulfate conjugates with strains A and B

The same conditions as described above were used for the incubation of T_1S, rT_3S and T_4S with strain A, and of the various conjugates with strain B. Final concentration of all conjugates was 5.0 μM, and 1-ml samples were drawn at 0 and 24 h. Isolation of the reaction products iodide, iodothyronine sulfate and unconjugated iodothyronine, respectively, was performed by chromatographic separation on Sephadex LH-20 columns for T_3S, rT_3S and T_4S, and on Sephadex G-10 columns for T_1S. The nature of the reaction products was confirmed by HPLC analysis as described elsewhere [6].

3.5. Identification of strains A and B

Identification of strains A and B was based on morphology, capacity to ferment various carbohydrates and determination of the glucose fermentation products. The carbohydrate fermentation capacity was determined according to Holdeman et al. [7] in peptone yeast extract medium (PY), 0.05% L-cysteine-HCl, 0.0002% resazurin and 0.03% agar in steam sterilized test-tubes (120°C, 15 min.) supplied with 1%(w/v) of the following filter-sterilized carbohydrates: amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose and xylose. A culture pH < 6.0 after 5 days of incubation was labeled as positive. The glucose fermentation products were determined by gas chromatography from 5-day incubates of the strains in PY broth with 1% glucose at 37°C under anaerobic conditions [8].

3.6. Sources of chemicals

T_1, T_3, rT_3 and T_4 were purchased from Henning Berlin GmbH, Berlin, FRG; [3′-^{125}I]T_1 and [3′,5′-^{125}I]rT_3 were prepared as described elsewhere [9]; [3′-^{125}I]T_3 and [3′,5′-^{125}I]T_4 were purchased from Amersham, U.K. Sephadex LH-20 and Sephadex G-10 came from Pharmacia Fine Chemicals, Uppsala, Sweden. Schaedler Broth was obtained from Oxoid, Basingstoke, U.K.

4. RESULTS

4.1. Hydrolysis of T_3S by strain A

Strain A bacteria hydrolyzed T_3S concentration-dependently. After 24 h of incubation, 78% of 0.1 μM, 73% of 1.0 μM and 47% of 5.0 μM T_3S had disappeared, with the reciprocal appearance of unconjugated T_3 (Fig. 1). Figures were corrected for spontaneous iodide production (4%) and hydrolysis observed in control incubations without bacteria. A linear increase in absorbance at 420 nm of the culture medium was observed during incubation of strain A with and without 5 μM T_3S (3.8 and 2.4 times after 24 h, respectively). The number of cfu did not change (mean 4.8×10^7/ml, standard deviation 0.9×10^7, 8 determinations). It is concluded that growth occurred due to an increase in bacterial cell mass, and not in cell number, and that growth in the presence of 5 μM T_3S was reduced to 65% of control values.

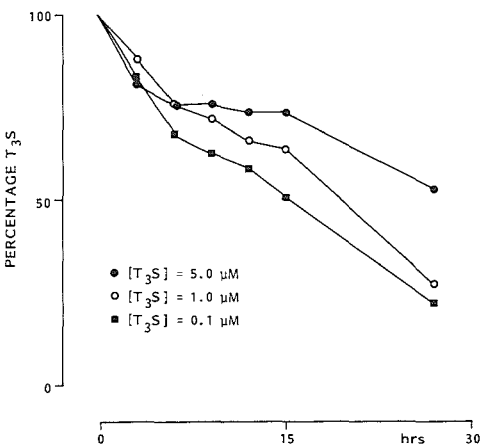

Fig. 2. Hydrolysis of 0.1 μM, 1.0 μ0M and 5.0 μM sulfate ester of 3,3′,5-T_3 (T_3S) by 10^7 bacteria/ml of strain A.

4.2. Hydrolysis of the different iodothyronine sulfates by strains A and B

Strain A hydrolysed 89% of 5.0 μM T_1S, 75% of 5.0 μM rT_3S and 64% of 5.0 μM T_4S in 24 h. Strain B hydrolyzed 50% of 5.0 μM T_1S, 29% of 5.0 μM T_3S, 45% of 5.0 μM rT_3S and 17% of 5.0 μM T_4S in 24 h.

No hydrolysis of the iodothyronine sulfates was observed during aerobic incubations with *Escherichia coli* or *Streptococcus* species.

4.3. Identification of strains A and B

Strain A is a small (4 μm), curved, non-motile, non-sporing, Gram-positive obligately anaerobic rod. The carbohydrates amygdalin, cellobiose, esculin, glucose, mannose and salicin were fermented. The following fermentation products were identified: formic acid (6 mM), acetic acid (2 mM), lactic acid (6 mM) and ethanol (3 mM).

On the basis of the glucose fermentation products, strain A belonged to the genus *Lactobacillus*. Fermentation of carbohydrates did not correspond to any of the species described by Holdeman et al. [7].

Strain B is a 5 μm, non-motile, non-sporing, Gram-positive obligately anaerobic rod. The carbohydrates fructose, glucose and ribose were fermented. Acetic acid (3 mM) and ethanol (43 mM) were the products.

The results did not allow the identification of strain B using the Anaerobe laboratory manual of Holdeman et al. [7]. The strain probably belonged to the genus *Eubacterium* or *Lachnospira*.

5. DISCUSSION

Sekura et al. have reported on the structure activity relationship of the sulfation of iodothyronines by rat liver phenol sulfotransferases I and IV and by a monkey hepatoma cell extract. They observed that 3′-T_1 and 3,3′-T_2 are better substrates for sulfation than T_3, while rT_3 and T_4 are poorly sulfated [10].

In studies using ^{131}I-equilibrated thyrodectomized rats, Escobar del Rey et al. found that the radioactivity excreted was equally distributed over feces and urine, the latter containing mainly iodide [11]. Later studies showed that the greater part of the iodothyronines excreted in rat bile is in conjugated form, predominantly as glucuronides, but also as sulfates [4]. The conjugates, especially the glucuronides, are less efficiently reabsorbed compared to native iodothyronines [12,13]. Therefore, it seems likely that intestinal hydrolysis of iodothyronine conjugates may enable enterohepatic retrieval of the parent compounds [5].

As early as 1959, hydrolysis of T_3S was demonstrated by Closon et al. in mixed bacterial suspensions [14]. However, these experiments were carried out under aerobic conditions, and are therefore less likely to be representative in vivo. In a different setting it was shown that *Bacillus faecalis* and a *Salmonella paratyphi* B species hydrolysed T_3S, whereas *Aerobacter aerogenes*, staphylococci and fungi did not have this potency [15]. Furthermore, in agreement with Closon et al. we detected no T_3S hydrolysis by the prominent aerobic intestinal strain *E. coli*, nor any hydrolyzing capacity of *Streptococcus* species. We therefore assume that if any substantial hydrolysis of iodothyronine sulfates occurs in vivo, this will be primarily effected by anaerobes. The numbers of strains A and B in the rat cecum ($> 10^8$/g) makes this more probable [5]. Morishita and Miyaki [16] found that *Lactobacillus* (strain A) and *Eubacterium* (strain B) species are major residents of the rat intestinal flora.

Closon et al. also found varying deiodination of T_3S or T_3 generated (2.5–44.5%) and also different amounts of triiodoacetic acid generated by deamination [14]. We could not reproduce either one of these results, and conclude that these findings may be caused by the different incubation conditions used, i.e. aerobic conditions, which enhance photo-oxidation and oxidative deamination.

The finding that sulfation is an important route for the inactivation of thyroid hormone, together with the presently described hydrolyzing activities of anaerobic intestinal bacteria, implicate sulfation as a reversible pathway. However, recent studies in our laboratory have also shown that sulfation facilitates the hepatic deiodination of various iodothyronines, suggesting that sulfation may also

be an important step in the irreversible elimination of thyroid hormone [17].

Therefore, the sulfatase activity we observed seems to come into play when the iodothyronines sulfated in the liver escape deiodination and are excreted in the bile. This happens specifically when deiodinase activity is impaired. Indeed, increased biliary excretion of T_3S is observed following administration of the deiodinase inhibitor propylthiouracil to rats, as well as in untreated hypothyroid rats [11,18,19].

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Appendix paper 3

Iodothyronine sulfate-hydrolyzing anaerobic bacteria isolated from human fecal flora

(Iodothyronine sulfatase; intestinal bacteria)

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1. SUMMARY

23 Bacterial strains isolated from human fecal flora were screened for hydrolysis of iodothyronine sulfates. Three obligate anaerobic bacterial strains possessed sulfatase activity. Two strains were identified as *Peptostreptococcus productus*, the other strain probably belonged to the genus *Eubacterium* or *Lachnospira*. In anaerobic incubations with growing bacteria up to 78% of the iodothyronine sulfates were deconjugated in 24 h. Hydrolysis was dependent on the bacterial strains and on the iodothyronine sulfates tested. This study extents previous observations of similar iodothyronine sulfatase activities associated with bacteria from rat intestinal microflora [5]. By analogy, hydrolysis of iodothyronine sulfates by anaerobic bacteria from human intestinal microflora probably represents an exo-enzymatic process.

2. INTRODUCTION

In the peripheral metabolism of thyroid hormones one of the possible metabolic pathways is hepatic conjugation with either glucuronic acid or sulfate, followed by excretion in the bile [1,2]. As compared with the parent compounds, the water-soluble conjugates are less easily reabsorbed through the mucosal cells lining the intestinal tract [3,4]. Biliary excretion will thus result in fecal elimination, unless intestinal hydrolysis of iodothyronine conjugates occurs, which may facilitate an enterohepatic circulation.

In a previous paper we reported on the iodothyronine sulfatase activity by two anaerobic bacterial strains from the rat cecal flora [5]. This study is an attempt to extent these observations to human fecal flora.

3. MATERIALS AND METHODS

3.1. Synthesis of iodothyronine sulfates

^{125}I-labeled iodothyronine sulfates of varying specific activity were synthetized essentially according to the methods described by Mol and Visser [6]. These included 3′-iodothyronine sulfate (T_1S), 3,3′-diiodothyronine sulfate (T_2S), 3,3′, 5-triiodothyronine sulfate (T_3S), 3,3′,5′-triiodothyronine sulfate (rT_3S) and thyroxine sulfate (T_4S).

3.2. Isolation of bacterial strains

Diluted human feces were cultured anaerobi-

* This study was presented in part at the 14th Annual Meeting of the European Thyroid Association (ETA) Rotterdam, 3–7 September 1984 [21].

cally on a non-selective medium (Schaedler broth) solidified with 2% agar in culture flasks [7]. Isolated bacteria were subcultured in culture-flasks or in test-tubes with the same medium containing 0.3% agar. Resazurin was added to the medium as indicator of anaerobic conditions.

Bacterial growth was estimated by the number of colony-forming units (cfu) of diluted subcultures on solid media after 48 h incubation at 37°C as well as by measurement of absorbance at 420 nm by the incubates.

Diluted fecal samples were plated on blood agar and incubated aerobically. Number of cfu was counted after 24 h incubation at 37°C. Gram-negative rods were identified with the API-system for Enterobacteriaceae and Gram-positive cocci with the API-system for streptococci (API Benelux, The Netherlands).

