ASPECTS OF PERIPHERAL THYROID HORMONE METABOLISM

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Aspecten van het perifere schildklierhormoon metabolisme

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CONTENTS

LIST OF ABBREVIATIONS

cAMP	cyclic adenosine 3',5'-monophosphate
D I/II	deiodinase type I/II
DCNP	2,6-dichloro-4-nitrophenol
diac	3,3'-diiodothyroacetic acid
DTT	dithiotreitol
FCS	fetal calf serum
GSH	glutathione (reduced)
IRD I/II	inner ring deiodinase I/II (catalysing 3- or 5-deiodination)
K_m	Michaelis constant
NBCS	new-born calf serum
ORD I/II	outer ring deiodinase I/II (catalysing 3'- or 5'-deiodination)
PCP	pentachlorophenol
PTU	6-propyl-2-thiouracil
$R_{(n)}$	reaction number corresponding with Table I and fold-out in back of the manuscript
RIA	radioimmunoassay
rT_3	3,3',5'-triiodothyronine (reverse T_3)
3'-T_1	3'-iodothyronine
3,3'-T_2	3,3'-diiodothyronine
3,3'-T_2S	3,3'-T_2 sulfate
T_3	3,3',5-triiodothyronine
T_3G	T_3 glucuronide
T_3S	T_3 sulfate
T_4	3,3',5,5'-tetraiodothyronine (thyroxine)
T_4G	T_4 glucuronide
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin
tetrac	3,3',5,5'-tetraiodothyroacetic acid
triac	3,3',5-triiodothyroacetic acid
TSH	thyroid-stimulating hormone (thyrotropin)
TU	2-thiouracil
V_{max}	maximal velocity

PREFACE

The research into thyroid function has a long history. The recognition of goiter as pathology of the thyroid gland dates back to the ancient world of Rome and Greece and possibly even to the early history of chinese medicine. In an excellent review of the historical aspects of the discovery of thyroid hormones and their biological action (1) Pitt-Rivers describes the growing awareness of the significance of iodine for thyroid function early in the 19th century.

The actual presence of organic iodine in the thyroid gland was demonstrated for the first time in 1896 by Baumann who called his concentrate of a thyroid extract "Iodothyrin". In 1914 Kendall isolated a crystalline material from thyroid hydrolysates and named it "Thyroxin" after thyroxindole, since he believed this substance to be an indole derivative. The compound proved to have biological activity in hypothyroid man and animals. The actual structure formula of the thyroxine molecule was disclosed by Harington in 1926. Only in 1952 the existence of 3,3',5-triiodothyronine was simultaneously demonstrated in beef thyroid and human serum by Gross and Pitt-Rivers and in rat thyroid by Roche et al. At the same time it appeared that this triiodothyronine was about three times as potent as thyroxine in the goiter prevention assay in rats. The original postulate by Gross and Pitt-Rivers (1953) that triiodothyronine originates by degradation of thyroxine in peripheral tissues and constitutes the principal active thyroid hormone was established by Sterling and Braverman in as late as 1970.

Since this time thyroid research has explosively expanded in many fields, such as thyroidal synthesis and secretion of iodothyronines, feedback regulations of the hypothalamic-pituitary-thyroid axis, goiter etiology and autoimmune mechanisms with associated hyper- and hypothyroidism, thyroid cancer, vascular transport proteins, nuclear thyroid hormone receptors with post-receptor biological effects and the broad field of peripheral thyroid hormone metabolism. This last subject in its turn can be subdivided into smaller areas, such as kinetic studies with isotope labeled thyroid hormones ,the processes involved in membrane transport, as well as the subcellular distribution of either locally pro-

duced or plasma-borne iodothyronines. But also, and this represents an important section in this thesis, the intracellular metabolism of the multiple thyroxine-derived metabolites. It is obvious that the above list is far from complete, but it gives an impression of the wide scope of current thyroid research.

This thesis is a compilation of various investigations in the field of peripheral thyroid hormone metabolism. The main objective of the presented work has been to obtain more knowledge about the physiology of the various metabolic pathways of iodothyronines. This objective was chosen in the hope to contribute to a better understanding of the complex metabolic adaptations in iodothyronine metabolism, known to be induced by several drugs, dietary changes or diseases. At first sight the rather diverse subjects of investigation in Section II of this thesis bear no apparent relation. However, in the next chapters a tentative linkage of the various results will be discussed.

Essentially three different lines of research have been followed. The first line consists of the investigation of dietary influences on peripheral thyroid hormone metabolism. Since the observation of Spaulding et al (2) that 800 kCal of dietary carbohydrates nullified the changes of human serum 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (reverse T_3) normally seen with hypocaloric nutrition, multiple reports mentioned similar effects with a variety of different dietary compositions, all paying special attention to the effect of carbohydrates. The lack of detailed information about the influences of the other two major components of food i.e. proteins and fat, prompted us to design the study described in Section II:1. It was found that dietary fat played an active role, opposite to carbohydrates and proteins, in altering the serum levels of both T_3 and reverse T_3.

The second line of research represents the in vitro experiments with monolayers of isolated rat hepatocytes. Kinetic studies with labeled iodothyronines in human beings and animals give information about the respective production and metabolic clearance rates. Moreover, with these techniques in the body, apart from plasma, two different pools for iodothyronines can be distinguished which vary in equilibration and disposal characteristics. However, the individual contribution of the various tissues to overall metabolism remains obscure, and rapid successive intracellular reactions cannot be detected. On the other hand in vitro studies with tissue homogenates or subcellular fractions lack the integrated function of the intact cell. We, therefore, felt that monolayers of isolated rat hepatocytes would be a suitable model for studying iodothyronine metabolism at the tissue level. The more so, since the liver is regarded to be very active and play a central role in thyroid hormone metabolism. Indeed, this

in vitro model, which is ever more widely used for all kinds of different studies, proved to be a useful tool in thyroid hormone research as well. The relevance and complementary function of studies with intact cells is well illustrated by the striking difference of 3,3'-diiodothyronine (3,3'-T_2) metabolism with either rat liver microsomes or isolated rat hepatocytes. It was found that with the former, 3,3'-T_2 is only very slowly deiodinated in the outer ring, whereas in intact cells the reaction had a much higher velocity (Section II:3). In fact, the recognition of this discrepancy led to the discovery that hepatic deiodination of iodothyronines is preceded, and in effect accelerated, by conjugation with sulfate. Detailed information about the discovery and the characteristics of this hitherto unknown metabolic pathway for 3,3'-T_2 and T_3 is presented Section II:3-6.

A general problem in studying peripheral thyroid hormone metabolism is the multiplicity of degradative routes. The common metabolic pathways for iodothyronines are deiodination, conjugation with either glucuronic acid or sulfuric acid, oxidative deamination of the alanine side chain and finally the possibility of cleavage of the ether linkage. Theoretically all iodothyronines can be subjected to all of these reactions, but preferential pathways have been found in the different tissues. Another complicating factor is the rapid intracellular succession of some of these reactions, prohibiting the estimation of their exact rate. Moreover, the observations described in this thesis make it clear that close interactions between the different degradative pathways exist. Taken together, we are confronted with a vast amount of possible pathways and metabolites with quite different characteristics.

The early experiments described in Section II:2, where thyroxine and reverse T_3 were incubated with rat hepatocytes, already brought these comlexities to our attention. For this reason we decided first to study the metabolic fate of the less iodinated iodothyronine 3,3'-T_2, since it could be expected that from this substrate fewer metabolites would originate. The results of these experiments are presented in Section II:4.

The outcome of this in vitro work, enhanced by the recent data about diiodothyronine metabolism in humans (3), has brought about the realization that thorough knowledge of the pathways of these "lower" iodothyronines considerably facilitates the interpretation of thyroxine, T_3 and reverse T_3 metabolism. Exemplary to this notion is the elucidation of the actual metabolism of T_3 in rat hepatocytes. In Section II:6 it is shown that deiodination of T_3 is accomplished by three sequential intracellular reactions i.e. sulfation, deiodination of the inner ring and finally outer ring deiodination of the 3,3'-T_2 sulfate generated. Similarly the significance of sulfation as initial and rate

limiting reaction for the metabolic clearance of T_3 and 3,3'-T_2 is indicated.

Finally, the third line of research investigated the hydrolytic potential of rat gut bacteria towards iodothyronine sulfates. Thyroid hormones preferentially appear in bile as conjugates with either glucuronic acid or sulfuric acid. Since the unconjugated form is more rapidly reabsorbed from the digestive tract, bacterial hydrolysis of the conjugates may well enhance an enterohepatic circulation. Whereas the β-glucuronidase activity of the intestinal microflora is well established, much less is known about its sulfatase potential. In Section II:7 we provide evidence for the existence of hydrolytic activity towards iodothyronine sulfates in 2 anaerobic strains of rat cecal bacteria.

Section I of this thesis attempts to integrate the current knowledge of peripheral thyroid hormone metabolism with the most relevant observations from our own work. In this respect special attention is given to the recent and important recognition of two tissue-specific pathways for iodothyronine deiodination. By this distinction the body tissues can be devided in having deiodinase activity which is either inhibited or unaffected by propylthiouracil (PTU). Whereas e.g. liver and kidney are considered to be PTU-sensitive, pituitary and cerebral cortex belong to the latter category. This tissue-specific deiodination in combination with our own observations, has inspired us to propose a tentative model, postulating a close metabolic interaction of the two tissue types in the modulation of iodothyronine serum levels. It is conceived that the serum levels of a number of iodothyronines are determined by production in the one type of tissue and, after "crossing" the plasma compartment, degradation in the other type of tissue. The basis for this model will be 3,3'-T_2 metabolism, since the rapid turnover of this metabolite enables the measurement of arterial and venous plasma gradients. Support for the above hypothesis was found in our preliminary observation that human brains seem to be an important site of 3,3'-T_2 production, whereas the liver very efficiently extracts this compound from the blood.

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SECTION I

ASPECTS OF PERIPHERAL THYROID HORMONE METABOLISM

What makes science so fascinating is the task of pushing ever closer to the unattainable goal of complete knowledge.

Owen Gingerich

CHAPTER 1

MECHANISMS OF PERIPHERAL THYROID HORMONE METABOLISM

After the secretion from the thyroid of mainly thyroxine (T_4) with small quantities of 3,3',5-triiodothyronine (T_3) these principal representatives of the iodothyronine family enter the body circulation. In contrast to the larger protein and peptide hormones as thyroid stimulating hormone (TSH) or adrenocorticotrophin (ACTH) which are soluble in aqueous solutions, thyroid hormones are rather small and hydrophobic molecules. They share this property with the multiple steroids in the body. To transport these compounds in sufficient amounts through the blood vessels, non-covalent binding to the plasma proteins is mandatory (1,2). The transport proteins for iodothyronines are thyroxine-binding globulin (TBG), prealbumin and albumin. Very recently a fourth T_4-binding protein has been identified in our laboratory: thyroxine-binding albumin (TBA) (2A). Whereas the bulk of the iodothyronines are bound to these proteins it has been well established that it is the small fraction of unbound molecules that is available for uptake and subsequent metabolism or biological activity in the various tissues (2). Since 99.96% of thyroxine and 99.6% of T_3 is bound to the plasma proteins it may be envisaged that during a single transit of blood through the tissue capillaries only a small fraction of the total plasma T_4 and T_3 content can "cross" to the tissue compartment. However, since the tissue cells possess binding sites for these hormones not only within the plasma membrane but also on their surface, binding to the tissue compartment is not equivalent with cellular uptake. The transfer of iodothyronines from the moving plasma to the static tissue compartments should be regarded as the dynamic resultant of many and rapid equilibrium reactions between iodothyronines and the different binding proteins (3). In fact, it has recently been calculated both in rat and man that of both total T_4 and T_3 plasma-to-tissue-flux in slowly exchanging tissues 12% and in fast exchanging tissues only about 3% is further

processed. The remaining 88 and 97% return unaltered to the plasma compartment (4-6). The fraction of this plasma-tissue shuttle that actually does cross the cellular membrane can not be discriminated by the tracer injection technique.

Recently the interest in cellular uptake of thyroid hormones has been greatly revived by the demonstration of an ATP-dependent carrier system for iodothyronines in rat hepatocytes (7). Since diffusion is characterized as a passive transport process directed to the lowest free hormone concentration, the energy dependence of the hepatocyte uptake system suggests the reverse i.e. transportation in the direction of a higher intracellular free hormone concentration. At present the exact intracellular free hormone concentration is unknown and may fluctuate in different (sub-)cellular compartments. Furthermore, the intriguing question arises why the cell would require a high concentration of unbound hormone where the principal cellular target, the nucleus, has the highest affinity for T_3 of all subcellular elements (8). A possible advantage of functioning at a higher intracellular free hormone concentration is that by means of an active and controlled transport system the cell would be able to auto-regulate the desired intracellular hormone concentration. It may also be speculated that only those subcellular compartments that require a higher concentration are connected to the energy dependent uptake system. The possible

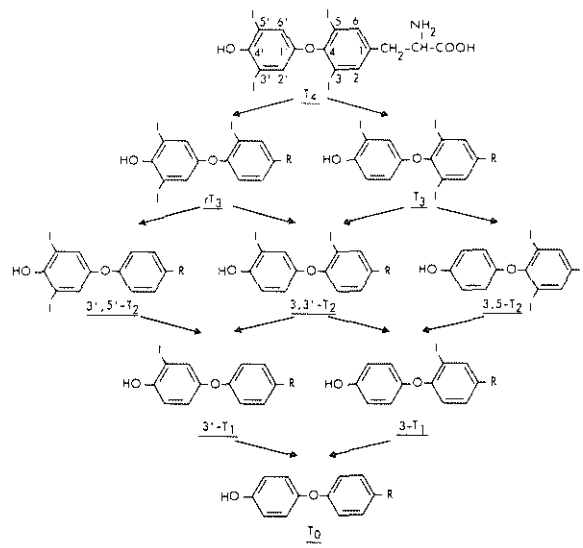

Fig.1. Thyroxine (T_4) and the cascade of other iodothyronines originated by either inner ring (↙) or outer ring (↘) deiodination. The outer or phenolic ring is characterized by the hydroxyl group in the 4' position. R represents the alanine side chain; in between is the inner- or tyrosyl ring. After removal of all iodine molecules the thyronine (T_0) skeleton remains. From ref. 15.

existence of different intracellular transport pathways and compartments is discussed in Chapter 4C.

Metabolism of iodothyronines in the peripheral tissues is characterized by 1) deiodination, 2) esterification of the phenolic hydroxyl group with either glucuronic acid (glucuronidation) or sulfuric acid (sulfation), 3) deamination and decarboxylation of the alanine side chain (oxidative deamination) and 4) ether link cleavage.

Although in theory all eight iodothyronines can be subjected to all these five processes, to the tranquility of the researcher in this field, this does not happen. It has become increasingly clear that different tissues have preferred metabolic pathways for the distinct iodothyronines. This notion has brought a new dimension to the understanding of thyroid hormone homeostasis. Former kinetic investigations were limited to plasma production rates and metabolic clearance rates of the various iodothyronines. There is now a growing interest in the contribution of the individual organ tissues to these processes.

A. Deiodination: a Tissue-Specific Pathway

Before thyroxine (3,3',5,5'-tetraiodothyronine, T_4) can exert its biological activity this main secretory product of the thyroid follicles has to be converted to 3,3',5-triiodothyronine (T_3) (8-10). The substitution of an iodine atom in the 5'-position with a hydrogen ion is essentially a reductive, enzymatic process called deiodination. Not only the iodine atom in the 5'-position of the outer ring is susceptible to deiodination but all four iodine atoms of thyroxine can subsequently be removed in a non-random fashion, giving rise to a total of 7 iodinated thyroxine metabolites (Fig.1). Several publications have reviewed the multiple aspects of these processes both in vivo and in vitro (11-19).

The in vivo conversion of T_4 to T_3, already suggested in 1953 by Gross and Pitt-Rivers (20), could definitely be established by Sterling (21) and Braverman (22) in 1970. Soon after the discovery of T_3 in human serum (23), in vitro generation of this hormone from T_4 was found to occur in rat kidney slices by Albright et al. (24). Later, this conversion was also demonstrated in other tissues of the rat as heart, liver, muscle, pituitary, brain and the thyroid itself (25-30). Similar activity was found in tissues of human origin as kidney, liver, heart, muscle, fibroblasts, polymorphonuclear leucocytes, lymphocytes, placenta and thyroid (31-37) as well as in tissues of other vertebrates (17). It was readily understood that these tissues showed considerable differences in deiodinative activity. In the rat, the liver and the kidney were found to have

the highest in vitro activity whereas e.g. muscle, spleen, lung and intestine have little deiodinating potential (27,38).

Since the first demonstration of predominant deiodinase activity in the microsomal fraction of rat liver homogenates (39,40) substantial effort was initiated to elucidate the mechanism and characteristics of these enzymatic reactions throughout the body. Due to their high activity, the liver and kidney deiodinases were the first to be scrutinized. Important advances have been the disclosure of the essential role of thiol groups as cofactor for outer ring deiodination (40) and the elucidation of the "ping-pong" kinetics of this reaction with rat kidney and liver microsomes in the presence of dithiotreitol (41,41A,42). They provided insight in the mechanisms of deiodination and the well-known inhibition by PTU (41A,43,44). The strong thiol dependency of deiodination suggested the existence of a SH-group containing endogenous cofactor (16,40,45-47). The advocated potential role of glutathione as principal endogenous cofactor, however, has been disputed (47-52A). Interestingly, a recent report suggests that not the total amount of glutathione in rat hepatocytes influences deiodination of iodothyronines (51) but that the ratio of oxidized (GSSG) versus reduced glutathione (GSH) is of importance (51A).

Similarly, considerable controversy has existed as to whether inner ring and outer ring deiodination were catalysed by one single or two distinct enzymes. It is now generally accepted that in liver both the phenolic and tyrosyl ring are deiodinated by the same enzyme (53,54). This deiodinase is a basic, integral membrane protein of the endoplasmic reticulum with a molecular weight of approximately 60.000 D (55,56). Further characterisation is retarded by the difficult solubilization of this tightly membrane bound enzyme (17,57,58), although very recently a 400-fold purification has been reported (59).

The discovery of thiol-dependent but PTU-insensitive T_4 to T_3 conversion in rat pituitary (60-63) in 1978 heralded the existence of an entirely different deiodinating enzyme system. Meanwhile, the characteristics (64-71) and the tissue occurrence (72-76) of these deiodinases, now commonly referred to as type II deiodination, have been extensively documented. Most data are derived from experiments with rat cerebral cortex or pituitary tissue but extracranial type II deiodinase activity has also been found in brown adipose tissue of the rat (76), both human and rat placenta (77,78) and possibly in cultured monkey hepatocarcinoma cells (79,80).

Several prominent differences between type I (D I) conventionally found in liver and kidney, and type II deiodinase (D II) can be indicated.

First, it is important to realize that, in contrast to the type I deiodinase in liver and kidney that catalyses both outer and inner ring deiodination, there

are two separate enzymes in the type II category required for these reactions. Both subtypes are present in brain cortical tissue (66,67,69,70,73,74) but outer ring type II deiodinase (ORD II) prevails in the pituitary (60,62,64,68,71). The latter plays an important role in maintaining thyroid hormone homeostasis through intra-pituitary T_4 to T_3 conversion (81).

Second, type I and II deiodinases follow different reaction mechanisms. In type I deiodination an essential thiol group of the enzyme is involved, that alternates (ping-pong) between the reduced state (-SH) and the oxidized state (-SI) (Fig.2) (69). PTU, as uncompetitive inhibitor, irreversibly inactivates the enzyme by forming a mixed disulfide with this reactive sulfur (43-44). Contrary to D I, type D II is not inhibited by preincubation with low concentrations iodoacetate (41A,69). Iodoacetate inhibits liver deiodination in a non-competitive manner (82) probably through inactivation of an essential SH-group in the enzyme (13,16,17,40). Therefore, enzymic thiol groups apparently are not obligatory to type II deiodination. This also explains the lack of PTU inhibition on this reaction as no enzyme inactivation via formation of mixed disulfides can occur. At present the exact reaction mechanism of type II deiodination has not yet been elucidated. The reaction kinetics derived from incubations with different substrate and DTT concentrations, are suggestive for a sequential-type reaction (69,83). This implies sequential formation of a reactive ternary complex of substrate (iodothyronines), cosubstrate (DTT) and the enzyme (Fig.2).

The third distinction between the two classes of deiodinases is their different susceptibility to inhibitors. Apart from PTU, type D I inhibitors as sodium salicylate, dicoumarol and amiodarone appear to leave D II unaffected (70), although iopanoic acid inhibits both enzymes (65,69,70,84).

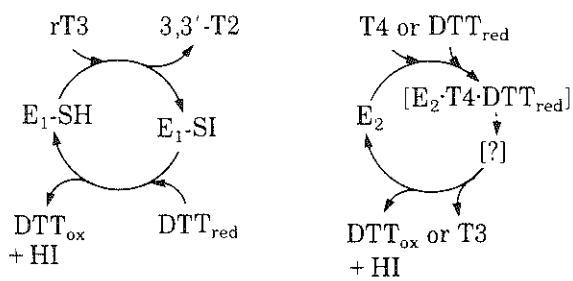

Fig.2. Pathways of outer ring deiodination. On the left the PTU-sensitive (type I, ORD I) deiodination of rT_3 which depends on the presence of an essential, reduced thiol group in the enzyme (E_1-SH). On the right the proposed PTU-insensitive (type II, ORD II) outer ring deiodination of T_4. The latter reaction does not require enzymic SH-groups and may be accomplished through a ternary complex of substrate (T_4), enzyme (E_2) and reduced cofactor, dithiotreitol (DTT). From ref. 69.

Table 1. DEIODINATION CHARACTERISTICS OF IODOTHYRONINES IN RAT LIVER AND CEREBRAL CORTEX

	Reaction	T_x[a]	K_m	V_{max} (min^{-1}.mg $prot^{-1}$)	V_{max}/K_m	DTT[b] mM	PTU[a]	Ref.
			CEREBRAL CORTEX					
1	$T_4 \rightarrow rT_3$	↓	37 nM	144 fmol	3.9	50	--	70
2	$T_4 \rightarrow T_3$	↑	14 nM	1.5 fmol	0.1	100	--	74
		hypo	1.1 nM	11 fmol	10	20	--	66,69
3	$T_3 \rightarrow 3,3'\text{-}T_2$	↓	5.5 nM	134 fmol	24	50	--	66,70,73
4	$3,3'\text{-}T_2 \rightarrow 3'\text{-}T_1$	↓					--	74
5A	$rT_3 \rightarrow 3,3'\text{-}T_2$		29 nM	37 fmol	1.3	20	↓	67
5B	$rT_3 \rightarrow 3,3'\text{-}T_2$	hypo	2.8 nM	6 fmol	2.1	20	--	69
			LIVER					
6	$T_4 \rightarrow T_3$	↓	2.3 µM	30 pmol	13	3	↓	266
7	$T_4 \rightarrow rT_3$	↓	1.9 µM	18 pmol	9	3	↓	266
8	$rT_3 \rightarrow 3,3'\text{-}T_2$	↓	0.06 µM	560 pmol	8730	3	↓	266
11	$T_3S \rightarrow 3,3'\text{-}T_2S$	?	4.6 µM	1050 pmol	228	5	↓	268
12	$3,3'\text{-}T_2S \rightarrow 3\text{-}T_1S$	?	0.3 µM	350 pmol	1170	5	↓	267

[a] The arrows indicate an increase (↑) or decrease (↓) of the reaction rate after thyroidectomy (T_x) or PTU. Hypo: condition in hypothyroid tissue.

[b] Concentration of dithiotreitol in the experimental incubate.

The reaction numbers correspond with the numbers in fig. 12 A and B. (fold-out in the back of the manuscript).

A fourth difference is found in the generally much higher substrate affinity for type D II, as reflected in the lower K_m values for these reactions. On the other hand, the reaction rates, with comparable DTT concentrations, are much lower. A survey of the available deiodination characteristics with both types of deiodinases is given in Table I. The reactions are numbered in order to match with the reaction numbers of the fold-out diagram in the back of the manuscript.

The fifth interesting but intricate discrimination is found in the enzymes' adaptation to hypothyroidism (Table I). After thyroidectomy type D I activity in rat liver and kidney rapidly declines (85-87). In contrast, hypothyroidism has a divergent influence on the two PTU-insensitive deiodinase subtypes i.e. inner ring deiodinase (IRD II) and ORD II. In the euthyroid adult rat cerebral cortex IRD II activity, responsible for T_4 to rT_3 and T_3 to 3,3'-T_2 conversion, prevails. After thyroidectomy this activity decreases, conversely an increase in ORD II, converting T_4 to T_3 and rT_3 to 3,3'-T_2, is found (66,67,69,73). It is of interest to note that, while thyroid hormone homeostasis generally is a slow and static process, increased ORD II activity regresses within 4 hours after T_3 substitution to hypothyroid rats (88). It has been suggested that in the pituitary these changes in enzyme activity are mediated through effects on deiodinase synthesis (89), though this notion was refuted by others (89A). It can be speculated that the hypothyroid adaptations, causing increased T_3 generation and diminished T_3 degradation, may serve to prevent cerebral T_3 depletion under this condition. However, this explanation probably is far too simple since the enzymatic changes after thyroidectomy also differ greatly with age, brain region (73,74) and during starvation (68,90).

B. Conjugation of Iodothyronines

Most of the 10 major conjugation reactions in man and animals were discovered in the nineteenth century (91), long before Kendall in 1914 isolated "thyroxin" from the thyroid gland. Five years later, in 1919, Kendall reported the biliary excretion within 50 hours of 43% of iodine from 200 µg thyroxine injected in a dog (92). This observation demonstrated for the first time that biliary secretion contributes to the disposal of thyroid hormone.

In the following years several investigators attempted to elucidate the nature of the iodine secreted in the bile. However, their methods lacked sufficient specificity to discriminate thyroxine from closely related but different compounds. The availability of radioactive isotopes and the development of

paper chromatography in the early 1940's permitted a more precise analysis of secreted products in bile. After the injection of small doses labeled thyroxine in rats, Taurog et al. in 1951 were the first to identify thyroxine glucuronide (T_4G), initially named "compound U", as major biliary secretion product (93,94). The quantitative importance of this disposal pathway was demonstrated in [^{131}I]T_4-substituted, thyroidectomized rats (95). In equilibrium conditions the radioactivity excreted was equally distributed over feces and urine, the latter containing mainly iodide (Fig.3). In bile, however, the majority of the radioactivity is excreted as iodothyronines conjugated mainly with glucuronic acid (67%) or to a lesser extent with sulfate (6%) (111). These observations designate conjugation as an important route in thyroid hormone metabolism. Nevertheless, its contribution may vary per species since lower excretions have been reported in man (96) and dog (97).

Most data on iodothyronine conjugation are obtained from in vitro studies with isolated, perfused rat livers or from animal experiments, mainly with rats and dogs. The obligatory collection of bile limits these studies in humans to rare occasions (98).

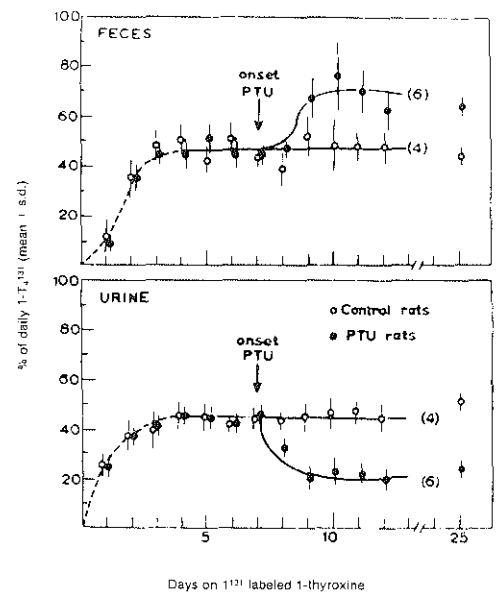

Fig.3. Excretion of radioactivity in feces and urine of thyroidectomized, 1 µg/day [^{131}I]T_4-substituted rats with or without treatment of 15 mg PTU/rat/day. In parentheses the number of animals per group. From ref. 95.

1. Glucuronidation

Glucuronidation, for the first time recognized in 1855, is the most widespread form of "conjugation" in mammalian metabolism (99). The reaction, coupling D-glucuronic acid with a variety of compounds, is catalysed by the enzyme UDP-glucuronyltransferase (EC 2.4.1.17). The enzyme, which appears in many heterogenous forms, is predominantly located in the endoplasmic reticulum of the liver cell. Extrahepatic glucuronidation, however, has been demonstrated in many tissues throughout the body. The reaction is highly dependent on the concentration of the "activated" form of glucuronic acid, uridine diphosphate D-glucuronic acid or UDP-glucuronic acid. The synthesis of this compound from D-glucose and uridine triphosphate is an energy-requiring process (99).

The preferential secretion of T_4 glucuronide in rat bile after tracer doses of labeled T_4 as originally observed by Taurog et al (93,94), has repeatedly been confirmed by others (100-103). Similarly it was found that when increasing amounts of unlabeled T_4 were added to the injected $^{131}T_4$, a greater proportion of thyroxine was secreted in bile in the unconjugated or free form. At very high concentrations, e.g. 0.1 to 20 mg, virtually all radioactivity appeared in the free T_4 fraction (99A,100-105). These experiments show that the liver can clear unphysiological amounts of thyroxine very efficiently by rapid biliary disposal of the unprocessed hormone. It has been suggested that this biliary clearance is mediated through active secretion (113). This mechanism may, quite feasibly, be operative in hyperthyroidism.

Conjugation with glucuronic acid has also been reported with T_3 and reverse T_3 (100,106-110) but only in minute amounts with the lesser iodinated iodothyronines as 3,3'-T_2 (110). In vitro glucuronidation of 3,3'-T_2 has been reported in rat hepatoma cells (162), but not in monkey hepatocarcinoma cells (193). This may be due to defective sulfation or a different glucuronyltransferase in the former cell line. At present no information exists about the characteristics of these compounds as substrates for UDP-glucuronyltransferase. In this respect it is of interest to note that halogen, and notably iodine, substitution in either the ortho- or para-position of a phenol molecule considerably enhances the affinity of the aglycon for UDP-glucuronyltransferase (126-128). It may, therefore, be anticipated that iodothyronines are potentially good substrates for glucuronidation and that the number and location of iodine molecules will have an important influence on the rate of conjugation. Since gluruconidation appears to play a significant contribution to thyroid hormone disposal, and possibly may even have a hormone conserving role in hypothyroidism (Chapter 3), more detailed knowledge about the characteristics of this reaction with the var-

ious iodothyronines may help to understand the complex shifts in serum concentrations induced by dietary modifications, drugs or diseases.

The significance of glucuronidation for thyroid hormone turnover is well illustrated by the effects of UDP-glucuronyltransferase inductors as phenobarbital, methylcholanthrene, polychlorinated biphenyls, benzpyrene and rifampicine (112-119,130,131). After at least 2 days administration to man or animals these compounds invariably induced increases in T_4 metabolic clearance rate, biliary secretion of T_4G, as well as raised fecal excretion and turnover of T_4. In humans (118,119) and intact rats (112,112A,116) T_4 levels were unchanged or only slightly depressed. Increased thyroid activity and T_4 production compensated for the augmented T_4 clearance (112,131). On the other hand the normal basal and TRH-stimulated TSH levels (118,119) suggest an adapted pituitary feedback. In T_4-substituted, thyroidectomized rats, however, the increased metabolic clearance caused a profound lowering of serum T_4 levels (112,116). In contrast, T_3 serum levels tended to be less affected by these compounds (116) and sometimes were even raised (117) in spite of a documented increased T_3 clearance rate (119). Moreover, a prolonged half life and raised thyroxine serum levels have been found in untreated Gunn rats, a species with genetically deficient UDP-glucuronyltransferase activity (125,129-132). These observations support the concept that glucuronidation contributes significantly to thyroid hormone disposal.

Extrahepatic glucuronidation has also been demonstrated for iodothyronines in hepatectomized dogs (102,110,111,120) and eviscerated rats (121). In spite of this extrahepatic production of glucuronides, surprisingly low (110,122) or lacking (100,107) serum levels of these conjugates are found, unless the bile duct is ligated (100,107). This phenomenon is explained by an extremely rapid hepatic clearance and biliary secretion of T_4 glucuronide (123-125). The estimated half life of this compound in the rat is about 13 minutes. Of particular interest is the glucuronidation of orally ingested thyroxine during transportation through the intestinal wall after its absorption from the gut lumen (134). The generated T_4G is rapidly and almost completely eliminated by the liver from the portal blood and secreted in the bile ducts (135). The shunting of absorbed thyroxine back into the intestinal lumen by this short circuit, at least in part, may explain why substitution of hypothyroid patients generally requires higher doses of thyroxine than endogenously produced in euthyroid persons. The existence of an enterohepatic circulation for iodothyronines and their conjugates has been advocated and disputed. This subject is amply discussed in the literature (92,111,136-139) and will be shortly mentioned in Chapter 3.

2. Sulfation

Sulfation, initially discovered in 1875, is a widely distributed conjugation form of inorganic sulfate with xenobiotics or endogenous compounds (91). The reaction occurs in most, if not all, animals and plants, is catalyzed by a group of heterogenous sulfotransferases and in higher mammals requires next to a suitable acceptor, the sulfate-donating cosubstrate PAPS or adenosine 3'-phosphate 5'-sulfatophosphate (140). The generation of this "activated sulfate" is an energy-requiring process and its concentration an important factor in the reaction rate (141).

Sulfotransferase activity seems to be present in most mammalian tissues, though considerable tissue and species variations with regard to substrate affinity and reaction rate exist (141). Many different forms of sulfate conjugation, e.g. $C.OSO_3^-$, $N.OSO_3^-$ or $C.NH.SO_3^-$, have been recognized. Since iodothyronines principally are sulfated in the phenolic hydroxyl group, our attention is focused on the family of phenol or aryl sulfotransferases (EC 2.8.2.1.) (142-147). From rat liver alone 3 forms have been purified to homogeneity. The enzymes appear in the 140.000 x g supernatant of rat liver homogenates and have a molecular weight of about 65 kD (142). The cytosolic origin makes these enzymes quite distinct from the membrane-bound glucuronyltransferases. However, as is exemplified by iodothyronine metabolism, their substrate specificities have a considerable overlap.

Sulfation of iodothyronines in vivo, though surmised for long (106), was demonstrated for the first time by Roche and coworkers in 1957 (148,153). After simultaneous injections of $^{35}SO_4^{2-}$ and 0.7 µg [3'-^{131}I] T_3 they identified in rat bile a compound bearing both labels and having the same chromatographic properties as synthetic T_3 sulfate (T_3S). Soon hereafter, the same compound was found in the plasma of similarly treated rats (149,154) and human beings (158). The isolated material was β-glucuronidase resistant but after incubation in 1N HCl at 100C for 10 min yielded $^{35}SO_4^{2-}$ and [3'-^{131}I] T_3 as hydrolysates. Later, the existence of iodothyronine sulfate esters was substantiated by enzymatic hydrolysis with a rather crude preparation called Mylase P (157) and partially purified commercial sulfatases (161,163, Section II:3,4).