3.3. Screening of iodothyronine sulfatase activity in bacteria

Iodothyronine sulfatase activity of the bacterial subcultures was screened by incubating a mixture of 9 ml broth (10 min steam-sterilized) containing 0.04% dithiothreitol and 10^7–10^9 bacteria/ml with 1 ml 5.0 μM T_3S in water (sterilized by 0.2 μm pore filtration). The final T_3S concentration in the medium amounted to 0.5 μM. The mixture was incubated for 24 h in a parafine sealed test tube at 37°C under anaerobic conditions. One ml sample was taken, treated with 1 ml of ethanol and centrifuged at $3000 \times g$ for 10 min. The supernatant was evaporated at 50°C under a stream of N_2. The residue was dissolved in 1 ml 0.1 N HCl. The extraction procedure thus employed resulted in a percentage of recovery of 84% ± 5% (mean ± SD, $N = 25$). After application to a 1 ml bed volume Sephadex LH-20 column (equilibrated in 0.1 N HCl) 3 compounds were isolated by successive elution with 0.1 N HCl, H_2O and ethanol/0.1 N NaOH (1/1, v/v), namely I^-, T_3S and native T_3, respectively. The radioactivity in the respective fractions expressed the quantity of the various products. The 24 h anaerobic incubation of 0.5 μM T_3S in bacteria-free medium at 37°C was used as a control.

3.4. Incubation with T_3S

The 3 T_3S hydrolyzing anaerobic bacterial strains were incubated with 0.5 μM of T_3S according to the procedures described above. During incubation 1 ml samples were drawn at 0, 5, 10, 14 and 25 h and analysed for hydrolysis. Strain H2 was incubated in broth during 40 h. Growth of these obligate anaerobic bacteria was stopped by admitting oxygen and addition of penicillin (1 mg/ml). After centrifugation of the bacterial suspension for 10 min at $10000 \times g$, the supernatant was taken and the pellet was resuspended in saline. The bacterial suspension, the resuspended cellular pellet and the supernatant were incubated with 0.5 μM T_3S during 40 h at 37°C. Samples were extracted and separation of the reaction products was performed on Sephadex LH-20 columns. Incubations of preheated suspensions, resuspended pellets or supernatants (20 min, 100°C) were performed as controls.

3.5. Incubation with other iodothyronine sulfates

The 2 strains with highest sulfatase activity were incubated with 0.5 μM of T_1S, T_2S, rT_3S and T_4S. Samples were taken at 24 h and analyzed for hydrolysis. Chromatographic separation was performed on Sephadex LH-20 columns for T_2S, rT_3S and T_4S and on Sephadex G-10 columns for T_1S. The nature of the reaction products was confirmed by HPLC analysis as described by Mol and Visser [6].

3.6. Identification of iodothyronine sulfate hydrolyzing strains

Identification of strains was based on morphology, their fermentation capacity of various carbohydrates and the determination of the glucose fermentation products. The carbohydrate fermentation capacity was determined according to Holdeman et al. [8] in peptone yeast extract medium (PY), 0.05% L-cysteine-HCl, 0.0002% resazurin and 0.03% agar in test-tubes supplied after steam sterilization (120°C, 15 min) with 1% (w/v) of the following filter sterilized carbohydrates: amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch,

sucrose, trehalose and xylose. A culture pH < 6.0 after 5 days of incubation was labeled as positive. The glucose fermentation products were determined by gas chromatography from 5 day-incubates of the strains in PY broth with 1% glucose at 37°C under anaerobic conditions [9].

3.7. Sources of chemicals

T_1, T_2, T_3, rT_3 and T_4 were purchased from Henning Berlin GmbH, Berlin, FRG. [3′-^{125}I]T_1, [3′-^{125}I]T_2 and [3′, 5′-^{125}I]rT_3 were prepared as described elsewhere [10]; [3′-^{125}I]T_3 and [3′, 5′-^{125}I]T_4 were purchased from Amersham, Amersham, U.K. Sephadex LH-20 and Sephadex G-10 were from Pharmacia, Uppsala, Sweden. Schaedler broth was obtained from Oxoid, Basingstoke, U.K. Penicillin was acquired from Specia, Paris, France.

4. RESULTS

4.1. Iodothyronine sulfatase-positive strains

Out of 20 obligate anaerobic and 3 facultative anaerobic bacterial strains (*Escherichia coli* and 2 strains of *Streptococcus faecalis*) isolated from human fecal flora, 3 anaerobic strains (H2, H8 and H25) hydrolysed T_3S.

4.2. Incubations of the strains H2, H8 and H25 with T_3S

In Fig. 1 results are shown of the hydrolysis of 0.5 μM T_3S by strain H2, H8 and H25 during 25-h incubations. Only very little spontaneous iodide production occurred during incubations (< 2%) and no hydrolysis of 0.5 μM T_3S was observed in control incubations without bacteria. During the first 14 h, the absorbance of the incubations of H2, H8 and H25 increased 2.4, 2.7 and 3.8 times, respectively, indicating that the strains were growing. Hereafter, growth of H2 and H8 stopped, but H25 continued to grow so that after 25 h the absorbance was 5 times the original value. The number of cfu of H2 and H8 did not change during the incubation period (3×10^7 and 2×10^7/ml), but the number of H25 increased from 1×10^8 to 9×10^8/ml. From the results it is concluded that T_3S was hydrolyzed during growth of strains H2, H8 and H25. Considering the cfu and absorbances of the incubates, H2 was the most potent iodothyronine sulfate-hydrolyzing strain.

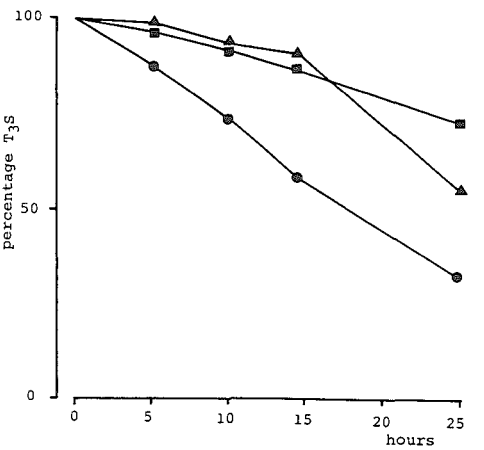

Fig. 1. Hydrolysis of 0.5 μM T_3S by 10^7–10^9 bacteria/ml of strain H2 (●), H8 (▲) and H25 (■) isolated from the human intestinal flora. For details, see text.

After incubation of 0.5 μM T_3S for 40 h with 4×10^7 bacteria per ml from a H2 culture inactivated with oxygen and penicillin, 19.6% of the substrate was hydrolysed. After centrifugation, the isolated cells (4×10^8/ml) and the supernatant hydrolysed 13.4 and 9.6% of the T_3S, respectively. The supernatant was concentrated 20-fold by freeze-drying, but this resulted in only a 2.3-fold increase in sulfate hydrolysis. These results suggest that the T_3S-hydrolyzing activity is heat-labile, localized on the bacterial surface or released in the medium, and readily inactivated by further manipulation.

4.3. Incubations of strains H2 and H8 with the other iodothyronine sulfates

In Table 1, the results are shown of the 24 h incubations of the various iodothyronine sulfates with strains H2 and H8 and compared with control incubations without bacteria. Strain H2 hydrolyzed up to 78% of the iodothyronine sulfates which again emphasized its large iodothyronine sulfate hydrolyzing capacities. In contrast strain H8 hydrolyzed only up to 28% of the iodothyronine sulfates. Spontaneous iodide production and non-

Table 1

Hydrolysis of 0.5 μM sulfate esters of the iodothyronines by two strains (H2 and H8) of *Peptostreptococcus productus*. Data represent mean values of 3 experiments. For details, see text.

Iodothyronine sulfate	% Hydrolysis		
	H2	H8	control
T_1S	76	15	0
T_2S	56	20	1
T_3S	78	27	1
rT_3S	75	28	0
T_4S	52	18	1

enzymatic hydrolysis amounted to less than 4% and 2%, respectively.

4.4. Identification of strains H2, H8 and H25

Strain H2 is a coccoid (2 μm), non-motile, non-sporulating Gram-positive, obligate anaerobic rod. Strain H8 is a small (1 μm), non-motile, non-sporulating Gram-positive, obligate anaerobic rod. Both strains fermented the carbohydrates amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, starch, saccharose, trehalose and xylose. The fermentation products from glucose which were identified were acetic acid (6 mM), lactic acid (2 mM) and succinic acid (3 mM) for strain H2 and acetic acid (14 mM), lactic acid (2 mM) and succinic acid (7 mM) for strain H8. On the basis of the morphology, strain H8 differs from H2, but both were identified as *P. productus*.

Strain H25 is a 5 μm, non-motile, non-sporing, Gram-positive, obligate anaerobic rod. The carbohydrates fructose, glucose and ribose were fermented. Acetic acid (2 mM) and ethanol (42 mM) were the fermentation products identified. The results did not allow identification of strain H25 using the anaerobe laboratory manual of Holdeman et al. [8]. The strain probably belongs to the genus *Eubacterium* or *Lachnospira*.

5. DISCUSSION

Conjugating enzymes such as glucuronyl-transferases and sulfotransferases are predominantly localized in hepatocytes [11,12]. Conjugation is an important pathway for the inactivation of several xenobiotics and endogenous compounds [13]. The hydrophylic nature of the conjugates also enables their elimination from the body with the urine or by excretion in the bile [13]. For iodothyronines both sulfation and glucuronidation are described [14]. The iodothyronines T_4 and rT_3 with 2 iodine atoms in the phenolic ring are mainly substrates for glucuronidation, whereas the iodothyronines $3'$-T_1 and $3,3'T_2$, with only 1 iodine molecule in the phenolic ring are predominantly sulfated. T_3 is equally sulfated as glucuronidated [15]. In the metabolism of thyroid hormones native iodothyronines as well as their conjugates are excreted in the bile. Iodide released by deiodinating pathways is mainly excreted in urine [1, 14–17]. Bacterial hydrolysis of several sulfated compounds has been reported in the literature [18]. Until recently, however, only a bacteria-dependent hydrolysis of T_3S was reported, although the sulfatase-producing strain was not isolated [19]. Iodothyronine sulfatase activity was recently described as a property of certain anaerobic rat cecal strains [5]. The availability of synthetic iodothyronine sulfates also enabled us to test this activity in human fecal bacteria.

We have shown rapid hydrolysis of iodothyronine sulfates by 3 of 20 bacterial strains isolated from human fecal anaerobic flora. Two strains (H2 and H8) were identified as *P. productus* which, according to Moore and Holdeman [20], belongs to the major residents of the human intestinal flora. The third strain (H25) resembles closely an iodothyronine sulfate-hydrolyzing strain (strain B) from the rat intestinal flora, which we described in a previous study [5]. A limited number (3×10^7/ml) of bacteria belonging to one of the *P. productus* strains hydrolyzed more than 50% of the thyroid-hormone sulfates (0.5 μM) within 24 h. In consideration of the much larger numbers of these bacteria present in the intestinal tract together with the much smaller prevailing concentrations of these sulfates, this would implicate a very active in vivo hydrolysis. Enzymatic hydrolysis of iodothyronine sulfates could also be demonstrated in isolated bacterial cells or supernatants acquired after centrifugation of the bacterial sub-

culture. These activities, however, turned out to be much lower as compared with activities of viable dividing cells in culture.