Roche and coworkers also compared the metabolic fate of T_3S and [3'-^{131}I] T_3 after intravenous administration in rats. Quite contrary to the glucuronide of T_4 which has an extremely rapid hepatic and biliary clearance (123-125), the sulfate of T_3 was found to have a much slower plasma disappearance rate than T_3 itself (150,151) (Fig.4). Furthermore, a marked difference in tissue-distribution was noted: T_3 accumulated faster in liver and kidney than T_3S. At the

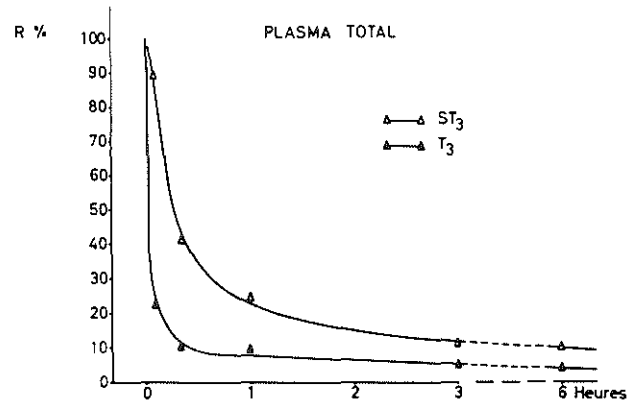

Fig.4. Plasma disappearance curves of radioactivity after the intravenous administration of 0.8 nmol labeled T_3 or T_3 sulfate (ST_3) in the rat. R indicates the percentage of the total radioactivity injected. From ref. 155.

same time, the fecal, biliary and urinary excretion of T_3 exceeded that of T_3S (151). The urinary radioactivity was invariably excreted as iodide, irrespective whether T_3 or T_3S was injected. This suggested deiodination of T_3S in rat tissues, although preceding deconjugation was not excluded. Interestingly, in spite of its polar nature, virtually no T_3S was detected in the urine. This is in keeping with the low urinary excretion of iodothyronines and their conjugates in euthyroid subjects observed by Faber et al (152A). Kidney clearance of sulfates is largely unexplained. It appears that for sulfated aglycons with a molecular weight below 350 D urinary excretion, and for compounds over 450 D biliary excretion prevails. Only molecules between 350 and 450 can be excreted via both ways (152). Correspondingly, T_3 (651 D) as a sulfoconjugate predominantly appears in bile. The low urinary concentration of T_3S is partly explained by the observation of Roche et al that in rat plasma this compound is largely bound to albumin. The free fraction or the association constant of T_3S with albumin was not determined, but would be of interest in the light of the slow plasma disappearance of this conjugate (151).

Clearly, the group of Roche has made a major contribution to the knowledge about iodothyronine sulfation. This work was explicitly compiled in a monograph by Closon in 1964 (155). However, it should be emphasized that most of their in vivo experiments have been carried out, for technical reasons, with hypothyroid rats, i.e. 3 weeks after thyroidectomy. Since this condition has profound implications for thyroid hormone metabolism in general and deiodination in particular, no attempt should be made to draw quantitative conclusions from their stu-

dies with respect to the physiological role of conjugation in intact animals. This is illustrated by a minimal presence of iodothyronine sulfates in bile of euthyroid, [^{125}I] T_4 injected rats, using HPLC analysis (103).

After the initial identification of T_3S by Roche in 1957, sulfation of other iodothyronines and in other species as dog (156,157), rabbit, fetal rat (155) and man (158) has been demonstrated. Though in the various species considerable and incomparable differences exist in the conjugation properties of iodothyronines, a unanimous observation seems to be the lacking of thyroxine sulfation in vivo in man (152A), rat (103,111,159) and dog (109), but also in vitro in perfused rat livers (160), monkey and rat hepatoma cells (161,162) and with preparations of rat liver aryl sulfotransferases I and IV (145). The only exception to this rule is the equal rate of glucuronidation and sulfation of T_4 in 2 days cultured rat liver cells (163). This proportionally high sulfation rate has tentatively been explained by a loss of glucuronyltransferase activity due to dedifferentiation of the cultured cells (Section II:6). At the same time it is equally clear that the lesser iodinated compounds as 3'-T_1, 3,3'-T_2 and T_3 are good substrates for in vivo (109,110,129,149,154,156,157) and in vitro (145,160,Section II:4,5,6) sulfation.

It is interesting to note that iodothyronines with 2 iodine molecules in the outer ring (i.e. T_4 and reverse T_3) are mainly substrates for glucuronidation, whereas iodothyronines with only 1 iodine molecule in the outer ring (3'-T_1 and 3,3'-T_2) are the best substrates for sulfation. T_3 is the only iodothyronine representative that appears to be equally well conjugated by both enzymes (Section II:6). It should be born in mind, however, that the iodothyronine sulfation rate estimated by biliary appearance, inevitably is underestimated since sulfated iodothyronines are rapidly deiodinated prior to excretion in the bile (this thesis).

C. Oxidative Deamination

Oxidative deamination is the denomination for a series of enzymatic reactions converting the alanine side chain of iodothyronines to the acetic acid form. Though this activity has been demonstrated in multiple tissues in the rat, it appears to prevail in liver and kidney (164). Tomita and Lardy elucidated the reaction sequence of oxidative deamination of various iodinated thyronines with sonicated rat kidney mitochondria (165). They showed that the alanine side chain ($R.CH_2.HCNH_2.COOH$) first is deaminated to the pyruvic acid ($R.CH_2.CO.COOH$) and subsequently converted via the acetealdehyde form

($R.CH_2.HCO$) to the acetic acid ($R.CH_2.COOH$) derivative. The first mention of iodothyronine acetic acid derivatives came from Harington and Pitt-Rivers who in 1952 chemically synthesized the acetic acid analogue of thyroxine, 3,3',5,5'-tetraiodothyroacetic acid or tetrac (166). Soon hereafter it was demonstrated that tetrac and 3,3',5-triiodothyroacetic acid (triac) possessed one tenth of the biological potency of T_3 in the rat goiter prevention test (167). The observation of the immediate increase in oxygen consumption in rat kidney slices induced by triac and its rapid clinical effect in hypothyroid patients led Thibault and Pitt-Rivers to speculate that triac might be the biological active form of thyroid hormone (168). This notion instigated a lot of investigation in search of the biological relevance of oxidative deamination.

In 1954 deaminated, pyruvic acid analogues of T_4 and T_3 were identified by the group of Roche in rat bile and urine (169). Later the in vivo occurrence of tetrac, triac and 3,3'-diiodothyroacetic acid (3,3'-diac) was demonstrated as well as their generation after the parenteral administration of the labeled parent compound in the rat (103,170,171), the mouse (172), the dog (109) and finally human beings (98,22,164,173-175). It also appeared from in vitro experiments that essentially all iodothyronines can be subjected to oxidative deamination (165,168,176-179). In Section II:4 it is shown that most likely 3,3'-diac and probably some intermediate products are formed in isolated rat hepatocytes. However, these metabolites are only detected in the presence of thiouracil or very high substrate concentrations. This accords with the observation that tetrac only appears in rat bile when 8.3 μg carrier T_4 was added to the injected [^{125}I] T_4 tracer (103).

The acetic acid derivatives are conjugated with glucuronic acid and sulfuric acid in much the same way as their parent iodothyronines. That is, tetrac and reverse triac are mainly glucuronidated, whereas the lesser iodinated compounds (e.g. 3,3'-T_2) are sulfated. Consonantly, triac is conjugated with both compounds (180-182). In spite of a high affinity of triac for cellular nuclei (183), its biological potency is negligible (1,164,185). The biological significance of oxidative deamination is further reduced by its minor contribution to overall thyroid hormone metabolism (164,186). The kinetics of tetrac and triac, rather similar to those of T_4 and T_3, have very recently been reviewed (19). Finally it should be mentioned that in man, dog and rat tetrac and triac again "analogous" to T_4 and T_3 are primarily deiodinated and to a lesser extent, are excreted as conjugates in the bile, subsequently appearing in the feces (182,187,188).

D. Ether Link Cleavage

Ether link cleavage is the fourth and probably smallest metabolic pathway of thyroid hormones. The reaction is oxidative in nature, probably peroxidase-catalysed and results in cleavage of the ether link between the two ring structures in the thyronine skeleton. The multiple processes involved have lately been discussed by Engler and Burger (19). They demonstrated that ether link cleavage is the major metabolic pathway for iodothyronines in human leucocytes in vitro, but also occurs in the rat in vivo (189). The presence of diiodotyrosine in serum of athyreotic, T_4-substituted patients suggest that ether link cleavage also takes place in human peripheral tissues (190). However, as stated before, the actual contribution to overall iodothyronine metabolism is small (186,190-192) but its significance may increase in diseases accompanied by severe leucocytosis (189).

CHAPTER 2

FACILITATED DEIODINATION AFTER SULFATION OF IODOTHYRONINES:

The Concerted Action of Two Metabolic Pathways

Until very recently deiodination and conjugation of iodothyronines were considered to be functionally distinct metabolic processes in peripheral thyroid hormone metabolism. The major function ascribed to deiodination seemed to be 1) the biological activation of the prohormone T_4 by its conversion to the active hormone T_3 2) the shunting of T_4 to the biologically inactive compound reverse T_3 and 3) the conservation of iodine through further deiodination of partly iodinated metabolites. Conjugation of iodothyronines, on the other hand, has invariably been associated with excretion of iodothyronines from the body, mainly by the fecal route and to a small degree in the urine. The studies presented in Section II cast a new light on this classical concept.

As outlined in the Preface we have chosen to study 3,3'-T_2 instead of T_3 or T_4 metabolism in rat hepatocytes since fewer metabolites of 3,3'-T_2 could be expected, enabling a closer look at the subsequent or simultaneous metabolic steps. It appeared that 3,[3'-^{125}I]-T_2 was far more rapidly degraded by the cells than its precursors T_3, reverse T_3 or T_4. The generation of mainly labeled iodide suggested very active outer ring deiodination. In the presence of the deiodinase inhibitor thiouracil, 3,3'-T_2 clearance remained unchanged and instead of iodide, the sulfoconjugate of 3,3'-T_2 accumulated in the medium. This suggested that sulfation acted as metabolic "escape" route in case the apparent preferential pathway, deiodination, was blocked. However, the velocity of 3,3'-T_2 deiodination in isolated hepatocytes was difficult to reconcile with the very low rate of deiodination of this compound with the deiodinase containing microsomal fraction of these cells. This apparent inconsistency led us to postulate an intermediate molecular conversion of 3,3'-T_2 which might induce facilitated deiodination. The identical rate of 3,3'-T_2 deiodination and sulfa-

tion in the presence of thiouracil, designated 3,3'-T_2S as a potential intermediate. To test this hypothesis biosynthesized 3,3'-T_2S was incubated with rat liver microsomes and proved to be very rapidly deiodinated in the outer ring. The enhanced reactivity of 3,3'-T_2S for this reaction in comparison with 3,3'-T_2 was explained by a 30 fold lower K_m (0.3 vs 9 μM) and a double V_{max} (188 vs 353 pmol.min^{-1}.mg $prot^{-1}$). The hypothesis of sulfation preceding deiodination of 3,3'-T_2 was corroborated by the finding that during sulfotransferase inhibition, 3,3'-T_2 deiodination and 3,3'-T_2 sulfation in the presence of thiouracil were affected to the same degree (Section II:3,4). From this point it required little imagination to postulate a similar mechamism for deiodination of other iodothyronines. The biological significance of T_3 made this hormone an attractive candidate for further study. The obligatory synthesis of T_3S, however, posed some problems as the biosynthetic method used for 3,3'-T_2S was not suitable. We adopted with some modifications, the method for chemical synthesis of T_3S originally described by Roche and coworkers (148,154). In essence, appropriate amounts solid T_3 and [3'-^{125}I]T_3 were incubated with 100 μl concentrated H_2SO_4 at -10C for one hour. The incubate was diluted with 900 μl chilled H_2O and immediately chromatographed on small Sephadex LH-20 columns (Section II:5).

After isolation of the appropriate fractions, the deiodination characteristics of [3'-^{125}I]T_3S with diluted rat liver microsomes were determined. As outlined in Section II:5, Visser et al showed by the subsequent appearance of 3,3'-T_2S and $^{125}I^-$ in the incubate, that T_3S is first deiodinated in the inner ring followed by rapid outer ring deiodination of the 3,3'-T_2S generated (Fig.5). Analogous to 3,3'-T_2, inner ring deiodination of T_3 was considerably enhanced in the sulfated molecule as evidenced by the two fold lower K_m (11 vs 5 μM) and 30 fold higher V_{max} (30 vs 1050 pmol.min^{-1}.mg prot.$^{-1}$). Interestingly, accelerated deiodination for sulfated T_3 is accomplished by a raised V_{max}, whereas for 3,3'-T_2 this is caused predominantly by an increased substrate affinity. In Section II:6 we present evidence for sulfation facilitated deiodination of T_3 in isolated rat hepatocytes. Although some sulfation-independent deiodination of T_3 can not entirely be excluded, it is assumed that T_3 degradation in the liver is mainly accomplished through preceding conjugation with sulfate. The sulfation induced accelerated degradation is not a phenomenon confined to iodothyronine metabolism. Rehfeld recently reported enhanced degradation of gastrin in rat cerebral tissues after sulfation of the tyrosine residues in this 34 aminoacid peptide (194).

In retrospect, our interest in the deiodinative properties of sulfated iodothyronines appeared not to be original. The possibility of enhanced inner ring deiodination of T_3S was seriously considered by Flock et al in 1960. They

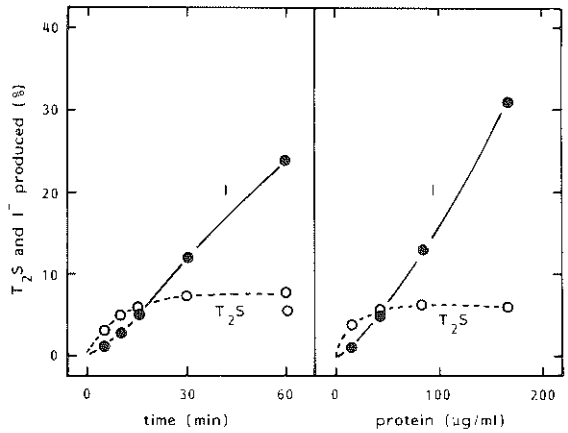

Fig.5. Production of 3,3'-T_2S (o) or radioactive I^- (●) during incubations of 0.5 µM "unlabeled" or [3'-^{125}I]T_3S, respectively, for 5-60 min with 42 µg microsomal protein per ml (left), or for 15 min with 16.8-168 µg protein per ml (right). 3,3'-T_2S and I^- are expressed as percentages of added T_3S. The time sequence of 3,3'-T_2S and I^- production suggests that T_3S is first deiodinated in the inner ring. From ref. 268.

founded their suggestion on the higher amounts of 3,3'-T_2S in comparison with T_3S in bile, plasma or urine of T_3 injected dogs (202). On the other hand Closon, working with Roche, and studying in vitro T_3S metabolism noted only 20% iodide production after 40 hours incubation of 1 µM [3'-^{131}I]T_3S with rat liver or kidney homogenates (155). In fact these investigators were forced to conclude that sulfation of T_3 protected against deiodinase activity. The discrepancy with our observation may in part be explained by the lack of sufficient thiol groups in their incubates, an essential condition for this type of deiodination. Sato and Robbins have also studied the influence of sulfate depletion on iodothyronine metabolism in 2 days cultured rat hepatocytes (51). Due to the presence of methionine and cysteine in the culture medium and the sulfate neogenesis through the cysteine-oxidizing pathway (195) in hepatocytes they were unable to detect the reduced deiodination in sulfate-depleted hepatocytes observed by us.

The significance of the sulfation-induced accelerated deiodination is still speculative. In general, as outlined in Section II:6, it may be assumed that sulfation of iodothyronines predisposes to a very rapid and irreversible degradation of the molecule. On the other hand conjugation with glucuronic acid appears at least not to enhance the rather poor deiodinative properties of T_3, and may in effect protect against irreversible degradation. Furthermore, it may be envisaged that no longer a strict separation between deiodination and non-deiodinative pathways can be maintained. Iodide production in in vivo ex-

periments is likely to be accomplished partly through prior sulfation of iodothyronines. The enhanced excretion of biliary or fecal radioactivity in [^{131}I]T_4-equilibrated, PTU-treated rats (95,196) (Fig.3) is compatible with an increased excretion of sulfated iodothyronines in the bile, analogous to our findings with isolated hepatocytes. In fact, an increased biliary excretion of 3,3'-T_2S and T_3S was found by Flock and Bollman in long-term thiouracil-treated [^{131}I]T_4 injected rats (197) and in Gunn rats fed butyl-hydroxydiiodobenzoate (BHDB), another substance known to interfere with deiodination (129). A similar observation was made by De Herder and Otten in rats injected with a tracer dose of [3'-^{125}I]T_3 30 minutes after the administration of 4 mg/kg PTU (Fig.6). Of academic interest is whether the products of oxidative deamination as tetrac and triac are also subjected to sulfation-preceded deiodination. The T_4 and T_3 resembling metabolic properties of these compounds (Chapter 1C) and the slow rate of triac deiodination (252) are suggestive for this notion.