Enterohepatic circulation of thyroid hormones has been studied by several authors [14, 17]. Differences between intestinal absorption of conjugated and unconjugated hormones focused the attention on intestinal hydrolysis of iodothyronine conjugates. The significant bacterial hydrolysis as demonstrated in this study may be an essential component of the enterohepatic circulation. To assess the importance of this mechanism further in vivo studies have to be performed.

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Appendix paper 4

Hydrolysis of iodothyronine glucuronides by obligately anaerobic bacteria isolated from human faecal flora

(Iodothyronine glucuronides; obligate anaerobes)

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1. SUMMARY

35 bacterial strains isolated from the human faecal flora were screened for hydrolysis of the glucuronides of 3,3′,5-triiodothyronine and 3,3′-diiodothyronine. Two Gram-positive obligately anaerobic strains possessed glucuronidase activity. These strains probably belong to the genus *Eubacterium*, but ethanol was produced in high concentrations during glucose fermentation, which makes final classification difficult. Considering the number of bacteria in the intestinal flora (> 10^8/ml) and the biliary excretion of iodothyronine conjugates, the strains must be able to hydrolyse a major part of the total daily intestinal supply of these iodothyronine metabolites. The study extends previous observations with faecal suspensions of human and rat origin [24]. The relevance of bacterial β-glucuronidase activity for a possible enterohepatic circulation of iodothyronines is discussed.

2. INTRODUCTION

Iodothyronines preferentially appear in bile as conjugates with either glucuronic acid or sulphate [1,2]. Hydrolysis of these conjugates by the intestinal microflora might enhance enteral re-absorption of the aglycones, thereby promoting an enterohepatic circulation. In a previous paper we reported on the iodothyronine sulphatase activity of 2 anaerobic bacterial strains from the rat faecal flora and of 3 strains isolated from the human intestinal flora [3,4]. In this study, the isolation of iodothyronine β-glucuronidase-producing bacteria from the human intestinal flora is described.

3. MATERIALS AND METHODS

3.1. Reference compounds

3,3′-diiodothyronine (T_2) and 3,3′,5-triiodothyronine (T_3), were obtained from Henning, Berlin, F.R.G. [3′-^{125}I]T_2 was prepared as previously described [5]. [3′-^{125}I]T_3 was purchased from Amersham, Amersham, UK. β-glucuronidase from *Helix pomatia* type IX, sulphatase from Abalone entrails type VIII and phenolphthalein glucuronide were obtained from Sigma, St. Louis, MO, U.S.A. Other reagents were of laboratory grade. Reversed-phase high-performance liquid chromatography (HPLC) was performed on a CP-Spher C18 column (Chrompack, Middelburg, The Netherlands) using a Model 6000 A solvent delivery system and a Model 680 automated gradient controller (Waters, Milford, MA, U.S.A.).

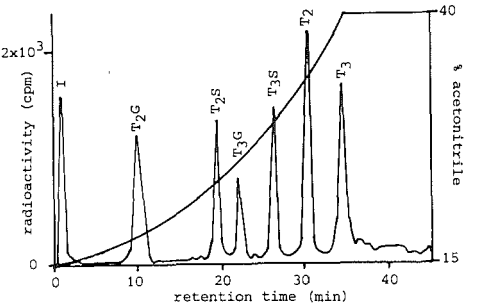

Fig. 1. Reversed-phase HPLC of a mixture of ^{125}I-labelled iodide, free T_2 and T_3, and their sulphate (S) and glucuronide (G) conjugates. The compounds were applied in 100 μl 15% acetonitrile in 0.02 M ammonium acetate (pH 4) and eluted with a gradient of up to 40% acetonitrile in ammonium acetate at a solvent flow of 0.8 ml/min.

3.2. Synthesis of iodothyronine glucuronide

^{125}I-labeled T_2 glucuronide (T_2G) and ^{125}I-labeled T_3 glucuronide (T_3G) were biosynthetised by incubating monolayers of isolated rat hepatocytes with [3′-^{125}I]T_2 and [3′-^{125}I]T_3 for 3 h in a protein-free Dulbecco medium [6]. For the isolation of the conjugates produced, HPLC analysis was performed. The medium was mixed with an equal volume of ethanol and centrifuged. After centrifugation for 15 min at 1000 × *g* the supernatant was evaporated at 50°C under a stream of N_2. The residue was dissolved in 100 μl of the starting elution solvent and applied to the HPLC system. A gradient of increasing concentrations of acetonitrile in 20 mM ammonium acetate (pH 4) was used. Radioactive 'peak' fractions were collected and separately pooled. Fractions were characterised by treatment with commercially available sulphatase and β-glucuronidase [6]. HPLC analysis of the hydrolysed products was compared with a separately performed HPLC analysis of the native iodothyronines. By these methods at least 7 peaks could be identified, namely iodide, 3,3′-diodothyronine glucuronide (T_2G), 3,3′-diiodothyronine sulphate (T_2S), 3,3′,5-triiodothyronine glucuronide (T_3G), 3,3′,5-triiodothyronine sulphate (T_3S) and the parent hormones T_2 and T_3. Fig. 1 depicts the sequential elution of a prepared reference mixture containing the iodothyronines and their conjugates.

Incubates with bacteria and T_2G or T_3G were analysed with standard Sephadex LH-20 chromatography [3,4]. One ml of the incubate was extracted with 2 ml ethanol and centrifuged at 3000 × *g*. The supernatant was evaporated at 50°C under a stream of N_2. The residue was desolved in 1 ml 1N HCl and added to small (1 ml bed volume) Sephadex LH-20 columns equilibrated in 0.1N HCl. By successive elution with 0.1N HCl, H_2O and a mixture of ethanol and 0.1 N NaOH (1/1, v/v) a good separation between I^-, glucuronide and parent iodothyronine was accomplished. Product quantities were reflected by the radioactivity in the respective fractions.

3.3. Isolation of bacterial strains

1 ml of a 10^{-8} dilution of 1 g faeces of a healthy subject was plated anaerobically in modified Roux flasks [7] with a non-selective medium (Schaedler broth, Oxoid, Basingstoke, U.K.) solidified with 2% agar and incubated during 48 h at 37°C. The dilution gives rise to 200 colonies. On basis of differences in type of colony, morphology and Gram-stain, strains were selected and subcultured in culture flasks or in test-tubes with the same medium containing 0.3% agar. Resazurin was added to the medium as indicator of anaerobic conditions.

Bacterial growth in incubates of strains was estimated by the number of colony-forming units (cfu) of diluted subcultures on solid media after 48 h incubation at 37°C as well as by measurement of absorption by the incubates at 420 nm.

Diluted faecal samples were plated on blood agar and incubated aerobically. The number of cfu was counted after 24 h incubation of 37°C. Gram-negative rods were identified with the API-system for Enterobacteriaceae and Gram-positive cocci with the API-system for streptococci (API Benelux, The Netherlands).

3.4. Screening of glucuronidase activity

3.4.1. Iodothyronine glucuronidase activity

Hydrolysis of iodothyronine glucuronides by the bacterial subcultures was screened by incubating a mixture of 2.5 ml broth (10 min steam-sterilised) containing 0.04% dithiothreitol

and 10^8–10^9 bacteria/ml with 0.5 ml of a tracer amount of [^{125}I]T_3G in water (sterilised by 0.2-μm pore filtration). The final T_3G concentration amounted to 5 pM. The mixture was incubated for 24 h in a paraffin-sealed test tube at 37°C under anaerobic conditions. Analysis of the reaction products was done by Sephadex LH-20 chromatography as described above. The 24-h anaerobic incubation of T_3G in bacteria-free medium at 37°C was used as a control. The T_3G-hydrolysing anaerobic strains were also incubated with 3 pM T_2G or T_3G in 10 ml broth according to the procedures described above. During incubation, samples were drawn at 0, 3, 6, 9, 12 and 26 h and analysed for hydrolysis. Again 24 h anaerobic incubations of T_2G and T_3G in bacteria-free media at 37°C were used as controls.

3.4.2. Phenolphthalein glucuronidase activity

Hydrolysis of phenolphthalein glucuronide (PG) was tested with bacterial suspensions in saline under aerobic conditions. Suspensions were obtained by washing of the bacterial strains from solid medium with saline and the absorbance was adjusted to $A_{420} = 2.0$ (approx. 2×10^8 bacteria/ml). An 0.9 ml aliquot of this suspension was incubated for 4 h at 37°C with 0.1 ml 10 mM PG, followed by the addition of 1.5 ml ethanol. After centrifugation (10 000 × g), 10 μl 2 N NaOH was added to 2 ml supernatant. Absorption at 500 nm was measured and compared with known concentrations of phenolphthalein.

3.5. Identification

Identification of strains was based on morphology, fermentation capacity for various carbohydrates and the determination of the glucose fermentation products. The carbohydrate fermentation capacity was determined according to Holdeman et al. [8] in peptone yeast extract medium (PY), 0.05% L-cysteine-HCl, 0.0002% resazurin and 0.03% agar in test-tubes supplied after steam-sterilisation (120°C, 15 min) with 1% (w/v) of the following filter-sterilised carbohydrates: amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose and xylose. A culture pH < 6.0 after 5 days' incubation was labelled as positive. The glucose fermentation products were determined by gas chromatography from 5-day incubates of the strains in PY broth with 1% glucose at 37°C under anaerobic conditions [9].

4. RESULTS

4.1. Glucuronidase-positive strains

Out of 32 obligately anaerobic and 3 facultatively anaerobic bacterial strains (*Escherichia coli, Proteus* sp. and *Streptococcus faecalis*) isolated from the human faecal flora, two obligately anaerobic strains (No. 4 and 21) hydrolysed 80 and 85%, respectively, of T_3G in 26 h. In the incubations with other strains less than 10% hydrolysis was observed and in control incubations without bacteria 8%. Additionally, strains were tested for hydrolysis of PG. Strains 3, 4 and 21 hydrolysed 3, 13 and 22%, respectively, of the substrate in 4 h. In the other incubates and in control incubations without bacteria, no hydrolysis of PG was observed.

4.2. Incubation of strains 4 and 21 with T_2G and T_3G

In Fig. 2 results are shown for the hydrolysis of

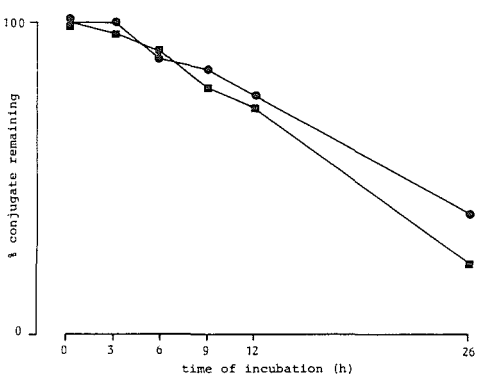

Fig. 2. Hydrolysis of 3 pM T_2G (●) and T_3G (■) by 10^7 bacteria of strain 4 per ml.

3 pM T_2G or T_3G by strain 4. Only little spontaneous iodide production occurred during incubation (< 2%) and 6% hydrolysis of T_2G and T_3G was observed in control incubations without bacteria. At 0 h, 19 h and 26 h of incubation with strain 4, the numbers of cfu were 6.5×10^7, 4.2×10^7 and 5.8×10^7 per ml, respectively.

During the first 9 h, the absorbance of the suspension did not increase ($A_{420} = 0.125$), but after 26 h it was 4 times the original value. The results with strain 21 were almost identical. From the experiments it is concluded that T_2G and T_3G are hydrolysed during growth of strains 4 and 21. Incubation of strains 4 and 21 with T_3 sulphate according to previously described methods [3,4] showed no hydrolysis of the substrate. The potent glucuronidase-producing strains 4 and 21, therefore, lacked any iodothyronine sulphatase activity.

4.3. Identification of strains 4 and 21

Strains 4 and 21 are 1-μm, non-motile, non-sporulating, Gram-positive, obligately anaerobic rods. The strains fermented cellobiose, fructose, glucose, lactose and maltose. With strain 4 the following fermentation products from glucose were identified: formic acid (4 mM), acetic acid (8 mM), lactic acid (4 mM) and ethanol (75 mM). The fermentation products of strain 21 were similar but additionally 0.4 mM propionic acid was produced in 3 observations.

The strains could not be identified using the Anaerobe Laboratory Manual of Holdeman et al. [8]. Although on the basis of morphology and fermentation of different carbohydrates they fit into the genus *Eubacterium*, the strong production of ethanol makes assignment to a particular species difficult.

5. DISCUSSION

Glucuronidation, first recognised in 1855, is the most widespread form of 'conjugation' in mammalian metabolism [10]. The reaction, coupling of D-glucuronic acid with a variety of compounds, is catalysed by UDP-glucuronyltransferases. They represent a class of closely related enzymes which are predominantly located in the endoplasmic reticulum of the liver cell [10]. The biliary excretion of an iodinated metabolite of thyroxine in dogs was reported by Kendall in 1919 [11]. In 1951, Taurog et al. were the first to identify thyroxine glucuronide (T_4G), initially named 'compound U', in rat bile [12,13]. Later, conjugation with glucuronic acid was also reported for T_3, reverse T_3 and lesser iodinated iodothyronines as T_2 [14–16]. Apart from the glucuronides, iodothyronines are also excreted to some extent into rat bile in the free, non-conjugated form or as sulphate conjugates [17,18]. Compared with the aglycones, conjugated iodothyronines are poorly reabsorbed in the intestinal tract [19–21]. Liberation of the free hormones by intestinal hydrolysis of the conjugates will, therefore, enhance their reabsorption.

β-glucuronidase activity of intestinal contents and of isolated intestinal bacteria has been described by several authors, including Lankhorst et al. [22] and Gadelle et al. [23]. Tests for iodothyronine glucuronidase activity, however, depend on the availability of substrates. In our laboratory it was possible to biosynthesise T_2G and T_3G in monolayers of isolated rat hepatocytes. Previous experiments showed that faecal suspensions of human or rat origin hydrolysed T_3G. Faeces from germ-free or intestine-decontaminated rats, however, were not able to hydrolyse iodothyronine conjugates, indicating the importance of the intestinal microflora for this process [24].

The present study demonstrates that two major residents (see below) of the human intestinal microflora [25], probably belonging to the genus *Eubacterium*, hydrolysed T_2G and T_3G. Although it was not yet possible to biosynthesise T_4G, it seems likely that the strains are also able to hydrolyse this conjugate, since they also hydrolysed the artificial substrate PG. This assumption is in agreement with our previous results with iodothyronine sulphatase-producing bacteria, showing that the iodothyronine sulphates T_2S, T_3S, rT_3S and T_4S were all hydrolysed by the same strains, although differences in substrate affinity were observed [3,4]. The in vitro hydrolysis of 80–85% of 3 pM T_3G and T_2G by $4–7 \times 10^7$ bacteria/ml in 26 h may implicate a very active in vivo hydrolysis. The results with PG (13–22% hydrolysis of 1

mM PG by 2×10^8 bacteria/ml is 4 h) even suggest that this requires only a small fraction of the total β-glucuronidase capacity of these bacteria. The biliary excretion of T_4, T_3 and T_2 in rats was estimated by Foldes et al. [26] to amount to 50.0 pmol T_4, 12.9 pmol T_3 and 2.5 pmol T_2 per h. Of this, 32.8, 30.0 and 31.7%, respectively, appeared as free hormone in the bile. The remaining fraction was conjugated with either glucuronic acid or sulphate. The numbers of bacteria of both strains 4 and 21 in the intestine in vivo, as estimated on the basis of the isolation methods, must exceed 10^8/g intestinal material. In conjunction with the above-mentioned findings, this suggests that complete hydrolysis of the intestinal supply of iodothyronine glucuronides must be possible during intestinal passage. As the intestinal microflora is also capable of iodothyronine sulphate hydrolysis [3,4] this implies that iodothyronines occur in the intestinal tract mainly in the free, non-conjugated form. These results are in agreement with our previous in vitro findings with suspensions of human and rat faeces [24]. Whether there will be sufficient in vivo reabsorption of the liberated iodothyronines is a subject of further investigations.

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Appendix paper 5

RAPID AND BACTERIA-DEPENDENT IN VITRO HYDROLYSIS OF IODOTHYRONINE-CONJUGATES BY INTESTINAL CONTENTS OF HUMANS AND RATS

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ABSTRACT

Faecal suspensions from healthy humans, conventional (CV), germ-free (GF) and intestine-decontaminated (ID) rats were tested for the in vitro hydrolysis of ^{125}I-labelled iodothyronine sulphates and 3,3',5-triiodothyronine glucuronide (T_3G). Whereas 20-fold diluted human and CV rat faecal suspensions hydrolyzed up to 90 % of the sulphates, no hydrolysis was observed in 5 times diluted faecal suspensions of GF and ID rats. These results add further weight to the assumption that intestinal iodothyronine sulphatase activity is of bacterial origin. Twenty times diluted human and CV rat faecal suspensions hydrolyzed approximately 80 % of the T_3G. In the 5 times diluted faecal suspensions of GF and ID rats up to 15 % hydrolysis of T_3G was still observed. It was concluded that the major part of the gastrointestinal iodothyronine glucuronidase activity is produced by bacteria. The remaining activity presumably originates from gastrointestinal mucosal cells.

KEY WORDS: IODOTHYRONINE-SULFATASE; IODOTHYRONINE-GLUCURONIDASE; INTESTINAL-CONTENTS

INTRODUCTION

Hepatic conjugation plays a role in the inactivation of numerous drugs and xenobiotics in man and animals (10). The subsequent biliary excretion of the conjugated compounds is an efficient way of elimination, as the hydrophylic nature of the conjugates precludes intestinal reabsorption (16). Conjugated compounds such as glucuronides or sulphates may, however, be hydrolysed by β-glucuronidase or sulphatase activities originating from intestinal micro-organisms (7, 11). Intestinal hydrolysis enables intestinal reabsorption of the parent compounds. This enterohepatic circulation has been described for several endogenous and exogenous products such as bile acids, bilirubin and steroid hormones (8, 16). The thyroid hormones and their metabolites are conjugated in the liver with either glucuronic acid or sulphate and are excreted with the bile (18, 20). In previous studies we demonstrated that iodothyronine sulphates are rapidly hydrolysed in vitro by sulphatase activities from human faecal or rat caecal anaerobic gut bacteria (4, 6). Also hydrolysis of T_3G occured when incubated with an anaerobic bacterial strain isolated from human intestinal microflora (3). In this paper we studied the in vitro hydrolysis of the iodothyronine conjugates by diluted faecal suspensions. In order to illustrate the bacteria-dependency of this process, incubations were also carried out with faecal suspensions from germ-free (GF) or intestine-decontaminated (ID) rats.

MATERIAL AND METHODS

Reference compounds. 3'-iodothyronine (T_1), 3,3'-diiodothyronine (T_2), 3,3,'5-triiodothyronine (T_3), 3,3,'5'-triiodothyronine (rT_3) and 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) were obtained from Henning Berlin, GmbH, Berlin West. [3'-^{125}I]T_3 and [3',5'-^{125}I]T_4 were purchased from Amersham, Amersham, U.K. [3'-^{125}I]T_1, [3'-^{125}I]T_2 and [3',5'-^{125}I]rT_3 were synthesised according to methods described elsewhere (21). Sephadex LH-20 and G-10 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampicillin was from Beecham, Worthing, U.K. and penicillin from Specia, Paris, France. β-glucuronidase from Helix pomatia type IX and sulphatase from Abalone entrails type VIII were obtained from Sigma Chemical Co, St. Louis, U.S.A. The other reagents were of laboratory grade.

Conjugate synthesis. ^{125}I-labelled sulphates of T_1, T_2, T_3, rT_3 and T_4 (T_1S, T_2S, T_3S, rT_3S and T_4S) were chemically synthesised according to Mol and Visser's method (19). This method consists of the reaction of anhydrous [^{125}I]iodo-

TABLE 1

Hydrolysis of iodothyronine-conjugates in 20 times diluted suspensions of human and rat faeces.

Substrate	Hydrolysis, %			
	Human faeces		CV rat faeces	
	Exp I	Exp II	Exp I	Exp II
T_1S(0.5 μM)	64	51	32	47
T_2S(0.5 μM)	86	67	46	52
T_3S(0.5 μM)	70	22	3	42
rT_3S(0.5 μM)	89	91	48	86
T_4S(0.5 μM)	86	88	41	78
T_3G(3.0 pM)		80		79

thyronine of varying specific activity with a mixture of N,N-dimethylformamide and chlorosulphonic acid (4/1, v/v) for 12 h at 20°C followed by purification on Sephadex LH-20 or G-10 (only for T_1S). The identity of iodothyronine sulphates thus synthesised was confirmed by high pressure liquid chromatography (HPLC) (19). ^{125}I-labelled T_3 glucuronide (T_3G) was biosynthesised by incubating monolayers of isolated rat hepatocytes with [3'-^{125}I]T_3 for 3 h in a sulphate and protein free Dulbecco medium. The medium was mixed with an equal volume of ethanol and centrifuged for 15 min at 3000 rpm. The supernatant was evaporated under a stream of N_2. The residue was dissolved in 0.1N HCl and applied to a small (1 ml bed volume) Sephadex LH-20 column equilibrated in 0.1N HCl. By successive elution with 0.1N HCl, 0.1M sodium acetate-buffer (pH 4), H_2O and ethanol/0.1 N NaOH (1/1, v/v) a good separation between I^-, T_3G, T_3S and T_3, respectively, was obtained. T_3G was identified using commercially available β-glucuronidase and T_3S was identified using commercially available sulphatase but also by HPLC analysis (19).