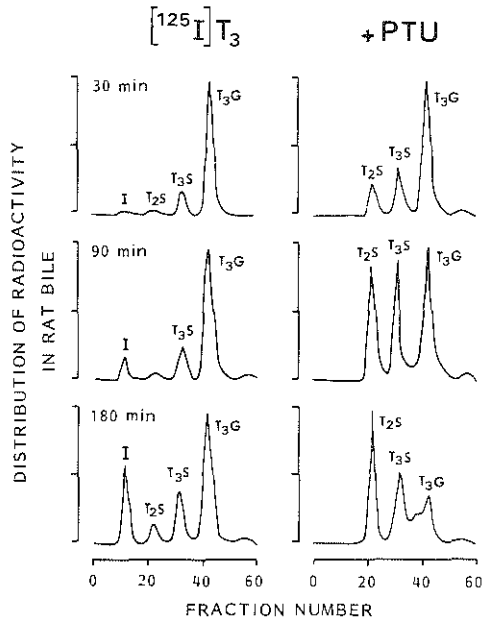

Fig.6. Distribution of radioactivity in rat bile. Rats were injected with a bolus of 25 µCi [3'-^{125}I]T_3, 30 min after the administration of either 1 ml 0.9% saline or the same solution containing 4 mg/kg PTU. HPLC analysis of bile samples at 30, 90 and 180 min revealed the depicted distribution of radioactivity in the iodide (I), 3,3'-T_2S (T_2S), T_3S and T_3G fractions. In rats pretreated with PTU a proportionally strong increase in biliary 3,3'-T_2S and, to a lesser extent, T_3S was observed. Iodide appearance was abolished by PTU. The total amount of radioactivity is not indicated on the y-axis since the specific activity in the bile samples decreased with that of plasma T_3. (De Herder and Otten, unpublished).

In the light of the tissue-specific type of deiodination mentioned in Chapter 1A the intriguing question arises whether sulfation also enhances type II deiodination of iodothyronines. At present this question is still open but should be relatively simple to answer by incubations of T_3S or 3,3'-T_2S with type II deiodinase containing tissues under the appropriate conditions. Indirect evidence from experiments with monkey hepatoma cells would suggest the answer to be negative. These cells appear to have a PTU-insensitive, but very active inner ring deiodinase, with type II-resembling characteristics (199,200) and only a low activity of PTU-sensitive, type I outer ring deiodination (199,200,201). The presence of 100 μM PTU during one hour incubation of these cells with T_3 or 3,3'-T_2 did not affect their clearance or the generation of 3,3'-T_2, 3'-T_1 and their sulfates (201).

Finally, a quite different but interesting observation linking sulfate and thyroid hormone metabolism was described by Tallgren in 1980 (203). In a large series of hypo-, eu- and hyperthyroid subjects serum sulfate levels were determined. There appeared to be a linear correlation between serum sulfate and T_4 ($r=0.88$) or the free thyroxine index ($r=0.75$). Several hypothetical but inconclusive explanations for this phenomenon were presented, including thyroidal influence of sulfate neogenisis or its tubular reabsorption in the kidneys as well as TSH mediated transmembrane transport of SO_4^{2-}. Unfortunately serum T_3 levels were not determined. It can be anticipated, however, that the correlation of serum sulfate with T_3 would be far less pronounced. This is expected especially in the hypothyroid range since T_3 levels tend to remain normal in the early phases of developing hypothyroidism (81). On the other hand, the low serum sulfate levels (many were below 0.1 mM) under hypothyroid conditions might reduce sulfation of T_3 (Section II:6) and thus protect this vital hormone from rapid and irreversible degradation.

CHAPTER 3

SULFATION OR GLUCURONIDATION OF T_3:
The Significance of Dual Metabolism

In Section II:6 it is demonstrated that in rat hepatocytes T_3 initially is metabolized by conjugation with either sulfuric or glucuronic acid. As judged by our in vitro experiments the rate of T_3 sulfation and glucuronidation in the cells is about equal. However, this may not be the case in vivo, since glucuronidation of T_3 in our hepatocytes was found to be more susceptible to variations in cell viability than sulfation. As the cell isolation procedure is a rather rude way of tissue treatment, it is feasible that in spite of the 4 hrs preincubation, total cellular glucuronidation is below its physiologic level.

Comparison of enzyme activities in the intact cell is hampered by many pitfalls. A major problem is the lack of information about the actual free substrate concentration(s?) in the subcellular compartment(s?). This especially goes for UDP-glucuronyltransferase and phenol-sulfotransferase since the former is membrane associated whereas the latter is cytosolic in nature. Furthermore, we do not know exactly how iodothyronines are transported in the cell to the different enzyme sites or via which mechanism they are excreted after metabolic conversion. All these steps may have a profound impact on the net metabolic rate. This problem has lately been approached by Koster and Mulder. These investigators developed a theoretical kinetic model for cellular metabolism of increasing substrate amounts via two separate intracellular pathways assuming identical uptake and back-transport of the compounds involved (204). It was concluded that the intracellular substrate concentration increases more than proportionally with the extra-cellular concentration when the reaction with the high affinity becomes saturated. This causes the conversion via the unsaturated route (with the low affinity conversion) to increase also more than proportionally.

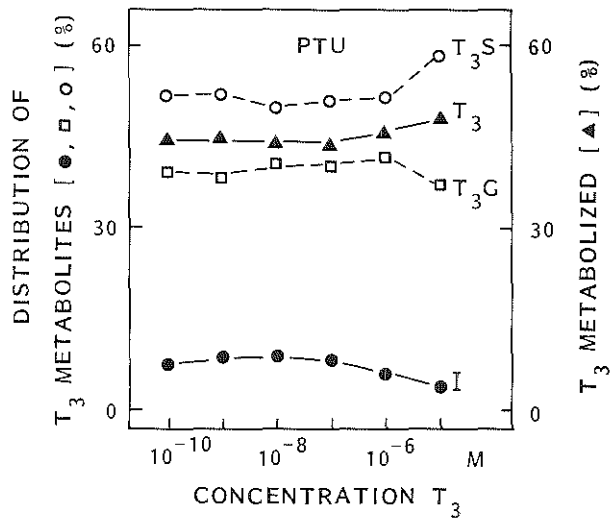

Fig.7. Influence of substrate concentration on T_3 metabolism in isolated rat hepatocytes. Monolayers of rat hepatocytes were incubated for 3 h at 37C with increasing amounts of T_3, [3'-^{125}I]T_3 and 10 μM PTU. The distribution of iodide (I), T_3S and T_3G is depicted on the left hand scale. The right hand scale indicates the percentage of metabolized T_3. From Section II:6.

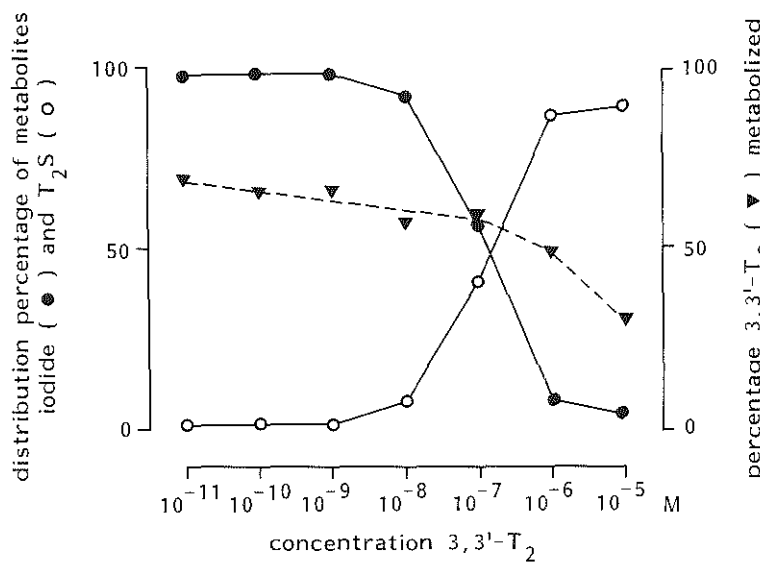

Fig.8. Influence of substrate concentration on 3,3'-T_2 metabolism in isolated rat hepatocytes. Monolayers were incubated for 30 min at 37C with increasing concentrations of 3,3'-T_2 and 3,[3'-^{125}I]-T_2. The distribution of the metabolites in the culture medium, iodide (●—●) and 3,3'-T_2S (o—o), is depicted on the left hand scale. The proportional clearance of 3,3'-T_2 (▼) is given on the right hand scale. From Section II:4.

We observed a comparable situation for T_3 metabolism in rat hepatocytes. Fig.7 shows that with increasing medium T_3 concentrations in the presence of PTU, the fractional clearance of this substrate and the production of T_3S and T_3G remains constant upto 10^{-6} M T_3. At 10^{-5} M, however, the production of T_3G declines whereas T_3S generation increases. This suggests saturation of T_3 glucuronidation and a proportional increase of T_3 sulfation. According to the proposed kinetic model for this type of metabolism a lower K_m of T_3 for UDP-glucuronyltransferase than for phenol-sulfotransferase should be expected. This may seem somewhat controversial since usually common substrates tend to have a lower K_m for sulfation than for glucuronidation. It is pointed out in Chapter 1B that the presence of an iodine atom in the phenolic ring may explain the enhanced avidity of T_3 for conjugation with glucuronic acid, as halogen substitution in phenol lowers its K_m for glucuronidation 30-fold (126-128).

In a similar way, the metabolism of 3,3'-T_2 and T_3 in rat hepatocytes can be compared. Fig.8 shows that the sulfation capacity of the cells for 3,3'-T_2 is saturated at approximately 10^{-5} M whereas T_3 sulfation at the same concentration is not. According to the above theoretical kinetic model this would implicate a lower K_m for sulfation of 3,3'-T_2 than for T_3. It may, therefore, be speculated that the better substrate properties of 3,3'-T_2 for sulfotransferase activity (145) at least in part are explained by a higher affinity for the enzymatic reaction. Confirmation of this hypothesis awaits determination of the exact reaction characteristics of 3,3'-T_2 and T_3 with purified enzyme preparations.

Closely associated with the interest in the quantitative aspects of hepatic T_3 sulfation and glucuronidation is the question with respect to the physiologic significance of this dual conjugation mechanism. As pointed out before, conjugation of xenobiotic or endogenous compounds, and T_3 is no exception, has mostly been associated with detoxification or biliary and urinary secretion of hydrophobic aglycons (152). It has however become increasingly clear that this simple notion in no longer tenable. Several steroids retain their biological activity after sulfation, also sulfated compounds sometimes are more actively metabolized than their unconjugated precursors (152), and even the formation of reactive carcinogenic intermediates after sulfation of xenobiotic compounds has been described (205). Although these alternative functions are mainly associated with sulfation, similar properties have been attributed to glucuronidation (99).

The recent discovery of sulfation-facilitated deiodination of iodothyronines endorses the view that conjugation with sulfate can induce enhanced metabolism of drugs or endogenous compounds. In spite of this adapted insight in metabolic functions, the classical concept of "detoxification" still holds true for sulfa-

tion of T_3. There are two ways by which sulfation can inactivate the biological potency of T_3. First, T_3S probably lacks any biological activity since, using the method described by Docter et al (206), we were unable to detect any specific binding of T_3S to purified rat liver nuclei (Otten, unpublished). Second, irreversible inactivation of the T_3 molecule due to accelerated deiodination results from the conjugation with sulfate. So sulfation appears to be the way by which the liver can very effectively eliminate T_3 from the body circulation without being dependent on urinary or fecal excretion.

Quite distinct seems to be the role of glucuronidation of T_3. Conjugation with glucuronic acid probably disables direct biological activity of T_3 in a similar way as sulfation, i.e. by prohibiting binding to the specific T_3 nuclear receptor. However, contrary to sulfation, glucuronidation does not seem to change much of the rather poor deiodinative properties of T_3. Evidence for this conception is presented in Section II:6. In essence: 1) accumulation of T_3G is not enhanced during incubation of T_3 with hepatocytes in the presence of PTU 2) inhibition of glucuronidation by cellular depletion of the essential UDP-glucuronic acid with galactosamine does not alter the rate of T_3 deiodination by the cells and finally 3) T_3G proved to be a very poor substrate for rat liver microsomal deiodinase (Otten, unpublished). For the latter experiment labeled T_3G was prepared biosynthetically by incubation of isolated rat hepatocytes for 16 h at 37C with 10 μM T_3 in the presence of [3'-^{125}I]T_3 without PTU. After the incubation T_3G was purified by the same chromatographic procedure as described in Section II:6. This material was incubated for 60 min at 37C at a concentration of 0.05 μM with 165 μg/ml rat liver microsomal protein. After the incubation only 2% of free iodide as a result of outer ring deiodination of T_3G was generated. Furthermore, 0.5 μM T_3G did not inhibit microsomal deiodination of 0.01 μM rT_3. The possibility of exclusive inner ring deiodination of T_3G, also seems unlikely since no substantial amount of the glucuronides of 3,3'-T_2 or 3'-T_1 were detected in the bile of T_4 or T_3 injected dogs (110,111,157) and rats (103,106).

Since both T_4 and T_3 are excreted in bile mainly as glucuronides, we may look at glucuronidation as a means of the liver to dispose of these hormones. In this respect it is noteworthy that UDP-glucuronyltransferase activity in rat liver cells can be enhanced directly by cyclic adenosine 3',5'-monophosphate (cAMP) and indirectly by glucagon stimulation of this second messenger (207). Induction of glucuronidation by compounds as phenobarbital or polychlorinated biphenyls has been associated with increased metabolic clearance and turnover of T_4 (Chapter 1B). In contrast to these compounds the induction of UDP-glucuronyltransferase activity by cAMP, in agreement with its nature, is

rapid and reaches its maximum within 2 hrs (207). This property of cAMP may explain in part the cAMP-induced changes in iodothyronine metabolism in isolated-perfused rat livers observed by the group of Hesch (208). Using T_4 as a substrate they noted a reduction of T_3 production in cAMP-treated perfused livers. Also a cAMP-enhanced metabolic clearance was found for T_3 or rT_3 as primary substrates, whereas 3,3'-T_2 clearance remained unchanged. These effects were ascribed to a stimulated uptake and degradation of T_3 via the non-deiodinative pathways. In the light of the unchanged clearance of 3,3'-T_2, which is accomplished through sulfation, and the known effects of cAMP on glucuronidation this phenomenon may now tentatively be ascribed to enhanced glucuronidation of T_3 and rT_3. Even the slightly reduced T_4/T_3 conversion rate may be envisaged through T_4 shunting via enhanced conjugation with glucuronic acid.

The essential difference between iodothyronine conjugation with either glucuronic or sulfuric acid is that after biliary secretion the glucuronidated aglycons, notably T_4 and T_3, have kept their original structure. This provides the possibility of an enterohepatic circulation for these hormones. Both T_4G and T_3G are poorly absorbed from the digestive tract (134,209), whereas for T_4 40-68% (210,211,212,215) and for T_3 100% absorption (214,215,217) has been reported. Hence, hydrolysis of these glucuronides by bacterial β-glucuronidase activity in the intestinal contents is likely to promote reabsorption of T_4 and T_3 (Fig.9). Although we have no intention of dealing extensively with the rather controversial enterohepatic circulation of iodothyronines (136,137,139), the potentially important function of T_3 glucuronidation will be discussed.

Glucuronidation of T_4 and T_3 results in biliary excretion of these hormones. Putting aside the above-mentioned possible enteral reabsorption after bacterial hydrolysis, biliary excretion will ultimately result in fecal elimination. Under normal conditions this natural loss of active hormones is balanced by the daily thyroidal production. Under conditions of documented increased fecal loss of thyroid hormones e.g. in steatorrhea in man (96) or during ingestion of highly absorbing dietary components as soyflour or fibre in rats (216,217) a compensating increase in thyroidal function was found, in extreme situations even producing goiter in rats (216). Conversely, with low residue diets only half of the normal amount of thyroxine substitution was needed in the rat goiter prevention assay (217). These data are suggestive of fecal mass and composition dependent thyroid hormone disposal. At the same time biliary and subsequent fecal excretion of glucuronides may also function as a rapid drainage system for superfluous hormones in hyperthyroidism (218).

In contrast to this disposal function of glucuronidation a hormone conserving task may be postulated in hypothyroid patients. Glucuronidation of T_3 es-

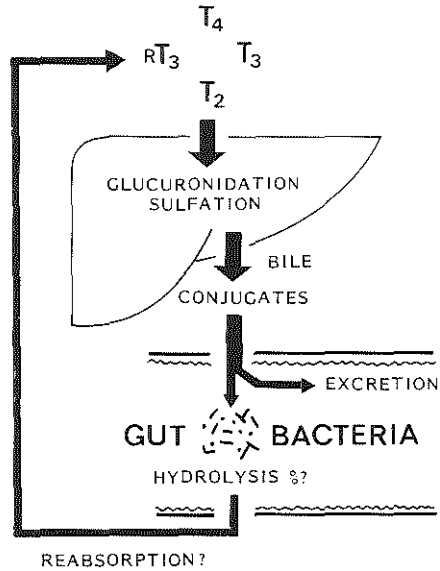

Fig.9. Diagram of the postulated enterohepatic circulation of iodothyronines. An unknown proportion of the conjugated iodothyronines is hydrolysed by the gut microflora. This hydrolysis promotes reabsorption from the intestinal tract.