Animals. Conventional and germ-free Wag/Rij rats (TNO Rijswijk, The Netherlands) were used. Intestinal tracts of conventional rats were decontamined by adding ampicillin (1 mg/ml) to the drinking water for 5 days before faeces collection.

Faeces collection. Freshly produced faeces were obtained from healthy laboratory workers and from 3 groups of rats: conventional rats (CV), intestine-decontaminated rats (ID) and germ-free rats (GF).

Incubations. Faeces were diluted 5 or 20 times in saline. To 9.0 ml of the suspensions 1.0 ml of 5.0 μM of T_1S, T_2S, T_3S, rT_3S or T_4S or tracer (30 pM) T_3G in sterilised water was added. The final substrate concentrations amounted to 0.5 μM, for the sulphates and to 3.0 pM for T_3G. Suspensions were incubated up to 24 h at 37°C. Preheated (30 min, 80°C) faecal suspensions were simultaneously incubated as controls. Penicillin (1 mg/ml) was added to the suspensions to prevent bacterial growth during incubations. Penicillin alone was not capable of conjugate hydrolysis.

Extractions. To 1 ml samples of the above incubations 1.5 ml of ethanol was added and the mixture was centrifuged for 10 min at 3000xg. The supernatant was taken for isolation of the reaction products by Sephadex chromatography. The recoveries of the metabolites were calculated from similarly performed extractions immediately after mixing faecal suspensions with iodothyronine sulphates or with the parent iodothyronines.

Chromatography. Supernatants were evaporated under a stream of N_2 at 50°C and the residues were dissolved in 0.1 N HCl. The reaction products I^-, conjugates and iodothyronines, were isolated by Sephadex LH-20 or G-10 chromatography by successive elution with 0.1 N HCl, H_2O and ethanol/0.1 N NaOH (1/1, v/v), respectively. A good separation of the products was obtained, the quantity of which was calculated from the radioactivity in the fractions.

Cultures. Faeces were diluted and cultured under anaerobic conditions on a non-selective medium (Schaedler broth; Oxoid, Basingstoke, U.K.) solidified with 2 % agar in culture flasks (22). Aerobic diluted faecal samples were plated on blood-agar. The number of colony forming units was counted after 48 hours incubation at 37°C.

RESULTS

A recovery of 72.2 ± 5.3 %, (mean ± SD, *n* 300) of the reaction products was obtained by a single ethanol extraction procedure. This recovery was independent of the nature of the substrates, the kind of faecal suspensions incubated or the incubation periods.

The numbers of obligately anaerobic bacteria cultured from human faeces and CV rat faeces were 4.5×10^{10} and 1.5×10^{10} and of facultatively anaerobic bacteria 1.6×10^7 and 4.3×10^6 per gram, respectively. The number of facultative anaerobes cultured from the ID rat feces was 1.4×10^8 per gram, and the number of obligate anaerobes was $< 10^5$ per gram. Neither obligate nor facultative anaerobes could be cultured from the GF rat faeces.

Faecal suspensions were incubated up to 24 h with the iodothyronine conjugates. The addition of penicillin prevented bacterial growth during incubations, but inactivated cells and bacterial products remained. After 2 h incubation of five times diluted human and CV rat faeces the iodothyronine conjugates were completely hydrolysed. These results were reproduced three times in separate experiments. Five times diluted faecal suspensions of GF and ID rats did not hydrolyse any of the iodothyronine sulfates, but 10 and 15 % of the T_3G was hydrolyzed in 24 h, respectively. Preheated human or CV rat faecal suspensions were not capable of conjugate hydrolysis in 24 h. From these results it is concluded that iodothyronine conjugate hydrolysis in the intestinal tract is an enzymatic process. Intestinal hydrolysis of iodothyronine sulphates and, at least in part, iodothyronine glucuronides is bacteria-dependent.

Considering the very rapid hydrolysis of the conjugates in the five times diluted suspensions of human and CV rat faeces, 20 times suspended faeces were incubated for 1 h with the substrates. In Table 1 the results are shown of two

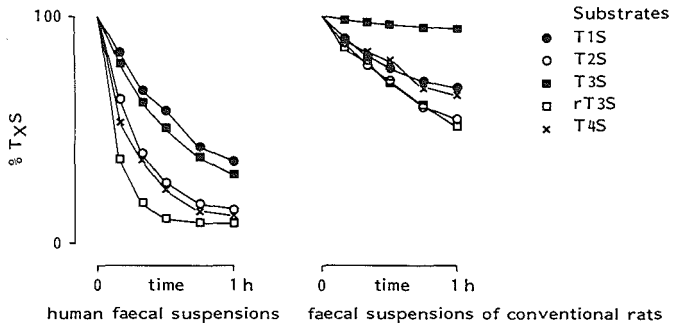

Fig. 1. Hydrolysis of 5.0 μM of the iodothyronine sulphates by incubations up to 1 h at 37°C with 20-fold diluted suspensions of human faeces and faeces of conventional rats.

experiments with the 20 times diluted suspensions of human and CV rat faeces. In experiment I samples were also drawn from the incubates at 0, 10, 20, 30, 45 and 60 min (Fig. 1). The results of the first experiment show that human faeces hydrolysed iodothyronine sulphates more efficiently than rat faeces, the latter hardly deconjugating T_3S. In the second experiment, showing also the deconjugation of tracer T_3G, hydrolysis of the iodothyronine sulphates by human and rat faeces was similar except for the conversion of T_3S. These results show that supraphysiological concentrations of iodothyronine sulphates and tracer T_3G are effectively hydrolyzed by high dilutions of human and CV rat faeces.

Suspensions of human faeces were diluted 10 times and centrifuged for 15 min. at 25,000x*g*. The supernatant was not capable of sulphate hydrolysis. Therefore, the enzymatic activity must be associated with the solid part of the feces, e.g. bacterial particles.

DISCUSSION

In vitro differences between conventional and germ-free rat caecal contents have been shown for the hydrolysis of the sulphate esters of several steroids (9). Procedures for in vivo studies of the effects of gut microflora on the metabolism of xenobiotics are discussed by Illing (14). Studies by Grantham et al. (12) on the metabolism of the carcinogen N-hydroxy-N-2-fluorenylacetamide showed substantial amounts of conjugated metabolites, glucuronides as well as sulphates, present in the caecum and faeces of germ-free rats. In control conventional rats, however, the major portion of metabolites occured in the non-conjugated form (12). The significance of intestinal hydrolysis of conjugated compounds towards an enterohepatic cycle was illustrated by the reduction of the recirculation of norethisterone by antibiotics in the rat (1).

Bacteria-dependent hydrolysis of T_3S by rat intestinal contents was reported by Closon in 1964 (2). In the present study we demonstrated the presence of iodothyronine sulfatase activity in faecal suspensions from conventional rats or healthy humans using five iodothyronine sulphates chemically synthesised in our laboratory. No sulphate-hydrolysis was observed in faecal suspensions of germ-free rats or intestine-decontaminated rats, although in the latter a rest flora of facultatively anaerobic bacteria remained. This indicates that the sulphatase activity of faecal suspensions from conventional rats or healthy humans is of bacterial origin. In previous studies we described that the obligately anaerobic bacterial strains from human faecal or rat caecal microflora were capable of hydrolyzing iodothyronine sulphates in vitro (4, 6). No iodothyronine sulphatase activity, however, could be demonstrated in aerobic or facultatively anaerobic bacteria. Therefore, considering the higher numbers of strictly anaerobes in the intestinal tract we conclude that the intestinal hydrolysis of iodothyronine sulphates is executed by anaerobic bacteria or their products. The reported microbial iodothyronine sulphatase differs from the commercially available sulphatase from Abalone entrails as the latter is incapable of the hydrolysis of T_4S and rT_3S (19).

Hydrolysis of steroid glucuronides by caecal contents of conventional rats in vitro was de-

scribed by Eriksson and Gustafsson (9). They noticed that small amounts of glucuronidase activity were still present in caecal contents and in intestinal mucosa from germ-free rats. It was suggested that intestinal glucuronidase activity originated both from intestinal bacteria and from cellular debris of mucosal origin liberated in the lumen (9). This concept is supported by the findings of a greatly reduced glucuronidase activity in colonic contents of rats after oral administration of antibiotics (15). Williams et al. (23) reported an optimum pH of 5 for the β-glucuronidase isolated from germ-free rat cecal contents which is typical for mammalian β-glucuronidase. However, an optimum pH of 6 was found for the β-glucuronidase isolated from conventional rat caecal contents, a value characteristic for the bacterial enzyme (23). Attempts to characterise the bacteria responsible for β-glucuronidase production showed that Escherichia coli and Clostridium, Peptostreptococcus and Staphylococcus species liberated the enzyme (11, 13, 17). We have found in vitro hydrolysis of T_3G by two anaerobic bacterial strains isolated from human faecal flora (3). In the present study we observed hydrolysis of T_3G by human or CV rat faecal suspensions. In GF or ID rat faecal suspensions this activity was greatly reduced. It might be concluded that the remaining glucuronidase activity is produced by the gastro-intestinal mucosal cells.

The possible contribution of the intestinal hydrolysis of T_3S or T_3G towards establishing an enterohepatic cycle for T_3 was already suggested by Closon (2). In this study we have demonstrated the in vitro hydrolysis of all iodothyronine sulphates and T_3G by intestinal contents making an enterohepatic circulation for different iodothyronines possible. Further in vivo experiments have to be performed to assess the existence and the importance of such a circulation and to evaluate its contribution towards the metabolism of iodothyronines.

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Appendix paper 6

ON THE ENTEROHEPATIC CYCLE OF TRIIODOTHYRONINE IN RATS; IMPORTANCE OF THE INTESTINAL MICROFLORA

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Summary

Until 70 h after a single iv injection of 10 uCi [^{125}I]triiodothyronine (T_3), normal rats excreted 15.8±2.8 % of the radioactivity with the feces and 17.5±2.7 % with the urine, while in intestine-decontaminated rats fecal and urinary excretion over this period amounted to 25.1±7.2 % and 23.6±4.0 % of administered radioactivity, respectively (mean±SD, n=4). In fecal extracts of decontaminated rats 11.5±6.8 % of the excreted radioactivity consisted of T_3 glucuronide (T_3G) and 10.9±2.8 % of T_3 sulfate (T_3S), whereas no conjugates were detected in feces from normal rats. Until 26 h after ig administration of 10 uCi [^{125}I]T_3, integrated radioactivity in blood of decontaminated rats was 1.5 times higher than that in normal rats. However, after ig administration of 10 uCi [^{125}I]T_3G or [^{125}I]T_3S, radioactivity in blood of decontaminated rats was 4.9- and 2.8-fold lower, respectively, than in normal rats. The radioactivity in the serum of control animals was composed of T_3 and iodide in proportions independent of the tracer injected, while T_3 conjugates represented <10 % of serum radioactivity. These results suggest an important role of the intestinal microflora in the enterohepatic circulation of T_3 in rats.

Thyroxine (T_4) is the main secretory product of the thyroid gland in animals and humans. Since it possesses little intrinsic biological activity, T_4 is generally considered a prohormone. Most T_4 is metabolized by deiodination, resulting in the formation of 3,3',5-triiodothyronine (T_3), the bioactive form of thyroid hormone, and 3,3',5'-triiodothyronine (reverse T_3, rT_3), which is biologically inactive (1,2). Both T_3 and rT_3 undergo further deiodination to the inactive metabolite 3,3'-diiodothyronine (3,3'-T_2).

Apart from enzymatic deiodination, iodothyronines are also substrates for UDP-glucuronyltransferases and phenol sulfotransferases (1,2). Deiodination of T_3 by rat liver (type I) iodothyronine deiodinase is facilitated by sulfation of the 4'-hydroxyl group of the substrate (2). Therefore, the proportions of T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) in bile vary under different conditions (3-8). In normal rats, mainly T_3G is excreted, and only small amounts of T_3S are found in bile. In thyroidectomized rats, which have low hepatic deiodinase activities (2), and after treatment of normal rats with the type I deiodinase inhibitor 6-propyl-2-thiouracil (PTU), biliary T_3S excretion is selectively increased (3-6,8). This implies that in the intact rat deiodination of T_3 by the type I deiodinase is also facilitated by sulfation (8).

In vitro, conjugates of T_3 are hydrolyzed by sulfatase and glucuronidase activities of obligately anaerobic intestinal bacteria (9,10). In contrast to

rapid deconjugation by diluted fecal suspensions from normal rats, no hydrolysis of T_3S and little hydrolysis of T_3G occur in such suspensions from germ-free and intestine-decontaminated rats (11). In accordance with other conjugated compounds, T_3 conjugates are poorly resorbed from the gastrointestinal tract of rats in contrast to T_3 itself (12-14). The presence of anaerobic intestinal bacteria, therefore, seems a prerequisite for the reabsorption of T_3 excreted as glucuronide and sulfate conjugates in the bile. The existence of such an enterohepatic circulation for iodothyronines in rats as well as in other animals and humans has been postulated by several authors (15-22) but negated by others (23,24).

In the present study we evaluated the effects of intestinal decontamination by antibiotics on the possible enterohepatic circulation of T_3 in rats. Firstly, radioactive products were analysed in feces collected from normal and decontaminated rats after intravenous (iv) injection of labeled T_3. Secondly, gastrointestinal absorption of T_3 and its conjugates was studied after intragastric (ig) administration to normal and decontaminated rats.

Materials and Methods

Materials

Ampicillin was obtained from Beecham (Heppignies, Belgium), neomycin from Pharmachemie (Haarlem, The Netherlands), and polymyxin B from Pfizer (Brussels, Belgium). [3'-^{125}I]T_3 was purchased from Amersham (Amersham, UK); for iv injection it was dissolved in 0.01 M NaOH in saline. Synthetic [^{125}I]T_3S and [^{125}I]3,3'-T_2S and biosynthetic [^{125}I]T_3G and [^{125}I]3,3'-T_2G were prepared according to previously described methods (9,25). For ig administration, compounds were dissolved in 1 ml 0.5 M $NaHCO_3$ to avoid hydrolysis by gastric acid. All other reagents were of laboratory grade.

Animals and diet

Three months-old male Wistar rats (TNO, Rijswijk, The Netherlands) were fed sterile pellet (SPF Pellet, Hope Farms, Woerden, The Netherlands). The experimental and control groups each consisted of 4 animals. During the experimental period rats were kept in metabolic cages with mesh wire bottoms to facilitate the separate collection of feces and urine and to reduce coprophagy. Intestinal decontamination was carried out by treatment of rats with antibiotics in the drinking water (1 g ampicillin, 1 g neomycin and 1 g polymyxin B per liter) from 12 days prior to the start until the end of the experiments. In the first experiment, feces was collected up to 70 h after the iv injection of 10 uCi [^{125}I]T_3. In the second experiment, 10 uCi [^{125}I]T_3, [^{125}I]T_3G or [^{125}I]T_3S were administered ig by means of a stomach tube. Blood samples (0.5 ml) were drawn from the tail vein at regular time intervals. Radioactivity was counted in samples of feces, urine and blood using a Nuclear Enterprises NE1600 gamma counter with 70 % efficiency.

Extraction of fecal material

Fecal material was extracted with 2-4 volumes of ethanol. The suspension was vortexed for 1 min and subsequently centrifuged for 10 min at 3000 x g. The supernatant was evaporated under a stream of N_2 at 50 C. Prior to HPLC analysis the residue was dissolved in 100 ul of mobile phase.

Isolation and quantitation of iodothyronine metabolites

Reversed-phase HPLC was performed on a 10 x 0.3 cm CP^{tm}-Spher C18 column (Chrompack, Middelburg, The Netherlands) with a 15-40 % concave gradient (nr. 7) of acetonitrile in 0.02 M ammonium acetate (pH 4), programmed by a model 680 automated gradient controller and delivered by a combination of model 6000A and 510 solvent pumps (Waters, Milford, MA, USA). The gradient was started at the time of injection and was completed in 30 min, followed by a 15 min isocratic elution with the 40:60 acetonitrile-ammonium acetate mixture. Solvent flow was

0.8 ml/min, and 0.5 min fractions were collected and counted for radioactivity. The HPLC system was calibrated using radioactive T_3, 3,3´-T_2, T_3S, 3,3´-T_2S, T_3G and 3,3´-T_2G as references (10).

Identification of serum radioactivity

Blood samples were centrifuged, and sera were stored at -20 C until further analysis. For this purpose, 0.25 ml of serum was mixed with 0.75 ml 0.3 N HCl and applied to a short Sephadex LH-20 column (0.75 ml bed volume), equilibrated in 0.1 N HCl. By successive elution with 0.1 N HCl, H_2O, and ethanol/0.1 N NaOH (1/1, vol/vol), fractions were obtained containing iodide, conjugates, and nonconjugated iodothyronines, respectively.

Intestinal flora

Two days before the experiments the composition of the intestinal flora was evaluated by microscopic examination of Gram-stained fecal smears and by culturing of fecal dilutions under aerobic and anaerobic conditions according to Hazenberg et al. (26). Instead of the described anaerobic medium Schaedler broth (Oxoid, Basingstoke, UK) was used.

Results

Clearance of iv injected [^{125}I]T_3

After iv administration of 10 uCi [^{125}I]T_3 to normal rats 15.8±2.8 % (mean±SD, n=4) of radioactivity was excreted with the feces and 17.5±2.7 % with the urine in 70 h. Over the same period, decontamined rats excreted 25.1±7.2 % of injected radioactivity with the feces and 23.6±4.0 % with the urine (Fig. 1). HPLC analysis of fecal extracts from normal rats showed that 7.6±3.8 % of excreted radioactivity consisted of iodide, 52.5±9.7 % of T_3, and 5.8±2.1 % eluted from the column with the same retention time as 3,3´-T_2. No conjugates of T_3 or 3,3´-T_2 were detected. In fecal extracts of decontaminated rats 12.5±8.7 % of excreted radioactivity consisted of iodide, 29.6±6.4 % of T_3, 11.5±6.8 % of T_3G, and 10.9±2.8 % of T_3S; 21.2±4.7 % eluted in the position of 3,3´-T_2 (Fig. 2). The remainder of fecal radioactivity in both groups was not eluted in well-defined peaks.

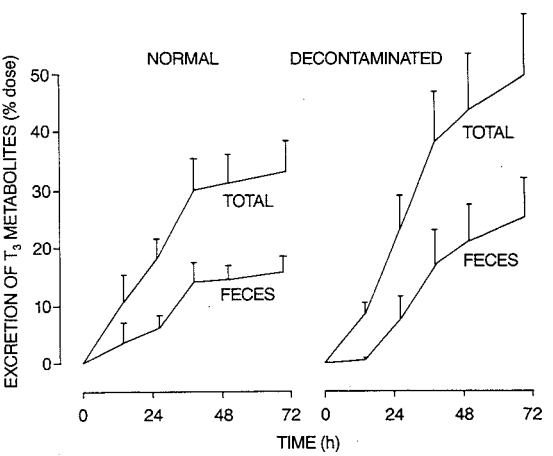

FIG.1

Cumulative excretion of radioactivity after i.v. injection of [^{125}I]T_3 to normal or decontaminated rats. The data are depicted as mean±SD percentage of the dose administered to groups of 4 rats.

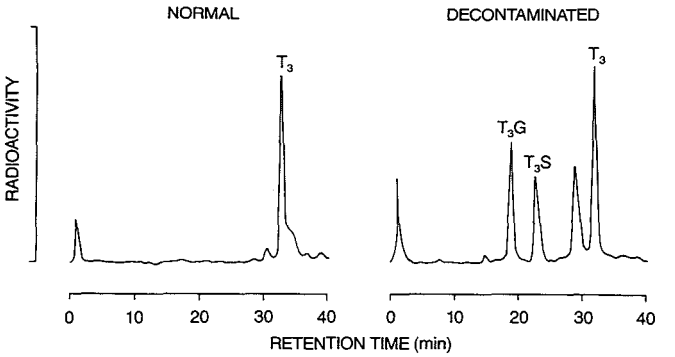

FIG.2

HPLC of radioactivity from pooled feces collected up to 38 h after iv injection of [^{125}I]T_3 to normal or decontaminated rats.

Absorption of ig administered T_3, T_3G and T_3S

Figure 3 shows the radioactivity in blood at regular intervals until 26 h after ig administration of ^{125}I-labeled T_3, T_3G or T_3S to normal and decontaminated rats. After ig administration of [^{125}I]T_3, radioactivity in blood was consistently higher in decontaminated rats than in controls. However, the reverse was the case after ig administration of the conjugates. As a measure of absorption, the area under the curve of blood radioactivity versus time (AUC) was estimated. In decontaminated rats given ig [^{125}I]T_3 the AUC was 1.5 times higher than in the controls. In contrast, the AUC in decontaminated rats that received ig [^{125}I]T_3G or [^{125}I]T_3S was 4.9- or 2.8-fold lower, respectively, than in the corresponding controls. Absorption of radioactivity was similar in normal rats receiving ig [^{125}I]T_3 or [^{125}I]T_3G but was substantially less in those treated with [^{125}I]T_3S.

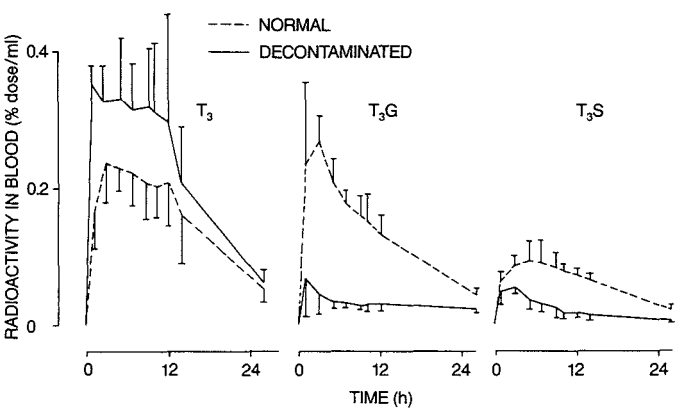

FIG.3

Radioactivity in blood (mean+SD % dose/ml) after ig administration of T_3, T_3G or T_3S to normal or decontaminated rats (n=4).