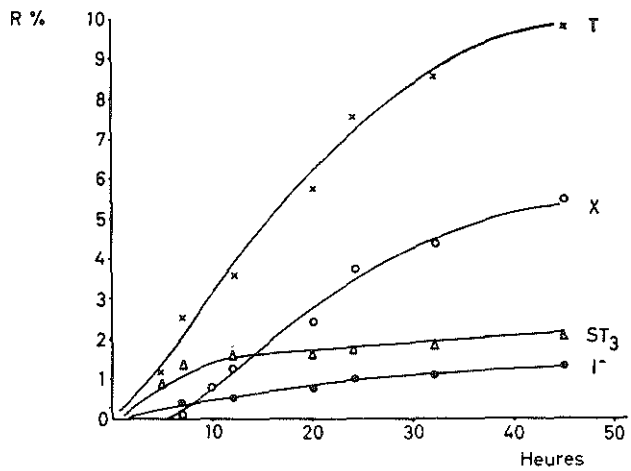

Fig.10. Distribution of biliary radioactivity after the intraduodenal administration of [3'-^{131}I]T_3S in hypothyroid rats. R represents the percentage of total administered radioactivity. T: total bile radioactivity; I^-: iodide; ST_3: T_3 sulfate; X: T_3 glucuronide. The appearance of T_3G indicates that T_3S has been hydrolysed and re-conjugated with glucuronic acid. From ref. 155.

sentially protects the molecule against sulfation-induced deiodination and, therefore, against irreversible loss of biological activity. In spite of T_3 depletion in hypothyroidism its metabolism via hepatic glucuronidation, sulfation and biliary excretion will continue, though possibly at a different rate. Due to the bacterial β-glucuronidase activity in the intestinal tract T_3G but also T_4G will be hydrolysed and by reabsorption (92,151,154) saved for the depleted body. Several conditions present in hypothyroidism will prolong the exposition of these glucuronides to the enteral bacteria and thus contribute to their hydrolysis and subsequent recovery. For instance, the low gut motility in hypothyroidism (139,219A) will a) increase the intestinal transit time and b) cause bacterial overgrowth in the ileum (219). Consonant with the above hypothesis is the reduced fecal excretion of ^{131}I in hypothyroid (218,218A,218B) and iodine deficient rats (220). In conclusion, glucurodination may have the paradoxical function of either disposal or salvage of thyroid hormones.

A similar mechanism may be postulated for iodothyronine sulfates in hypothyroidism. Roche et al have observed a strong increase in biliary T_3S excretion in T_3 injected hypothyroid rats (106,154). This is probably caused by the reduced deiodinase activity in hypothyroid rat liver (45) prohibiting complete deiodination of T_3S. Sulfatase activity in gut flora towards iodothyronine sulfates has been demonstrated by Closon and Roche as early as 1959 (221). The hydrolytic activity was caused by an exo-enzyme of an unidentified bacterial strain (155). Recently De Herder et al isolated two anaerobic, gram-negative rods from rat cecal contents which showed sulfatase activity towards 3,3'-T_2S and T_3S (222,Section II:7). Since in hypothyroid rats increased amounts of T_3S are excreted in the bile these sulfatase producing strains may in a similar way contribute to the vital recovery of T_3. This postulate is supported by the 28% absorption of 4 µg labeled T_3S infused in the duodenum of hypothyroid rats (223). T_3S hydrolysis seemed plausible since part of the radioactivity was recovered in bile as T_3G (Fig.10). Whether this mechanism is also operative in human subjects is uncertain since it is unknown whether in hypothyroid man the liver deiodinase activity is reduced. Also, as discussed in Chapter 2, the low sulfate serum levels in hypothyroid patients (203) will reduce sulfation of iodothyronines in man and in this fashion prevent loss of scarce T_3.

CHAPTER 4

THE INTERACTION OF DIFFERENT TISSUES IN THYROID HORMONE METABOLISM:

The Proposal of a Hypothetical Model

As with any complex mechanism, knowledge advances
by the gradual refinement of oversimplifications.

Panksepp

As outlined in Chapter 1 serum levels, as well as production and metabolic clearance rates of iodothyronines give no information about the site of their generation or degradation. Since considerable tissue differences in iodothyronine metabolism have been demonstrated, the interest in the contribution of the various organs to peripheral thyroid hormone metabolism is rapidly increasing. The more so, since the complex changes in iodothyronine serum levels induced by fasting, altered dietary composition, diseases or drugs are incompletely understood. A major contribution to the growing interest in tissue-specific thyroid hormone metabolism has been the realization that plasma T_3 is not totally exchangeable with tissue T_3 (225) and the discovery of a PTU-insensitive (type II) deiodinase in rat cerebral tissues by the group of Larsen. This enzyme proved to have quite distinct physicochemical characteristics in comparison with the "classical" liver and kidney deiodinases (Chapter 1A). Despite the fact that some PTU-sensitive outer ring deiodinase is present in rat cerebral tissues, the predominance of type II deiodination caused these tissues to be referred to as PTU-insensitive. This tissue-specific deiodination in combination with several incompletely understood phenomena has brought about a growing dissatisfaction with the concept that hepatic outer ring or 5'-deiodinase is the sole regulator of fluctuating T_3 and reverse T_3 serum levels in what erroneously is called "The low T_3 Syndrome".

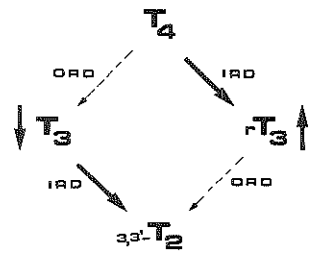

Fig.11. The original concept of the regulation of iodothyronine serum levels. T_4 is degraded in mainly the liver to either T_3 or reverse T_3. 3,3'-T_2 originates from both these products. According to this model inhibition of outer ring deiodination (----), e.g. by fasting, illness or drugs, would cause a decrease of T_3 and increase of reverse T_3 serum levels.

In this model the liver is the central organ in controling iodothyronine serum levels. Total body production of T_3 depends for about 80% on outer ring deiodination of T_4 in extrathyroidal tissues. Since the metabolic clearance rate of T_3 shows little changes in fasting or diseased individuals, reduction of outer ring deiodination conceivably is responsible for the lowered T_3 serum levels under these conditions. Also the usually increased serum reverse T_3 levels are explained by the same reduction of outer ring deiodination and an essentially unaltered production rate (Fig.11). However, several observations both in vivo and in vitro are hard to reconcile with this simple theory and suggest far more complex regulatory mechanisms. To illustrate this point some examples are given.

1) According to the above concept the liver (and/or the kidneys) would be both the supply and clearance organ of reverse T_3. Several studies, however, show that in spite of documented hepatic inner ring deiodination of T_4 (163,227), no rT_3 is released from perfused rat livers (160,208,226), rat liver slices (227) or cultured hepatocytes (163). This is probably best explained by rapid intracellular outer ring deiodination of all reverse T_3 generated.

2) During the initial period of caloric restriction in man serum T_3 lowers and reverse T_3 shows a reciprocal rise (228). After 4 weeks, however, reverse T_3 normalises, whereas T_3 remains low. Also, the dietary study in Section II:1 demonstrated separate influences on T_3 and rT_3 serum levels by dietary constituents. Moreover, serum 3,3'-T_2 levels remained remarkably constant in spite of considerable fluctuations of the common precursors T_3 and rT_3. All these observations are hard to explain merely by fluctuations of hepatic outer ring deiodinase activity.

3) The theory does not account for the multiple alternative metabolic pathways for iodothyronines. Especially conjugation with glucuronic acid and sulfa-

tion-facilitated deiodination contribute significantly to thyroid hormone metabolism and are believed to be the principal processes in hepatic T_3 handling (Section II:6).

4) Despite the fact that hepatic outer ring deiodination is entirely blocked by pharmacological concentrations PTU (Section II:4), T_3 generation in L-thyroxine-substituted, PTU-treated hypothyroid patients is not completely inhibited (229).

5) The theory further depends on the existence of 2 separate enzymes for outer and inner ring deiodination. However, recent investigations favour the opinion that both processes are catalysed by the same hepatic enzyme (17).

6) Also other factors as incomplete plasma-tissue T_3 exchange (225), active transport of iodothyronines into rat hepatocytes (7) and the documented deiodinase activity in rat kidney plasma membranes (230) suggest far more intricate regulatory systems.

The gradual realization of these inconsistencies and the different deiodinase properties in PTU-insensitive tissues has instigated in our laboratory a new conception about iodothyronine metabolic pathways. This notion will be presented here as a hypothetical model. A schematic diagram is given on a fold-out page in the back of the manuscript (Fig.12 A,B).

In spite of the rather complex drawing, the model clearly is an oversimplification of the actual situation. The major presupposition is the subdivision of the body in three compartments: the PTU-insensitive (type II) and the PTU-sensitive (type I) tissues, connected by the plasma compartment. The type I tissues are represented by the liver in order to include biliary excretion of thyroid hormones. The concept is essentially based on the production of 3,3'-T_2 and reverse T_3 in type II tissues and their subsequent degradation in the liver after "crossing" via the plasma compartment. For T_4 and T_3 a more complex situation exists. The model will be introduced by successive discussion of the pathways of 3,3'-T_2, reverse T_3, T_3 and T_4 in the presence or absence of PTU.

We would like to mention that the opportunity of reading a preprint of the excellent review about iodothyronine deiodination by Engler and Burger (19) as well as the foreknowledge of their work on the generation of the various diiodothyronines (231) have had an important complementary function in the support of our contention.

A. Metabolism of 3,3'-T_2

As outlined in Section II:4 a significant proportion of iodothyronine degra-

dation is channelled via the generation of 3,3'-T_2. Serum levels of this metabolite are rather low (ranging in the literature from 23 to 137 pmol/l) despite the relatively high production rate (38 to 76 nmol/day). This is caused by a very rapid metabolic clearance rate, with 621 to 1116 l/day the highest of all iodothyronines (19). Since 3,3'-T_2 has no metabolic activity, this compound has received rather limited attention. However, it is the rapid turnover that makes 3,3'-T_2 an interesting metabolite. Due to this property it is one of the few iodothyronines for which an anterio-venous gradient in certain tissues can be estimated. Faber et al. (232) as well as Bauer and coworkers in our laboratory (233) found the 3,3'-T_2 concentrations in the hepatic vein to be only 60% of arterial values. Faber et al. calculated a hepatic clearance of 14 nmol/day accounting for almost half of the total 3,3'-T_2 clearance (34 nmol/day). Moreover, they also showed that the proportional liver clearance of 40% was independent of the plasma 3,3'-T_2 concentration (234). This latter phenomenon has been discussed in Section II:4 and reflects the high sulfation capacity of the liver which is not saturated at in vivo serum concentrations.

The various observations correspond well with our in vitro experiments, showing that 3,3'-T_2 in hepatocytes is rapidly metabolized by sulfation before undergoing accelerated outer ring deiodination. In the upper diagram (A) this rapid proces is indicated by a fat arrow directing 3,3'-T_2 from the plasma compartment into the liver cell with subsequent sulfation (R_9) and outer ring deiodination (R_{12}). Plate B shows that hepatic clearance is not affected by PTU but outer ring deiodination (R_{12}) is, resulting in a high output of 3,3'-T_2S in the bile. Support for this mechanism has been provided by the strong increase in biliary 3,3'-T_2S in PTU-pretreated, [3'-^{125}I]T_3-injected rats (Fig.6) (De Herder and Otten, unpublished). The rapid clearance of 3,3'-T_2 by the liver raises the question where this metabolite might be generated. Faber et al, by measuring 3,3'-T_2 concentrations in renal arterial and venous blood of 20 normal subjects, calculated a net renal 3,3'-T_2 production of 2,4 nmol/day, accounting for only 6% of the total 34 nmol generated daily. No significant arterial-venous gradient was measured in the femoral vessels, eliminating the extremities and therefore the muscles as important source of 3,3'-T_2 production. It was found by Laurberg and Weeke that 3,3'-T_2 serum levels in PTU or methimazole-treated hyperthyroid patients did not differ significantly (235). Since hepatic 3,3'-T_2 clearance is not affected by PTU or methimazole (Section II:4), this observation suggests mainly PTU-independent production of 3,3'-T_2. In another study 3,3'-T_2 serum levels decreased 18% in T_4-substituted, hypothyroid patients treated with PTU (254). This seems to be in close agreement with the 16% of plasma 3,3'-T_2 being derived from reverse T_3 (231), which according to

our model in euthyroid conditions, is exclusively mediated via PTU-sensitive deiodinase. The kidneys probably contribute to this part of 3,3'-T_2 production (232). These observations point at PTU-insensitive tissues as potential source of plasma 3,3'-T_2.

Fig.12 shows the possibility of 3,3'-T_2 generation via two pathways in these tissues. The thickness of the arrows in indicative for the expected conversion rate based on the V_{max}/K_m ratio's of the various reactions from the literature (Table I). Especially conversion of T_3 to 3,3'-T_2 (R_3) is reported to proceed very rapidly (66,70), whereas further inner ring deiodination (R_4) of 3,3'-T_2 to 3'-T_1 is much slower. It is hypothesized that 3,3'-T_2 is mainly generated in PTU-insensitive tissues from either locally produced (via R_2) or plasma-borne T_3. Since both R_2 and R_3 are PTU-insensitive, 3,3'-T_2 production will not be affected by this compound. The contribution of pathway R_{5A} (PTU-sensitive outer ring deiodination of rT_3) will be modest compared with pathway R_3 and absent in the presence of PTU (diagram B). Reaction 5B is only significant in hypothyroid tissues (69). This concept is corroborated by the study of Engler et al. who showed that 84% of plasma 3,3'-T_2 is derived from T_3 (19,231). If indeed PTU-insensitive tissues are the main source of plasma 3,3'-T_2 then an uphill arterio-venous gradient of this metabolite in the cerebral circulation should be expected. To verify this concept we have compared in anesthesized humans the 3,3'-T_2 serum concentrations in arterial blood, and in blood from the internal jugular vein, the predominant efferent vessel of the brain. In two subjects studied so far the 3,3'-T_2 content in the jugular vein was raised by 29 and 36% (Otten, Avezaat , Visser and Hennemann, unpublished). This observation firmly supports the postulate that plasma 3,3'-T_2 is generated from T_3 in PTU-insensitive (e.g. cerebral) tissues and further indicates that the brain may contribute significantly to peripheral thyroid hormone metabolism.

B. Metabolism of Reverse T_3

Similar to 3,3'-T_2, reverse T_3 also has a rather rapid turnover. If the lower reported RIA values for serum rT_3 (average 0.3 nmol/l) are considered to be true (236), the total daily production is calculated at approximately 30 to 45 nmol with a metabolic clearance rate between 82 and 108 l/day (15,19). Despite these figures and contrary to 3,3'-T_2 this clearance rate appears to be too low and the blood flow too high to allow the measurement of arterio-venous plasma gradients in the various tissues (232). Therefore, the actual proof for generation or clearance of rT_3 in a specific organ is hard to obtain. It has

been pointed out in the introduction of this chapter, that the liver probably does not contribute to plasma rT_3. On the other hand perfused livers (160,208,226) and hepatocytes (163, Section II:2) rapidly metabolize rT_3 giving rise mainly to iodide and small amounts of 3,3'-T_2 as well as some unidentified glucuronides and sulfates (160). The production of 3,3'-T_2 is unexpected in the light of its rapid hepatic clearance but may be due to the artificial in vitro conditions. With T_4 no 3,3'-T_2 production was noted in perfused livers (208). On the basis of these observations, we are at present inclined to consider the liver as an important clearance organ for rT_3.

In Section II:2 it is suggested that hepatic clearance of this metabolite is accomplished mainly through direct outer ring deiodination. This is concluded from the fact that inhibition of deiodination by PTU, again contrary to 3,3'-T_2, drastically reduces rT_3 degradation in rat hepatocytes. This conception is represented in the diagram by pathway R_8. The likely, but modest formation of rT_3G has been left out for ease of survey. Diagram B now makes clear why PTU (237-240), but not methimazole (237), causes an increase in serum rT_3: the major pathway of rT_3 degradation (R_8) is blocked.

At this point we would also like to point out that the total body production rate of rT_3 probably exceeds the production rate generally indicated in the literature (see above). Reverse T_3 generated from T_4 (R_7) in the liver is not secreted into the vascular compartment, but rapidly degraded by outer ring deiodination (R_8). Therefore, rT_3 produced via this pathway will not contribute to plasma rT_3 and the production rate (defined as: Serum rT_3 x Metabolic Clearance Rate) will be underestimated. This phenomenon may exist for all other iodothyronines as well. Thyroxine is the only exception since this hormone has only one source: the thyroid gland. The higher the intracellular catabolism of a generated metabolite, the greater the underestimation of its production rate. This may also apply to the 3,3'-T_2 originated from rT_3 in the liver (R_8), since the high rate of subsequent sulfation (R_9) results in only small amounts of extracellular 3,3'-T_2 (Section II:2, 208,226). To indicate that the tracer injection technique neglects this hidden production the terms "extrinsic" or "plasma" production rate seem more appropriate.

Considering the facts mentioned and analogous to 3,3'-T_2, we postulate that a major portion of plasma rT_3 is formed in type II tissues whereas the clearance takes place in the liver. The theoretical basis for this thesis is, that R_1 (PTU-insensitive) proceeds very rapidly in euthyroid rat cerebral cortex (66,70), whereas the much slower R_{5A} (67) will cause accumulation of rT_3 which is then shunted to the vascular compartment. As stated before, this hypothesis is difficult to substantiate since at present RIA's are not sensitive enough to

detect the expected small arterio-venous concentration gradient in the high flow vessels of the cerebral circulation. The only indirect indication for this PTU-insensitive production in vivo is the equivalent rT_3 production rate in PTU or methimazole-treated hyperthyroid patients (235). In this study the difference in peripheral activity of both drugs towards type I tissues is reflected by a 50% lower metabolic clearance rate in the PTU-group causing significantly elevated rT_3 levels. Interestingly, extra-hepatic production of rT_3 has, on quite different grounds, been suggested before (11).