Figure 4 shows the Sephadex LH-20 fractionation of serum radioactivity in combined samples obtained 0-6 h after ig administration of $[^{125}I]T_3$ or $[^{125}I]T_3G$ or 0-8 h after ig administration of $[^{125}I]T_3S$ to either normal or decontaminated rats. The results show that T_3 and I^- were the major radioactive compounds in the circulation irrespective of which tracer was administered and whether or not rats were pretreated with antibiotics. Less than 10 % of serum radioactivity eluted from Sephadex LH-20 in the conjugate fraction, and this was not further investigated. The serum radioiodide levels determined on Sephadex LH-20 were confirmed by measurements of the TCA-soluble radioactivity (not shown). The identity of radioactivity eluting from Sephadex LH-20 in the iodothyronine fraction was established by HPLC as authentic T_3. The low serum radioactivity in decontaminated rats after ig administration of $[^{125}I]T_3S$ consisted predominantly of I^-.

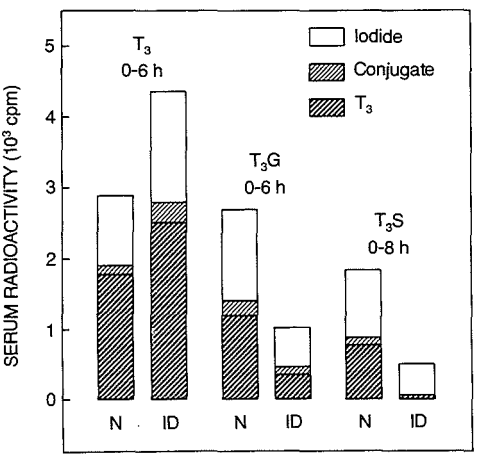

FIG.4

Sephadex LH-20 fractionation of radioactivity in 0.25 ml pools of serum collected 0-6 h after ig administration of $[^{125}I]T_3$ or $[^{125}I]T_3G$ or 0-8 h after ig administration of $[^{125}I]T_3S$ to normal (N) or intestine-decontaminated (ID) rats.

The HPLC analyses of fecal extracts from rats that had received ig $[^{125}I]T_3G$ are shown in Fig. 5. These results demonstrate that virtually all radioactivity excreted by normal rats consisted of nonconjugated T_3, whereas part of the radioactivity excreted by decontaminated rats still existed in the form of T_3G. In other experiments (not shown) it was found that after ig administration of $[^{125}I]T_3S$ to normal rats only nonconjugated T_3 was identified in the feces, whereas in decontaminated rats no deconjugation was observed and all fecal radioactivity appeared in the form of intact T_3S.

Intestinal microflora

Total numbers of obligately anaerobic and facultatively anaerobic bacteria in feces of normal rats were 1.5×10^{10} (range 1.0×10^{10} - 1.8×10^{10}) and 7.4×10^6 (range 1.9×10^6 - 1.6×10^7) per gram fecal material, respectively. Decontaminated rats were selected for the experiments if Gram-stained fecal smears were negative for the presence of microorganisms. Culturing of feces showed that the numbers of obligately anaerobic bacteria in feces of decontaminated rats were reduced to $<10^2$ per gram (detection level) and that facultatively anaerobic bacteria were present in numbers ranging from $<10^2$ to 4.5×10^4 per gram fecal material.

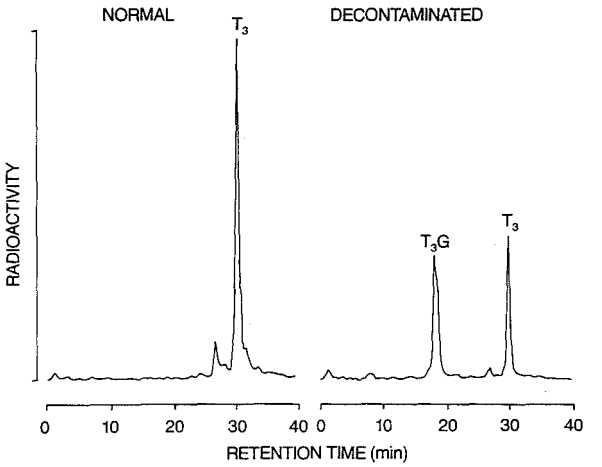

FIG.5

HPLC of radioactivity from pooled feces collected up to 26 h after ig administration of [^{125}I]T_3G to normal or decontaminated rats.

Discussion

Thyroid hormones are conjugated at the phenolic hydroxyl group with either sulfate or glucuronic acid. Conjugated and to some extent also nonconjugated iodothyronines are excreted in the bile (3-8,22). Roche and coworkers were the first to identify T_3S and T_3G in the bile of thyroidectomized rats after iv or subcutaneous (sc) injections of radiolabeled T_3 (3,4). We have recently also studied the biliary clearance of iv injected tracer T_3 in rats (8). In the first 4 h of bile sampling, on average 74 % of excreted radioactivity was in the form of T_3G, only 8 % as T_3S and still lower amounts were excreted as nonconjugated T_3 and iodide (8). Direct secretion of iodothyronines into the gut via a nonbiliary pathway has also been postulated (15,24). In recent studies using a mathematical model of T_3 turnover in intact rats a significant mesenteric secretion of T_3 was implied (21,22). As yet, however, direct experimental evidence for this excretory pathway is lacking.

It has been shown that T_3S and T_3G are poorly absorbed from rat intestine in contrast to nonconjugated T_3 (12-14). This is in accordance with the findings that in mesenteric venous blood no conjugates of T_3 could be detected (22). In normal rat feces no conjugated T_3 is found after iv injection of radiolabeled T_3 (Fig. 2; refs. 22,27). Under steady-state conditions using constant sc or intraduodenal infusion of tracer T_3, conjugated T_3 is found in the small but not in the large intestine (21,22).

Extensive deconjugation of T_3S and T_3G has been observed in fecal suspensions from normal rats (10,11,28). However, no hydrolysis of T_3S and little hydrolysis of T_3G were found in fecal suspensions from germ-free or decontaminated rats (10,11). Several obligately anaerobic bacterial strains capable of hydrolysing T_3S and T_3G have been isolated from rat and human intestinal microflora (9,10). Therefore, biliary excretion of conjugated T_3 is not necessarily an irreversible pathway for the elimination of T_3, since liberation of the hormone in the gut by bacterial glucuronidases and sulfatases would enable its subsequent reabsorption. The existence of such an enterohepatic circulation in rats and humans has been discussed by several authors but has not been estab-

lished (15-24). This putative cycle could contribute significantly to the so-called slowly-equilibrating extravascular T_3 pool deduced from plasma T_3 turnover studies (29).

It was the purpose of this study to further characterize the enterohepatic circulation of T_3 in experiments using normal and decontaminated animals. In the feces of decontaminated rats, but not in the feces of normal rats, T_3G and T_3S were found after a single iv injection of radiolabeled T_3. Fecal excretion of radioactivity until 70 h was 1.5 times higher in decontaminated than in normal rats. The almost complete elimination of intestinal bacteria in the decontaminated rats lead to a diminished intestinal capacity to hydrolyse T_3 conjugates. As T_3 is mainly excreted in the conjugated from in the bile, these conjugates will not be absorbed and will thus appear in the feces.

Our findings in both normal and decontaminated rats are in agreement with previous studies, showing equal clearance of radioactivity with the feces and the urine after iv administration of radiolabeled T_3 to normal rats (27,30). The increased excretion of radioactivity in the urine of decontaminated rats compared with controls in our study was surprising, since the interruption of the enterohepatic cycle would be expected to reduce the availability of T_3 for deiodination. An explanation for this phenomenon may be the possible reduction in bile-flow in decontaminated rats due to exhaustion of bile acids. This may lead to a compensatory increase in alternative, non-biliary pathways of T_3 metabolism, i.e. deiodination and subsequent urinary I^- excretion. Furthermore, decontaminated rats probably have longer intestinal transition times, resulting in a more efficient resorption of any T_3 liberated in the gastro-intestinal tract (see below). The data in Fig. 5 demonstrate that hydrolysis of T_3G in the intestine is not completely prevented by decontamination probably due to the glucuronidase activity of mucosal cells (11,31).

Chung et al. (32) found a higher absorption of T_4 in the isolated colon from germ-free rats compared with normal rats, but they found no difference in T_4 absorption in the isolated ileum of these animals. A higher absorption of T_4 in the isolated ileum from germ-free rats was found in the absence of intestinal contents. It was suggested that resorption of T_4 was partly prevented by binding to indigestible food particles and bacteria (32). In our studies, rats had free access to food and drinking water. We have found that radioactivity in blood is higher after ig administration of radiolabeled T_3 to decontaminated rats compared with normal rats. This may be explained analogous to the results obtained by Chung et al. for T_4 (32). Also, a slower intestinal transition time, as has been documented for germ-free animals (33), may result in a better absorption of T_3.

Decreased intestinal glucuronidase and sulfatase activities after treatment with antibiotics explain the 4.9- and 2.8-fold reduced absorption of radioactivity after ig administration of T_3G and T_3S, respectively (Fig. 3). Very little serum radioactivity was recovered in the conjugate fractions, and almost all radioactivity eluted as nonconjugated T_3 and I^- (Fig. 4). The effect of decontamination on the resorption of T_3 from T_3G represents an underestimation of the actual intestinal hydrolysis of the conjugate due to residual glucuronidase activity of the mucosa (see above). Physiologically, T_3G is a more important intermediate than T_3S in the enterohepatic circulation of T_3, since under normal conditions T_3G is the predominant biliary excretion product (8) and, moreover, resorption of T_3 is greater from T_3G than from T_3S (Fig. 3).

These two studies again emphasize the relevance of intestinal hydrolysis of iodothyronine conjugates excreted in the bile for the enterohepatic circulation of iodothyronines in the rat. This activity is predominantly of microbial origin as it is virtually absent after intestinal decontamination. Further studies are required to establish the importance of the enterohepatic circula-

tion to overall thyroid hormone kinetics and metabolism.

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SUMMARY

Thyroid hormone plays an important role in the development and metabolic processes of higher organisms. The metabolism of the hormone in mammals involves several organs or cell systems. In principle, iodothyronines may undergo four metabolic processes, of which deiodination and conjugation are considered major reactions and ether link cleavage and oxidative deamination and decarboxylation are considered minor metabolic processes.

In this thesis the relevance of an enterohepatic circulation for the metabolism of triiodothyronine is described. The manuscript consists of two sections. In section I relevant data from our research together with the current knowledge of peripheral thyroid hormone metabolism are integrated. Section II consists of publications dealing with the factual studies and data.