C. Metabolism of T_3

For obvious reasons T_3 metabolism has attracted a lot of investigation. Several review articles have dealt with this subject (11-13, 241). It is generally accepted, that in healthy individuals and animals some 20% of the 34-52 nmol T_3 produced daily, is directly secreted by the thyroid gland (15,19). The remaining 80% originates from T_4 by outer ring deiodination in peripheral tissues. However, for the same reasons as with rT_3 the exact production site can not be discriminated by arterio-venous plasma gradients. On the other hand, in vitro experiments strongly suggest that the liver contributes significantly to plasma T_3 production. Many studies describe the production of T_3 from T_4 in perfused rat livers (208,226,242), rat liver slices (227,243) and in human and rat hepatocytes (250, Section II:1). This hepatic production of T_3 from T_4 is mediated by a PTU-sensitive deiodinase (R_6). In thyroidectomized, T_4-replaced rats inhibition of this reaction by PTU causes only 50-70% decrease of serum T_3 generation (244-247). This contrasts with the almost complete inhibition of T_4 to T_3 conversion in kidney or liver homogenates of PTU-treated rats (41,248), and suggests an alternative, PTU-insensitive production of T_3.

When these data are taken into consideration the following conclusions may be made: a) in the euthyroid state the major part of serum T_3 is generated in type I (PTU-sensitive) tissues, b) the liver and quite possibly the kidneys (251-253) have an important contribution to this T_3 production, c) a smaller but significant amount of serum T_3 is produced in type II tissues. At present it can not be discriminated whether this T_3 originates from cerebral tissues or from the extracranial brown adipose tissue (76). The importance of this pathway is illustrated by the recent report that the contribution of type II tissues to plasma T_3 production may increase to 100% in hypothyroid rats (249).

According to the present concept T_3 metabolism takes place in both types of tissues. In the liver it was demonstrated (Section II:6) that T_3 initially is

conjugated with UDP-glucuronate or sulfate (R_{10}). Of the respective products T_3G is predominantly secreted in the bile, whereas T_3S is successively deiodinated in the inner ring (R_{11}) and the outer ring (R_{12}). In the presence of PTU, however, T_3S accumulates and should be increased in the bile. This expectancy was confirmed by De Herder and Otten (Fig.6). From this figure also a second feature becomes apparent. The other sulfate conjugate, 3,3'-T_2S, found to increase in bile of PTU treated-rats after [^{125}I]T_3, appears later but in more massive amounts than T_3S. This 3,3'-T_2S cannot be formed from T_3S in the liver since R_{11} is completely inhibited (Section II:5,6). The observation, therefore, is another indication for extra-hepatic PTU-insensitive production of 3,3'-T_2 and subsequent sulfation in the liver (Section II:4) or other, extra-hepatic tissues (202).

The second degradative pathway of T_3 is, of course, inner ring deiodination in type II tissues (R_3) yielding 3,3'-T_2 and small amounts of 3'-T_1 (R_4) (66). The fact that short-term pretreatment of PTU does not affect the T_3 clearance in rats (244,249) may be envisaged since all T_3 degradative pathways R_3, R_{10} and glucuronidation are not influenced by this agent. The diminished production of T_3 from T_4 in the liver (R_6) will therefore cause reduced plasma T_3 levels. However, in long-term PTU-treated animals this reasoning may not entirely be tenable since under these conditions a slightly reduced (14%) T_3 clearance was found (218B).

An interesting aspect about T_3 metabolism now emerges. If indeed the postulated pathways exist, the liver seems to be playing a double role: on the one hand it is responsible for a major part of T_3 plasma production whereas on the other hand it is involved in the plasma clearance of T_3. This may seem somewhat controversial, but, in fact, it would provide a potentially flexible mechanism for metabolic adaptation and regulation of plasma T_3. Van Doorn and Van der Heide came to a similar conclusion based on their observations with [^{125}I]T_4 and [^{131}I]T_3-equilibrated rats (218B). The immediate question arises how the liver cell could accomplish this dual task. Two speculative theories may be put forward.

1) The existence of separate intracellular pathways and compartments for iodothyronines and/or their metabolites. From recent studies it has become increasingly clear that iodothyronines do not exchange freely between the plasma and the various subcellular fractions of living cells (81,218A,B,225), Some tissues depend heavily on locally (i.e. in the cell itself) generated T_3, whereas in others plasma-borne T_3 predominates. Furthermore, the ratio between the two sorts of T_3 is different in some subcellular fractions. In the diagram this is provisionally indicated for the nucleus of the two cell types: the hepatocyte

nucleus obtains its T_3 mainly from the plasma, whereas the nucleus in cerebral or pituitary cells is occupied by a larger proportion of locally generated T_3. This phenomenon has been mentioned to show that "compartmentation" in cells exist and that, therefore, the possibility of separate intracellular pathways is plausible. This seems to be an interesting field for future research.

2) The liver is not an organ composed of functionally identical hepatocytes. The hepatocytes in each acinus of the liver are subdivided into three zones dependent on the extent to which their blood supply is oxygenated (261). In zone 1, where the portal and arterial blood perfuse first, the oxygen availability is highest, whereas zone 3 receives less well oxygenated blood. Marked differences in hepatocyte enzyme composition in the three zones have been detected (261). Therefore, it is quite feasible that the cells in the different zones accomplish different tasks with regard to iodothyronine metabolism, e.g. the generation or the clearance of T_3. The hypoxia-induced reduction of T_4 deiodination but intact T_3 generation in rat liver slices illustrates this possibility (262). The question may be addressed in the future with the use of fluorescent monoclonal antibodies directed against hepatic deiodinase or by studying iodothyronine metabolism in isolated rat hepatocytes at different pO_2.

D. Metabolism of Thyroxine

In the rat, after secretion from the thyroid gland, T_4 is distributed over the plasma compartment (26%), a fast pool made up mainly by the liver and the kidneys (17%) and a slow pool, e.g. muscle tissue (57%) (5). As discussed in Chapter 1 these hormonal contents of the tissues should not be regarded as a static condition but as a continues entry and efflux of molecules. A small portion of this shuttle (3% in fast pools and 12% in slow pools) is subjected to further metabolism. It was calculated that the amount of T_4 degraded in both pools is about the same i.e. 2 nmol/day (5).

As shown in the diagram, there are many ways by which T_4 can be metabolized. In general, a distinction can be made between deiodination in type I and type II tissues, whereas also conjugation with glucuronate and oxidative deamination occurs. These pathways have all extensively been discussed in Chapter 1. It has been estimated that 80% of the total daily T_4 production is metabolized by monodeiodination, yielding approximately equal amounts of T_3 and rT_3 (19). Since the fast and slow pools mentioned above, are not equivalent to type I and II tissues, the proportion of T_4 deiodinated in either PTU-sensitive or insensitive tissues is not exactly known. However, the roughly 50% PTU-induced reduction of

urinary iodide in T_4 substituted thyroidectomized rats injected with T_4 randomly labeled with ^{131}I (257), suggests that both tissues have an equal contribution to deiodination of T_4.

Interestingly, this inhibition of PTU-sensitive deiodination, which constitutes some 40% of T_4 degradation, has only a modest influence on T_4 serum levels and metabolic clearance. In multiple studies with T_4-substituted humans or rats, PTU caused either no change (229,239,246,255) or a small increase in plasma T_4 (196,238). It appeared that, identical to T_3, the T_4 metabolic clearance rate is either unchanged (244,257-259) or slightly reduced, mainly during prolonged PTU administration (197,218B,245,256) and in hyperthyroid patients (196). Apparently the alternative pathways compensate for the loss of PTU-sensitive deiodination.

On the basis of the presented metabolic model a tentative prediction of the short-term PTU-influence on the metabolite composition in bile of [^{125}I]T_4-injected rats can be made: T_4G may be unaltered or slightly increased, T_3G is expected to be lower due to the low plasma T_3 levels, conversely rT_3G will increase. The major proportional rise in radioactivity, however, is expected in the 3,3'-T_2S and T_3S fractions. These expectations are only partly confirmed by the study of Flock and Bollman, who investigated the biliary metabolite composition in thiouracil treated,[^{131}I]T_4-injected rats (197). In these animals a modest decrease in T_4G and T_3G was compensated by an increase of rT_3G leaving the total amount of glucuronides essentially intact. On the other hand only a small rise from 6 to 12% of iodothyronine sulfates was noted. The discrepancy may be explained by the prolonged duration of thiouracil administration (> 3 wks) which must have caused hypothyroidism (198). Experiments with T_4, analogous to those described for T_3 (Fig.6), are required for confirmation of the above supposition.

E. Concluding Remarks

The presented model for thyroid hormone metabolism contains many incertitudes, gaps and speculations, and clearly is an oversimplification of the actual situation. It is emphasized that the model has not been designed in a attempt to answer all questions regarding fluctuations in iodothyronine serum levels. The main purpose behind this concept has been to present some evidence that multiple tissues may closely interact in maintaining of what is called "the euthyroid state". By this notion also a better understanding of the intricate

changes in iodothyronine serum levels induced by drugs, diseases or diets, might be obtained.

In our model we have purposely only discussed PTU as interfering agent. This compound selectively inhibits type I deiodination in the body, thus enabling the estimation of the remaining type II deiodinase activity. Other conditions known to affect deiodination as fasting or illness are far more difficult to interpret.

A major contribution to the conception of our tentative model has been the elucidation of 3,3'-T_2 metabolism in rat hepatocytes. Ever since, we have repeatedly experienced that thorough knowledge of the metabolism of this relative unimportant metabolite is very helpful in understanding the sometimes enigmatic fluctuations of the more notorious iodothyronines. It can be speculated that in the future elucidation of the metabolism of the lower iodothyronines will prove to be essential for understanding of the more complex conditions mentioned above.

CHAPTER 5

FUTURE ASPECTS

In the last two decades the research of peripheral thyroid hormone metabolism has enjoyed considerable attention and progress. A lot of information emerged about the kinetic and metabolic properties of the various iodothyronines as well as their enzyme-inducing potency. However, the complex changes induced by alterations in dietary composition, different diseases or drugs are largely unexplained, and require a lot of future investigation.

As hypothesized in Chapter 4 the composition of thyroid hormone levels in the plasma seems to be determined by the concerted action of multiple tissues. By this notion it may be envisaged that different diseases can cause different changes in thyroid hormone homeostasis. This will depend on many variables such as the extent of the disease, i.e. which organs are affected by which noxious agent and to what degree, but also on changes in tissue perfusion, the presence of toxic or interfering metabolites and more general conditions as tissue oxygenation, caloric supply and body temperature. A prerequisite for a proper understanding of the pathobiological changes is a full comprehension of euthyroid organ physiology. At present this is far from complete.

Whereas the liver has been rather extensively investigated as perfused organ, very little information exists about similar experiments with other organs known to be active in thyroid hormone metabolim e.g. the kidneys and the brain. Perhaps also less obvious tissues as muscle and fat should be scrutinized, since even if their metabolic rate is slow, they may, due to their larger mass, contribute significantly to overall production or degradation of iodothyronines. This will require further investigation of the subcellular deiodinating and conjugating properties of the respective tissues in combination with the net production or clearance of iodothyronines in isolated (re-)perfused organs or limbs.

The use of PTU or thiouracil as selective type I deiodinase inhibitors, may prove to be very helpful in these tissue-directed investigations. It is in fact astonishing, that until now so little turnover studies of the lower iodothyronines in PTU-treated subjects have been published. In this context the discovery of a selective type II deiodinase inhibitor would be greatly wellcomed. Simultaneously, the question arises if the common type I and II inhibitor iopanoic acid, is capable of abolishing all deiodinating activity in the body and whether still other thyroxine degrading systems exist (263-265).

Furthermore, in vivo inhibition of glucuronidation with D-galactosamine or sulfation with pentachlorophenol (PCP) in the rat, may contribute to a better estimate of the physiologic relevance of these pathways. Summing up of the above organ-directed investigations could provide a more integrated understanding of thyroid hormone metabolism and eventually of the interfering action of dietary alterations, drugs and diseases.

Lastly, the possible existence of separated intracellular pathways for iodothyronines has already been mentioned. This problem is primarily associated with T_3 production and degradation, since for this hormone intracellular compartmentation (218B) and distinct nuclear and cytoplasmic equilibration in the liver (8,260) has been demonstrated. The approach to this intriguing possibility is not yet clear.

Despite the steady progress in understanding of peripheral thyroid hormone metabolism, it seems a long way before this knowledge will ultimately result in clinically applicable therapeutic measures.

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SECTION II

APPENDIX PAPERS

Metabolism 1980; 29: 930.

PAPER 1

THE ROLE OF DIETARY FAT IN PERIPHERAL THYROID HORMONE METABOLISM

M.H. Otten, G. Hennemann, R. Docter, T.J. Visser

Short term changes in serum 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (reverse T_3, rT_3) were studied in four healthy nonobese male subjects under varying but isocaloric and weight maintaining conditions. The four 1500 kcal diets tested during 72 hr, consisted of: I, 100% fat; II, 50% fat, 50% protein; III, 50% fat, 50% carbohydrate (CHO), and IV, a mixed control diet. The decrease of T_3 (50%) and increase of rT_3 (123%) in the all-fat diet equalled changes noted in total starvation. In diet III (750 kcal fat, 750 kcal CHO) serum T_3 decreased 24% (NS) and serum rT_3 rose significantly 34% ($p < 0.01$). This change occurred in spite of the 750 kcal CHO. This amount of CHO by itself does not introduce changes in thyroid hormone levels and completely restores in refeeding models the alterations of T_3 and rT_3 after total starvation. The conclusion is drawn that under isocaloric conditions in man fat in high concentration itself may play an active role in inducing changes in peripheral thyroid hormone metabolism.

SINCE THE BEGINNING of the past decade a lot of attention has been paid to peripheral thyroid hormone economy.[1] Among the many stimuli and conditions in man and animals introducing a preferential decrease in 5'-deiodinase activity resulting in a decrease of serum 3,3',5-triiodothyronine (T_3) and an increase of serum 3,3',5'-triiodothyronine (reverse T_3, rT_3), fasting is one of the most extensively studied.

A great number of dietary and kinetic models with a great variety of changes in calorie intake, dietary compositions, and body weights, have been used to further investigate the possible role played by each of the three basic elements of food: carbohydrates, proteins, and fat.

An interesting phenomenon during experimental dietary changes is the important contribution of oral carbohydrates (CHO) in restoring or preventing these alterations in peripheral thyroid hormone metabolism. Under total fasting conditions in man serum T_3 starts to decline within 24 hr to reach a plateau 50%–60% below control levels in about 4–6 days.[2,3] rT_3 shows a reciprocal rise of 58%–107%,[4–7] while in most reports of short term experiments thyroxine (T_4) shows little or no change.[7] Refeeding after total starvation with as little as 100 kcal of CHO causes a partial normalization of serum T_3 concentrations.[8] Moreover, refeeding with 800 kcal CHO, in spite of the still subnormal total amount of calories, fully restores T_3 to control levels[8] or 800 kcal CHO, per se, fails to induce T_3 or rT_3 changes in nonfasted normal subjects.[5] On the other hand a normocaloric diet with a high CHO content mimicked the rise in T_3 and decrease in rT_3 normally seen in overfeeding.[9]

While in the recent literature much data and speculation appeared about intracellular mechanisms influencing these presumed enzymatic deiodinations in relation to glucose metabolism,[10,11] far less detailed and quantitative documentation exists about the influences of oral proteins and fat on peripheral thyroxine metabolism in man. As most studies use (1) the refeeding model of the fasted state[4,8,12–14] or (2) hypocaloric diets[5,15,16] we were interested in the direct influence in nonobese subjects of approximately weight maintaining diets strictly composed of maximally 2 constituents on a 50%–50% basis. One isocaloric diet of mixed composition would serve as a control for the influence of the small caloric reduction. The rather arbitrary amount of 1500 kcal was chosen for comparison with other studies using amounts of approximately 1400 or 800 kcal.[4,5,8,13]

MATERIALS AND METHODS

Studies were performed in four healthy nonobese male volunteers aged 27–31 yr, on 4 different occasions with at least 1 mo interspace. In all studies after obtaining two baseline blood samples a 1500 kcal (6276 kJoule) mixed diet regimen was kept during 24 hr in order to rule out the possible influences of substantial individual variations. In the next 72 hr a 1500 kcal regimen with free water intake was maintained as well, but the fat (F), protein (P), and carbohydrate (CHO) content in the 4 studies differed as follows: I, 100% fat (F, 164 g; P, 5 g; CHO, 3 g); II, 50% fat with 50%

protein (F, 82 g; P, 168 g; CHO, 7 g); III, 50% fat with 50% CHO (F, 73 g; P, 6 g; CHO, 167 g); IV, mixed diet (F, 70 g; P, 76 g; CHO, 137 g).

Blood samples were taken twice daily during the 72-hr diet period. After this period usual caloric intake was resumed and 3 more blood samples were taken up to 72 hr after termination of the dietary regimen. During the whole experiment normal daily activities in the hospital or laboratory were allowed. The following measurements were done: serum T_4[17], T_3[18], rT_3[19], 3,3'-diiodothyronine (T_2),[20] thyrotropin[21] as duplicates in the same radioimmunoassay, T_3-resin uptake (Triosorb, Abbott Laboratories, Chicago Ill.) as well as routine hematologic and chemical laboratory analyses. Every subject served as his own control and statistical analyses were performed using the Student's paired t test.

RESULTS

The diets were well tolerated. The serum concentrations of sodium, potassium, calcium, magnesium, phosphate, SGOT, SGPT, and alkaline phosphatase as well as total protein, cholesterol, phospholipids, triglycerides, and hematologic values did not change during any of the diet periods. The important changes in creatinine, uric acid, and bilirubin are listed in Table 1. Only 0 and 72 hr values are depicted. Serum creatinine rose significantly in diets I (40%), II (19%), and IV (9%) but not in the fat/CHO diet. Serum uric acid showed significant rises in all four periods (I, 59%; II, 30%; III, 30%; IV, 11%). Serum bilirubin only increased, though in a considerable amount, in diet periods I (112%) and III (46%). On the other hand, serum glucose showed a fall predominantly in periods I and II (not shown). A rise in serum urea (66%, $p < 0.001$) only occurred in the protein/fat diet.

Serum T_4, TSH, T_2, and T_3-resin uptake did not change significantly during any of the experiments. Important changes, however, were noted in T_3 and rT_3 levels especially during periods I and II. These changes, as depicted in Table 2, equalled in diet period I (100% fat) the changes observed during total starvation in man. Serum T_3 fell significantly 50% and 41% in the first two periods, respectively. There was a nonsignificant decrease of 24% in period III and 16% in the 1500 kcal mixed diet. Serum rT_3 rose as much as 123% in diet I, higher than most data about total fasting in man. The rise in rT_3 in the fat/protein and fat/CHO diet was about the same (maximally 29% and 34%, respectively) but with a higher significance during the diet containing 750 kcal CHO and 750 kcal fat. The increase of rT_3 in the 1500 kcal mixed control diet was 23% and only significant on one occasion.

Serum creatinine correlated inversely with serum T_3 ($r = -0.75$, $p < 0.001$) and positively with serum rT_3 ($r = 0.67$, $p < 0.001$) only in the fat diet. Serum uric acid was significantly correlated with changes in rT_3 (I: $r = 0.73$, $p < 0.001$; II: $r = 0.61$, $p < 0.005$; III: $r = 0.55$, $p < 0.005$) except in the mixed diet. Only in diet I there was also an inverse correlation with serum T_3 ($r = -0.84$, $p < 0.001$). There was no important correlation of serum glucose with serum T_3 or rT_3 during any of the diet periods.

DISCUSSION

The present study confirms the finding of Danforth et al.,[9] that under isocaloric conditions variations of diet composition can drastically alter peripheral thyroid hormone metabolism. The constant T_4 and minimal change of TSH levels suggest that, as in fasting,[3] the hypothalamo–hypophyseal control is not responsible for

Table 1. Alterations in Serum Creatinine, Uric Acid and Bilirubin During 4 Isocaloric Diets† with Different Composition*

		Creatinine		Uric Acid		Bilirubin	
	Hours	μmole/l	Increase (%)	mmole/l	Increase (%)	μmole/l	Increase (%)
Diet I	0	90 ± 18	40	0.39 ± 0.08	59	7.8 ± 2.4	112
	72	126 ± 3	$p < 0.05$	0.62 ± 0.04	$p < 0.001$	16.5 ± 4.9	$p < 0.01$
Diet II	0	86 ± 7	19	0.37 ± 0.07	30	11.5 ± 3.1	4
	72	102 ± 6	$p < 0.005$	0.48 ± 0.05	$p < 0.01$	12.0 ± 2.9	NS
Diet III	0	96 ± 4	5	0.37 ± 0.06	30	11.3 ± 1.9	46
	72	101 ± 11	NS	0.48 ± 0.02	$p < 0.02$	16.5 ± 2.6	$p < 0.05$
Diet IV	0	97 ± 8	9	0.37 ± 0.05	11	11.0 ± 2.0	25
	72	106 ± 7	$p < 0.01$	0.41 ± 0.06	$p < 0.005$	14.0 ± 3.0	NS

*Values are mean ± SD and percentage increase of basal value.

†All diets contained 6.276 kJ (1.500 kcal) divided as follows: Diet I, 100% fat; Diet II, 50% fat and 50% protein; Diet III, 50% fat and 50% CHO; Diet IV, 40% fat, 20% protein, and 40% CHO.

Table 2. Changes in Serum Total T_3 and rT_3 Before, During, and After 4 Diets With Different Composition in 4 Healthy Nonobese Subjects*

	Diet I¶				Diet II¶				Diet III¶				Diet IV¶			
	T_3		rT_3		T_3		rT_3		T_3		rT_3		T_3		rT_3	
Hours	ng/dl	Decrease (%)	ng/dl	Increase (%)	ng/dl	Decrease (%)	ng/dl	Increase (%)	ng/dl	Decrease (%)	ng/dl	Increase (%)	ng/dl	Decrease (%)	ng/dl	Increase (%)
0	123 ± 16		19.7 ± 2.8		109 ± 17		21.5 ± 1.8		102 ± 18		18.7 ± 2.2		122 ± 24		19.5 ± 2.8	
24	99 ± 20	20	27.5 ± 2.3‡	40	97 ± 18	11	23.6 ± 3.2	10	108 ± 7		23.1 ± 0.6§	24	116 ± 34		21.7 ± 5.6	11
48	75 ± 7†	39	35.2 ± 6.6§	79	75 ± 15§	31	27.7 ± 6.1	29	92 ± 18	10	23.6 ± 1.0§	26	125 ± 38		24.0 ± 3.1‖	23
56	65 ± 8†	47	43.9 ± 9.2‡	123	64 ± 13†	41	26.6 ± 3.6‖	24	84 ± 14	18	25.1 ± 3.0§	34	118 ± 24		22.1 ± 1.5	13
72	62 ± 6†	50	43.9 ± 6.2‡	123	65 ± 20§	40	25.0 ± 2.9	16	78 ± 20	24	22.3 ± 1.8‡	19	102 ± 11	16	20.8 ± 1.9	
End of diet period; resumption of usual caloric intake																
8	67 ± 7†	46	34.8 ± 5.3†	77	70 ± 18§	36	26.2 ± 1.8‡	22	78 ± 27	24	21.7 ± 1.7‖	16	103 ± 4	16	22.8 ± 2.3	
24	78 ± 3§	37	28.3 ± 5.6‖	47	83 ± 26	24	21.9 ± 1.9		92 ± 23	10	18.8 ± 2.6					
72	108 ± 16§	12	23.1 ± 3.4‡	17	93 ± 13	16	18.0 ± 1.8		98 ± 23		17.3 ± 4.2		120 ± 12		19.0 ± 1.7	

*Data shown represent mean ± SD and percentage increase or decrease of basal value.
†Significantly different from 0 hr value, $p < 0.001$.
‡Significantly different from 0 hr value, $p < 0.005$.
§Significantly different from 0 hr value, $p < 0.01$.
‖Significantly different from 0 hr value, $p < 0.05$.
¶For composition of diets, see Table 1.

these changes. However, in the 1500 kcal all-fat diet the decrease of T_3 and the increase of rT_3 were far more pronounced than in the 80% fat 20% protein isocaloric diet used by Danforth et al.,[9] (T_3 decrease 50% versus 33% and rT_3 increase 123% versus 30%, respectively). These changes, as depicted in Table 2, equalled in diet period I (100% fat), in spite of adequate caloric intake, the changes observed during total starvation in man. A logical explanation for the dramatic alteration in peripheral thyroid hormone metabolism in diet I would, of course, be the total deprivation of oral carbohydrates and proteins. In that case, the conclusion could be that energy supply in the form of 1500 kcal oral fat is totally inert in the complex process of changing peripheral thyroid hormone metabolism. On the other hand, it also cannot be excluded on basis of this observation, that the consumed amount of fat in diet I does play more than a mere passive role in these regulating processes.

Another interesting and quite unexpected observation is the significant increase of rT_3 up to 34% ($p < 0.01$) and, though not significant, the decrease of serum T_3 by up to 24% in diet III. This diet contained 750 kcal of CHO and 750 kcal of fat. The essential role of CHO in maintaining normal serum T_3 and rT_3 levels is well established. Spaulding et al.[5] noted in a 800 kcal all-CHO diet no significant changes in serum T_3 and rT_3. Moreover, Azizi[8] found a complete recovery of both values after refeeding starved subjects with the same amount of CHO.

One might argue that in our study an increase in rT_3 was also present in the 1500 kcal mixed control diet. However, this increase was less pronounced, only significant on one occasion and did differ ($p < 0.02$) at 56 hr from diet III. We are inclined to contribute the changes in diet IV to the slight caloric reduction.

The discrepancy that serum rT_3 remains normal on a 800 kcal all-CHO diet[5,8] while there is a significant increase in rT_3 (34%, $p < 0.01$) in our diet period III (F/CHO) can only be explained by an effect of the added quantity of 750 kcal fat in the latter. In spite of the relatively small number of persons investigated in all published studies, including ours, it is tempting to speculate that fat in large amounts could have an influence on peripheral thyroid hormone metabolism. This presumed influence then apparently would interfere with the well known stabilizing potency of carbohydrates on peripheral deiodination. On the other hand, the also well documented higher potential of oral CHO[8] to maintain normal peripheral deiodination in comparison with oral protein is still demonstrated in the significant difference ($p < 0.05$) in serum T_3 decrease between diet II (F/P) and diet III (F/CHO). The "protective" effect of protein against the postulated influence of fat is apparently stronger for rT_3 than T_3 levels. This is compatible with the findings of Azizi,[8] who in his starvation-refeeding model noticed no changes in the low T_3 levels and a partial recovery of rT_3 levels on 800 kcal protein. Similarly, Spaulding et al.[5] noted a decrease in serum T_3 only and no change in serum rT_3 concentrations on a 800 kcal diet composed of 80% fat and 20% protein. Of further interest to the possible influence of fat on peripheral thyroid hormone metabolism in man are the in vitro observations of Harris et al.[23] in fresh rat liver homogenates. They found a decreased T_4 to T_3 conversion in the liver homogenates of 2-day starved rats. This decrease was fully restored by oral CHO, partially restored by oral protein, but not by oral lipid. These data are compatible with the findings in our study.

The mechanism by which fat can induce these changes in peripheral iodothyronine conversion remains purely speculative. The possible role of a gastroenterohepatic regulatory mechanism has been advocated.[13,22] The different secretions of various gastrointestinal hormones induced by oral ingestion of fat, amino acids, glucose, or fasting have been documented on several occasions.[24,25] A possible influence of one of these gastrointestinal hormones on peripheral thyroid hormone metabolism, however, has not yet been established.

This study clearly demonstrates further that the metabolic influences, merely by changing the composition of an isocaloric diet, are far more complex than just an alteration in peripheral thyroid hormone metabolism. Many mechanisms during fasting are directed towards fuel economy.[26,27] The demonstrated correlations of serum T_3 and rT_3 with the various metabolic parameters indicate that changes induced by dietary manipulation might be very complex.

The significant correlation in changes of rT_3 and T_3 with uric acid and creatinine, respectively, in long-term hypocaloric diets has been observed and discussed by us before.[16] The present data provide evidence that the close association between rT_3 degradation and uric acid metabolism also exists in short term isocaloric changes of dietary composition.

In conclusion, we are inclined to believe that dietary fat plays a more active role in changes in peripheral thyroid hormone metabolism than has been assumed until now. The mechanism by which fat could induce these changes are yet to be elucidated but could be mediated by gastrointestinal hormones. Protein seems to have more stabilizing potency for changes in rT_3 than T_3 levels induced by fasting or an isocaloric fat diet, while carbohydrates especially maintain a normal serum T_3 level when consumed in combination with the same amount of fat.

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PAPER 2

IODOTHYRONINE METABOLISM IN ISOLATED RAT HEPATOCYTES

Initial results

M.H. Otten, H. Bernard, J. Blom, R. Docter, G. Hennemann

Abstract

Freshly isolated rat hepatocytes have been used to study iodothyronine metabolism at the tissue level. Approximately 2 x 10^6 cells were incubated at 37C with different amounts of thyroxine (T_4), 3,3',5'-triiodothyronine (rT_3) and 3,3'-diiodothyronine (3,3'-T_2). The culture media were subsequently assayed for 3,3',5-triiodothyronine (T_3), rT_3 and 3,3'-T_2 content. T_4 metabolism was characterized by a very low production of T_3. Within 60 min at a concentration of 10^{-8} M both rT_3 and 3,3'-T_2 were rapidly cleared from the medium for 35 and 80%, respectively. An important observation was the influence of thiouracil, an inhibitor of deiodination, on these clearance rates. Whereas 3,3'-T_2 disappearance was hardly affected, rT_3 clearance was reduced from 35 to 8%, indicating that hepatic deiodination of rT_3 constitutes a major degradative pathway for this metabolite with little metabolic alternatives. This observation tentatively explaines why the metabolic clearance of rT_3 is reduced in conditions with associated reduced hepatic deiodinase activity and raised rT_3 serum levels, as e.g. starvation, illness or deiodination-inhibiting drugs. The unimpeded clearance of 3,3'-T_2 is explained by rapid conjugation with sulfate preceding deiodination.

Introduction

This paper describes the initial experiments with freshly isolated rat hepatocytes in the study of thyroid hormone metabolism. At the time these experi-

ments were initiated, in 1978, the importance of the extra-thyroidal conversion of the prohormone thyroxine (T_4) to the biologically active hormone 3,3',5-triiodothyronine (T_3) and the inactive metabolite 3,3',5'-triiodothyronine (reverse T_3, rT_3) was already fully appreciated. Moreover, the liver and kidneys were believed to be the principal tissues in which these processes take place. Furthermore, in addition to the determination of iodothyronine serum levels under varying conditions, the interest in production and clearance rates of these compounds was growing rapidly. Thyroid hormone kinetics in vivo, be it in man or in rat, are studied with injections of labeled iodothyronines (1,2). Since this technique depends on sequential collection of plasma samples, the individual contribution of the different tissues to the production or clearance of the numerous iodothyronines remains obscure. Moreover, rapid successive intracellular reactions can not be detected by this method. Therefore, it was decided to study iodothyronine metabolism at the tissue level with the use of monolayers of isolated rat hepatocytes. It was hoped that with this model a more detailed impression of thyroid hormone handling by liver cells could be obtained.

Methods

Monolayers of freshly isolated rat hepatocytes were prepared by the method of Berry and Friend with minor modifications (3). The technical details are given in Section II:4. The overall cellular vitality after isolation, as estimated by trypan blue exclusion, was over 85%. The incubation period was started 4 hours after the cell isolation. The cells (2 x 10^6/dish) were incubated at 37C under atmospheric condition with 4 ml complete Hams' F_{10} culture medium, which contained 10% fetal calf serum (FCS) and varying amounts of L-thyroxine (T_4), 3,3',5'-triiodo-L-thyronine (rT_3), or 3,3'-diiodo-L-thyronine (3,3'-T_2) (Henning Berlin GmbH). Every experiment was performed in 8-fold. In later experiments the culture dishes were placed on a slightly angled, slowly rotating dish to improve cellular interaction with the culture medium. This method considerably enhanced the metabolic rate of the incubated iodothyronines. After incubation, the culture media were stored at -20C until further duplicate analysis by specific radioimmunoassays for either T_3 (4), rT_3 (5) or 3,3'-T_2 (6) content. In parallel incubations with rT_3 or 3,3'-T_2 varying concentrations of 2-thiouracil (TU) (Sigma), an inhibitor of deiodination, were added. The non-protein-bound fraction of rT_3 and 3,3'-T_2 in the culture medium was estimated by equilibrium dialysis. The influence of culture medium protein content on

hepatic iodothyronine metabolism was investigated by measuring the 3,3'-T_2 production in 10^{-7} M rT_3 incubates with or without 10% FCS.

Results

T_4 metabolism

In the first experiments the cells were incubated with 10^{-6} M T_4 for 2,4 and 16 h, and the media analysed for T_3 and 3,3'-T_2 content. Fig.1 shows the production curves. The appearance of T_3 in the culture medium was, even at 16 h, very low i.e. 8 nmol/l which is less than 1% of the added amount of T_4. For 3,3'-T_2 the production was even lower and amounted to only 10% of T_3. Therefore, further experiments were conducted with rT_3 and 3,3'-T_2 which showed a faster metabolic rate.

Reverse T_3 metabolism

The metabolic clearance rate of rT_3 was estimated in incubations containing 5 x 10^{-9}, 10^{-8} or 10^{-7} M of this compound. Fig.2 shows the molar and proportional disappearance of rT_3 from the culture medium with or without 100 µM TU. At the lower concentrations rT_3 showed an almost linear clearance from the culture medium. At 60 min approximately 35 to 40 percent had disappeared. With 10^{-7} M rT_3 a lower 60 min clearance of 19% was noted. In the presence of TU, however, irrespective of the rT_3 concentration, only a circa 10% decrease was observed.

Production of 3,3'-T_2 from rT_3

The appearance of 3,3'-T_2 in the culture medium was assessed from incubates which contained 10^{-8}, 10^{-7} or 10^{-6} M rT_3 with or without TU. The respective production curves of 3,3'-T_2 are depicted in Fig.3. In spite of the exponential concentration increase only a very moderate rise in 3,3'-T_2 production was noted. The data are corrected for rT_3 cross-reactivity. In the presence of 100 µM TU and irrespective of the rT_3 concentration, the generation of 3,3'-T_2 was reduced to 10% of the highest production rate. The rather constant 3,3'-T_2 production at higher rT_3 concentrations was also demonstrated in the incubations of

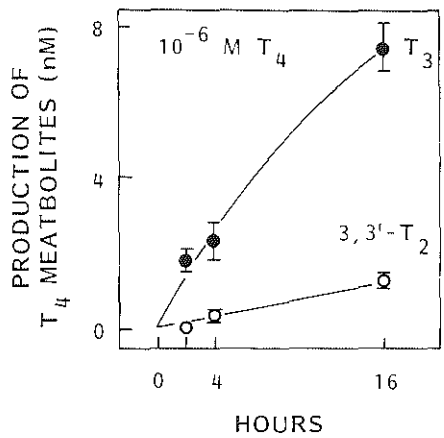

Fig.1. Production of T_3 and 3,3'-T_2 from T_4 by rat hepatocytes. 10^{-6} M T_4 was incubated with monolayers of isolated rat hepatocytes in 8-fold at 37C upto 16 h in culture medium with 10% FCS. At 2, 4 and 16 h samples of the culture medium were analysed in duplicate by RIA for T_3 and 3,3'-T_2 content. Values are given in nmol as means ± SEM.