In incubations with isolated rat hepatocytes, the deiodination of 3,3',5-triiodothyronine (T_3) by the type I deiodinase enzyme is greatly facilitated after sulfation. In order to establish the physiological relevance of the pathway of successive sulfation and deiodination, we have studied the biliary clearance of radioactive T_3 in pentobarbital-anesthetized rats. Until 4 h after intravenous (iv) administration of T_3, on average 22% of the radioactivity has been excreted in the bile, predominantly in the form of T_3 glucuronide (T_3G). In rats treated with the type I deiodinase inhibitor 6-propyl-2-thiouracil (PTU), biliary excretion of radioactivity has increased to 36% of the dose in 4 h, which is entirely explained by an increased excretion of T_3 sulfate (T_3S) and 3,3'-diiodothyronine sulfate (3,3'-T_2S), while excretion of T_3G is not influenced by PTU. This effect of PTU can be prevented by additional treatment of the rats with 2,6-dichloro-4-nitrophenol (DCNP), implicating the involvement of a DCNP-sensitive phenol sulfotransferase in the sulfation of iodothyronines (*Appendix paper 1*).

In contrast to nonconjugated iodothyronines, which are easily absorbed from the intestinal tract, the hydrophilic nature of their conjugates precludes intestinal absorption. However, biliary excretion of iodothyronine conjugates is not necessarily a pathway for irreversible elimination, if intestinal hydrolysis of these glucuronides and sulfates results in the release and subsequent absorption of the aglycon.

Several strains of obligately anaerobic bacteria belonging to the major residents of the intestinal microflora of man and rat have been shown to produce iodothyronine sulfatase or glucuronidase activities *in vitro* (*Appendix*

papers 2-4). These activities have also been found in diluted fecal suspensions of humans and conventional (CV) rats, but no hydrolysis of the iodothyronine sulfates occurs in fecal suspensions of germ-free (GF) and intestine-decontaminated (ID) rats. A residual hydrolysis of T_3G in these suspensions is presumably due to glucuronidase activity originating from gastrointestinal mucosal cells (*Appendix paper 5*).

The fecal excretion of radioactivity after a single iv injection of radiolabeled T_3 has been studied in CV as well as ID rats. It amounts to 16% of the dose in 70 h in CV rats, while in this time ID rats have excreted 25% of the injected radioactivity. The excreted radioactivity of CV rats is only composed of T_3, whereas up to 22% of the excreted radioactivity of ID rats consists of conjugates of T_3 (*Appendix paper 6*).

The almost complete elimination of anaerobic intestinal bacteria in the decontaminated rats leads to a diminished intestinal capacity to hydrolyze T_3 conjugates. Since T_3 is mainly excreted in the conjugated form in the bile, these conjugates will not be absorbed and will thus appear in the feces. The reduced absorption of T_3 in ID rats as compared with CV rats after the intragastric (ig) administration of radiolabeled T_3G or T_3S supports the above conclusions (*Appendix paper 6*).

In conclusion, intestinal hydrolysis of iodothyronine conjugates excreted in the bile is relevant for the enterohepatic circulation of iodothyronines in the rat. This activity is predominantly of microbial origin as it is virtually absent after intestinal decontamination. The importance of the enterohepatic circulation for overall thyroid hormone kinetics and metabolism, not only in rats, but also in humans still has to be established.

SAMENVATTING

De schildklier, een orgaan van gemiddeld 40 gram en gelegen in de hals, reguleert de basale stofwisseling en stimuleert groei en ontwikkeling. Dit orgaan scheidt de stof thyroxine aan het bloed af. Het thyroxine molecule bestaat uit een skelet van kool-waterstof verbindingen waaraan vier jodium atomen gekoppeld zijn. Daarom wordt het ook wel afgekort weergegeven als T_4. Deze stof is echter biologisch inactief. Pas nadat er 1 jodium atoom afgesplitst wordt, ontstaat het biologisch actieve hormoon 3,3',5-trijodothyronine of T_3. 20% van de hoeveelheid T_3 in het bloed is afkomstig uit de schildklier. De overige 80% wordt gevormd door omzetting van het prohormoon T_4 naar T_3 in buiten de schildklier gelegen weefsels, welke daarom ook wel perifere weefsels genoemd worden. Dit omzettingsproces wordt dejodering genoemd en kan door bepaalde eiwitten of enzymen versneld worden. De totale hoeveelheid T_3 in het bloed wordt normaliter binnen nauwe grenzen gehouden door enerzijds aanmaak en anderzijds afbraak.

Het T_3 kan worden afgebroken door enzymatische dejodering in de perifere weefsels, bijvoorbeeld in de lever. Hierdoor ontstaan onder andere de biologisch inactieve stoffen 3,3'-dijodothyronine of 3,3'-T_2 en jodide. Ook kan T_3 door enzymen gekoppeld worden aan glucuronzuur of aan sulfaat. Hierdoor ontstaan respectievelijk het T_3 glucuronide (T_3G) of het T_3 sulfaat (T_3S). Deze processen worden ook wel conjugatie reacties genoemd. De biochemische eigenschappen van het T_3 veranderen door deze reacties zodanig, dat het molecule makkelijker in water oplosbaar wordt en beter door de lever in de gal kan worden uitgescheiden. Door onderzoek in gekweekte rattelevercellen is reeds aangetoond dat het molecule T_3S beter gedejodeerd kan worden dan T_3 zelf. Om te onderzoeken of dit ook in levenden lijve zo gebeurt, werd radioactief gemerkt T_3 ingespoten in de bloedbaan van ratten. Vervolgens werd de uitscheiding van radioactiviteit in de gal bestudeerd. Na behandeling van deze ratten met 6-propyl-2-thiouracil (PTU), een stof die het dejoderende enzym in onder andere de lever remt, nam de uitscheiding van radioactiviteit in de gal toe. Dit viel te verklaren op basis van een toename van de uitscheiding van T_3S en 3,3'-T_2 sulfaat, terwijl de uitscheiding van het in de gal in overmaat aanwezige T_3G niet veranderde. Naar aanleiding van deze resultaten kunnen we concluderen dat ook onder fysiologische omstandigheden het T_3 in gesulfateerde vorm preferentieel gedejodeerd wordt.

T_3G en T_3S worden in de darm slecht geabsorbeerd, terwijl T_3 juist

gemakkelijk in de darm wordt opgenomen. Onderzocht werd of bacteriën uit de darm in staat waren om deze verbindingen van T_3 met glucuronzuur of met sulfaat te verbreken. De darm van gezonde mensen en vele andere dieren wordt bewoond door grote aantallen bacteriën (miljarden per gram darminhoud). Uit een aantal experimenten bleek dat relatief geringe hoeveelheden bacteriën, afkomstig uit de excreta van mensen en ratten, in korte tijd grote hoeveelheden T_3G en T_3S konden afbreken tot T_3 en respectievelijk glucuronzuur of sulfaat. De daarvoor verantwoordelijke bacterie-stammen werden geïdentificeerd. Zij behoren alle tot de zogenaamde obligaat anaerobe darmflora, dat wil zeggen dat deze bacteriën alleen in zuurstof-loos milieu goed gedijen. Waarschijnlijk oefenen deze micro-organismen hun effekt uit door middel van enzymen.

De hoeveelheid darmbacteriën kon fors terug gebracht worden door ratten gedurende enkele dagen te behandelen met antibiotica in het drinkwater. Suspensies van faeces van deze zogenaamde gedecontamineerde ratten of ID ratten hydrolyseerden T_3S niet en T_3G slechts in geringe mate. Dit laatste werd waarschijnlijk veroorzaakt door enzymen, welke door darmwandcellen met de ontlasting zijn uitgescheiden.

Na een injectie van radioactief gemerkt T_3 in de bloedbaan werd de uitscheiding van radioactiviteit in de faeces van CV en ID ratten bestudeerd. De ID ratten scheidden in dezelfde tijdsspanne meer radioactiviteit in de ontlasting uit dan CV ratten. In de ontlasting van de ID ratten werd ook nog T_3G en T_3S gevonden, terwijl de ontlasting van CV ratten alleen maar T_3 bevatte. Door de antibiotica worden de anaerobe darmbacteriën vrijwel compleet vernietigd. Daardoor kunnen de in de gal uitgescheiden conjugaten van T_3 niet meer volledig gehydrolyseerd worden en verschijnen in de faeces.

Wanneer radioactief gemerkt T_3G en T_3S juist direct in de maag van CV en ID ratten werden toegediend, dan bleek dat de absorptie van T_3 in ID ratten minder was dan die in CV ratten. Wanneer radioactief gemerkt T_3 in de maag werd toegediend namen ID ratten in dezelfde tijd zelfs meer radioactiviteit op dan CV ratten. Ook hieruit blijkt dus weer dat conjugaten van T_3 eerst gehydrolyseerd moeten worden. Vervolgens kan het vrijgekomen T_3 opgenomen worden.

Het T_3 kan dus een kringloop volgen welke in de bloedsomloop begint en eindigt, met als tussenstations de lever, gal en darm. Deze kringloop wordt ook wel enterohepatische kringloop genoemd. Het lijkt aan de hand van de resultaten gerechtvaardigd te concluderen, dat zo'n enterohepatische kringloop voor T_3 bestaat in de rat. Echter de stand van zaken laat het nog niet toe deze conclusie door te trekken naar de mens, alhoewel er ook indirecte bewijzen bestaan voor het bestaan van een enterohepatische kringloop van T_3 bij de mens. Zo is bij patiënten met bepaalde darmziekten, of die met bepaalde medicijnen behandeld worden de opname van T_3 in de darm verminderd. Bij

deze patienten wordt een verhoogde produktie van T_3 gevonden om het verlies via de darm te compenseren. Mogelijk kan de enterohepatische kringloop ook als reservoir voor schildklierhormoon dienen. Verlies van het hormoon met de ontlasting zou dan alleen optreden, wanneer het in overmaat in het lichaam voorhanden is.

NAWOORD

Velen hebben een bijdrage geleverd aan de totstandkoming van dit proefschrift. Zonder daarin anderen te kort te willen doen zou ik enkelen met name willen noemen.

Marten Otten gaf de aanzet tot het onderzoek, dat van 1982 tot en met 1989 met tussenpozen op de Medische Faculteit van de Erasmus Universiteit te Rotterdam werd verricht.

De experimenten werden deels op het Laboratorium voor Schildklierhormoon Onderzoek van de afdeling Inwendige Geneeskunde III onder leiding van Theo Visser en Prof. Dr. G. Hennemann en deels op het Laboratorium van de afdeling Medische Microbiologie (later afdeling Immunologie) onder leiding van Maarten Hazenberg uitgevoerd.

De technische bijdragen van Fred Bonthuis, René van der Hul, Ton Oosterlaken, Alma Pennock-Schröder, Yvonne Steinvoort en de hulp van Roel Docter en het personeel van het Laboratorium voor Schildklierhormoon Onderzoek waren daarbij onontbeerlijk.

De figuren werden verzorgd door medewerkers van de Audiovisuele Dienst van de Erasmus Universiteit.

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