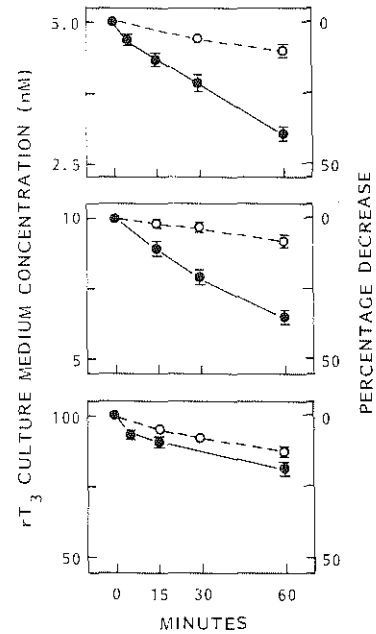

Fig.2. Influence of thiouracil on rT_3 clearance by hepatocytes. Hepatocyte monolayers were incubated at 37C upto 60 min with 5 x 10^{-9}, 10^{-8} and 10^{-7} M rT_3 in culture medium containing 10% FCS. Parallel incubations were performed with 100 µl TU(o). The culture media were analysed by RIA for rT_3 content at the given times. The values are expressed in nmol/l and represent means ± SEM of at least 4 different experiments. The points without SEM represent means of 3 experiments or less.

10^{-7} M rT_3 with or without 10% FCS. Fig.4 shows the remarkable small differences in the respective 3,3'-T_2 production curves. Since the non-protein-bound fraction of rT_3, for all concentrations used, amounted to 6.0 ± 1.4%, the medium substrate concentration, available for cellular uptake, might have been as much as 16-times higher in the protein-free incubates. The production of 3,3'-T_2 in relation to the clearance rate of 10^{-8}, 10^{-7} and 10^{-6} M rT_3 is depicted in Fig.5. At the lowest concentration tested only a minor part (22%) of the rT_3 cleared, reappeared in the medium as 3,3'-T_2. This discrepancy became even more pronounced at higher rT_3 concentrations when only 9% (10^{-7} M) and 1.3% (10^{-6} M) was recovered as 3,3'-T_2.

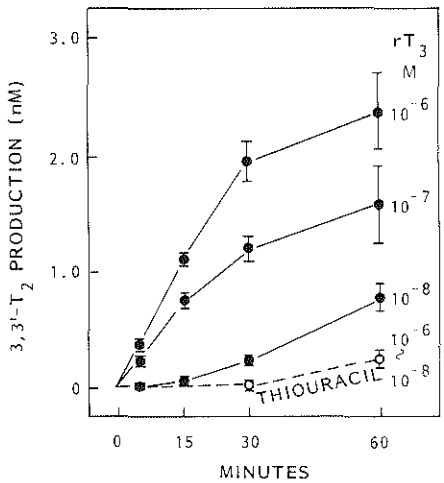

Fig.3. Production of 3,3'-T_2 from rT_3 by monolayers of isolated rat liver cells. Cells were incubated at 37C upto 60 min with 10^{-8}, 10^{-7} or 10^{-6} M rT_3. In parallel experiments 100 µM TU was added. The culture media from 8 parallel incubations in every experiment were analysed for 3,3'-T_2 content by RIA. Values are expressed in nmol/l and represent means ± SEM of at least 3 different experiments.

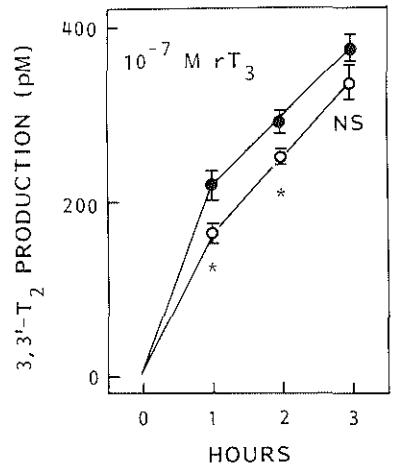

Fig.4. Production of 3,3'-T_2 from rT_3 by hepatocytes in incubations with (o) or without (•) 10% fetal calf serum. Monolayers of rat hepatocytes were incubated at 37C for 3 h with 10^{-7} M rT_3. The production of 3,3'-T_2 was measured by RIA. Values are expressed as pmol/l and represent the mean ± SEM of 8 parallel incubations.

Table 1. Disappearance of 3,3'-T_2 from the culture medium. Influence of thiouracil.

Thiouracil		3,3'-T_2 DECREASE (% ± SD)		
µM	n	30 min	n	60 min
0	18	60 ± 7	15	80 ± 7
10	7	51 ± 8*	5	72 ± 11*
100	11	51 ± 8+	8	73 ± 5*

Difference from control: * $p<0.05$
+ $p<0.01$

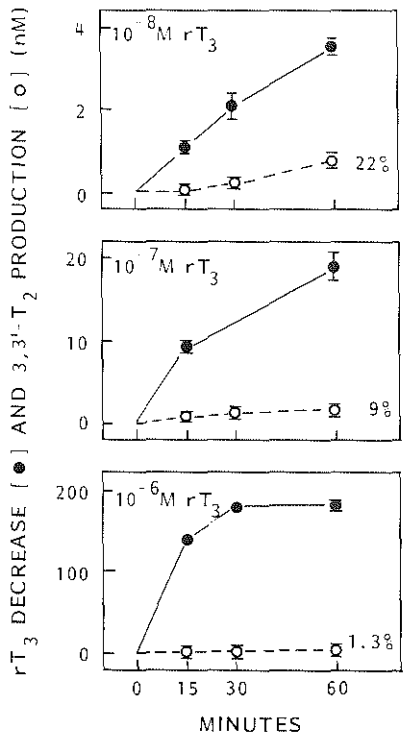

Fig.5. Production of 3,3'-T_2 in relation to the clearance rat of rT_3 by isolated hepatocytes. Monolayer rat hepatocytes were incubated at 37C for 60 min with 10^{-8}, 10^{-7} and 10^{-6} M rT_3 in culture medium with 10% FCS. The media of 8 parallel incubations per experiment were analysed for rT_3 and 3,3'-T_2 content by RIA. Values are expressed as nmol/l and represent the means ± SEM of at least 3 different experiments. When no SEM is indicated the value is derived from means of one or two experiments.

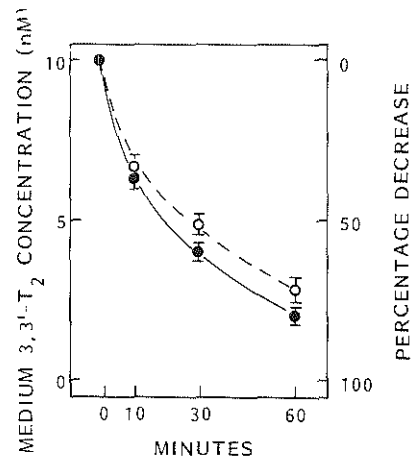

Fig.6 Thiouracil influence on 3,3'-T_2 metabolism by rat liver cells. Monolayers of hepatocytes were incubated at 37C for 1 h in the presence of 10^{-7} M 3,3'-T_2 with (○) or without (●) 100 μM TU. Media in 8 fold were analysed by RIA for 3,3'-T_2 content. The values are expressed as nmol/l and represent the means of at least 4 different experiments.

3,3'-T_2 metabolism

The metabolic rate of 3,3'-T_2 proved to be much faster than that of rT_3. At an initial medium concentration of 10^{-8} M, 3,3'-T_2 decreased by 60 and 80% after 30 and 60 min, respectively (Fig.6). In the presence of 10 or 100 μM TU, different from rT_3 again, only a small reduction of this fractional clearance was observed (Table I). The non-protein-bound fraction of 3,3'-T_2 was estimated to be 12.9 ± 2.2%.

Discussion

These initial experments showed that rat hepatocyte monolayers can be used as a model to study iodothyronine metabolism at the tissue level. We have focused our attention in this study on rT_3 and 3,3'-T_2 metabolism. The higher metabolic rate of these compounds allowed shorter incubation times than with T_4. Apart from this practical aspect, incertitudes about cellular changes during culture were thus avoided.

Both rT_3 and 3,3'-T_2 are rapidly cleared from the culture medium. Their rate of disappearance, however, was not identical. At a substrate concentration of 10^{-8} M, 35% of rT_3 and 80% of 3,3'-T_2 disappeared within only 60 minutes of incubation. This difference may, at least in part, be explained by the lower non-protein-bound fraction of rT_3 (6%) in comparison with 3,3'-T_2 (13%). The relevance of the free substrate concentration in this model was demonstrated in later experiments where a close correlation with the metabolic clearance rate was observed (Section II:4). Of further interest is the influence of the presence of thiouracil on the medium clearance of rT_3 and 3,3'-T_2. At 10^{-8} M the disappearance of 3,3'-T_2 was only mildly affected by thiouracil, whereas rT_3 clearance fell from 35 to 8%. These results have puzzled us for a long time, especially when subsequent experiments showed that thiouracil actually did inhibit outer ring deiodination of 3,[3'-^{125}I]-T_2. The phenomenon could later be explained by the discovery that 3,3'-T_2 is first conjugated with sulfate before it is subjected to accelerated outer ring deiodination. Inhibition of the latter process, therefore, will result in the accumulation of 3,3'-T_2 sulfate (3,3'-T_2S) and this compound is not detected by the 3,3'-T_2 radioimmunoassay (Section II:5).

Simultaneously, the notable reduction of rT_3 clearance by thiouracil suggests that for this metabolite no such intermediate metabolic step occurs and that deiodination is a direct and quantitatively important degradative pathway. This notion has later been supported by experiments with outer ring labeled reverse T_3. Chromatography of the medium on Sephadex LH-20 revealed no accumulation of rT_3 conjugates in the presence of PTU. Also no reduction of rT_3 outer ring deiodination was noted in sulfate depleted hepatocytes (Otten, unpublished). Interestingly, the decreasing fractional clearance of rT_3 from the culture medium with increasing substrate concentrations (Fig.2) suggests saturation of the hepatic rT_3 deiodinating capacity. The concentration range in question (10-100 nM) corresponds remarkably well with the apparent K_m value for in vitro outer ring deiodination of rT_3 with rat liver microsomes, i.e. 0.06 μM (14).

In Chapter 4 (Section I) it is postulated that rT_3 is predominantly generated in extra-hepatic tissues and cleared by the liver. Since apparently hepatic rT_3 metabolism largly depends on direct outer ring deiodination with little alternative, degradative pathways, inhibition of deiodinase activity will inevitably result in a reduction of the metabolic clearance rate of rT_3. This accords with the reduced rT_3 clearance rate during starvation (7) or systemic illness (8-10), both conditions being associated with diminished liver deiodinase activity (11,12). The demonstrated lack of important alternative degradation of rT_3 in the liver corroborates the concept that the raised serum rT_3 levels in the above situations are explained by reduced outer ring deiodinase activity and unimpeded rT_3 production.

If indeed hepatic outer ring deiodination of rT_3 is a direct major clearance pathway, an equivalent production of 3,3'-T_2 would be expected, since 3,3'-T_2 is a poor substrate for rat liver microsomal deiodinase. However, only a very modest 3,3'-T_2 production from rT_3 was noted. This "metabolic gap" is explained by the excellent substrate properties of 3,3'-T_2 for rat liver sulfotransferase activity (13) and the large sulfation capacity of the hepatocytes. Most of the 3,3'-T_2 generated from rT_3 will be conjugated immediately with sulfate and deiodinated in the outer ring prior to cellular secretion. The rather constant 3,3'-T_2 production at higher rT_3 concentrations can be explained by the high capacity of the hepatic phenol sulfotransferases which is far from being saturated at the 3,3'-T_2 concentrations measured (Section II:4).

More detailed information about the contribution of glucuronidation and sulfation to overall hepatic rT_3 metabolism and deiodination can be expected from further incubations of labeled rT_3 with hepatocyte monolayers and subsequent chromatographic analysis of the culture media.

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PAPER 3

SULFATION PRECEDING DEIODINATION OF IODOTHYRONINES IN RAT HEPATOCYTES

M.H. Otten, J.A. Mol, T.J. Visser

Abstract. *In man and animals iodothyronines are metabolized by deiodination and conjugation with glucuronic acid or sulfate. Until now these processes have been regarded as independent reactions. However, in the present study a close interaction of these pathways was observed in the hepatic metabolism of 3,3'-diiodothyronine and 3,3',5-triiodothyronine. Studies with rat hepatocytes and liver microsomes indicated that sulfation of the phenolic hydroxyl group facilitates the deiodination of these compounds.*

The thyroid gland secretes mainly thyroxine (3,3',5,5'-tetraiodothyronine, T_4), which is the precursor of the active form of thyroid hormone, 3,3',5-triiodothyronine (T_3). Some 80 percent of the total T_3 production originates from outer ring deiodination of T_4 in peripheral tissues, especially the liver. Inner ring deiodination of T_4 yields 3,3',5'-triiodothyronine (reverse T_3, rT_3), a biologically inactive compound. Both T_3 and rT_3 are further deiodinated to 3,3'-diiodothyronine (3,3'-T_2). A second important metabolic pathway for iodothyronines is conjugation with either glucuronic acid or sulfate. These principal pathways, deiodination and conjugation, have usually been regarded as functionally distinct processes. Here we present evidence for a close association of these processes in the hepatic metabolism of iodothyronines.

The metabolism of 3,3'-T_2 and T_3 was studied with monolayers of rat hepatocytes, prepared as described previously (*1*). Whereas hepatic T_3 metabolism is rather complex, 3,3'-T_2 metabolism is easier to follow as it is subject only to outer ring deiodination and sulfation (*2*). Initially, therefore, we focused on the metabolism of 3,3'-T_2 by incubating 3,[3'-^{125}I]T_2 with hepatocytes in monolayers. The medium was analyzed by chromatography on Sephadex LH-20. A good separation was obtained between $^{125}I^-$, produced by outer ring deiodination, and 3,3'-T_2 sulfate (T_2S) and unprocessed 3,3'-T_2. At substrate concentrations of 10 n*M* 3,3'-T_2 or less, iodide was the principal product observed (> 90 percent). At higher 3,3'-T_2 concentrations or during coincubation with 6-propyl-2-thiouracil (PTU), an inhibitor of deiodination, increasing amounts of T_2S accumulated in the medium at the expense of I^- formation (*2*). The rate of outer ring deiodination of 3,3'-T_2 in rat liver cells is similar to that of rT_3 (*3*). This is a surprising observation because 3,3'-T_2, in comparison to rT_3, is a poor substrate for outer ring deiodination by microsomal deiodinase activity (*4*). We, therefore, suspected that in intact hepatocytes deiodination of 3,3'-T_2 is preceded, and in effect accelerated, by sulfate conjugation. If indeed sulfation precedes deiodination of 3,3'-T_2, inhibition of the

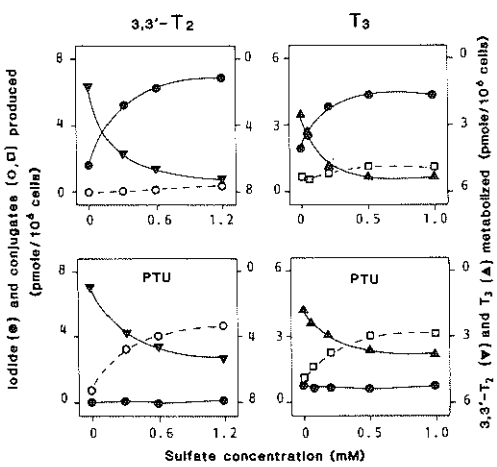

Fig. 1. Sulfate dependence of outer ring deiodination of 3,3'-T_2 and T_3 by monolayers of isolated rat hepatocytes. Approximately 2×10^6 hepatocytes were incubated for 60 minutes with sulfate- and protein-free Dulbecco's balanced salt solution to reduce the cellular sulfate content (*13*). Hereafter, incubations were performed with 4 ml of Dulbecco's medium containing 10 n*M* unlabeled plus 2 μCi of ^{125}I-labeled 3,3'-T_2 or T_3 and increasing sulfate concentrations. Identical incubations were conducted in the presence of 100 μ*M* PTU (lower panels) to inhibit deiodination. The 3,3'-T_2 incubations contained 0.5 percent bovine serum albumin. After 30 minutes for 3,3'-T_2 and 180 minutes for T_3, 100-μl samples were taken from the medium. After protein precipitation with ethanol, the supernatants were evaporated. The residues were dissolved in 0.1*N* HCl and chromatographed by subsequent 0.1*N* HCl and 0.1*N* NaOH elution on small (0.75 ml) Sephadex LH-20 columns. By this method a good separation was obtained between iodide and the conjugated and free iodothyronines, respectively. From the radioactivity in the various fractions, before and after incubation, the respective production and clearance rates were calculated. The results are expressed as picomoles produced or metabolized by 10^6 cells. The conjugates of 3,3'-T_2 and T_3 were identified by hydrolysis with sulfatase or β-glucuronidase (Sigma). Whereas 3,3'-T_2 was exclusively sulfated, T_3 was also glucuronidated. In the absence of PTU, T_3 glucuronide was the major conjugate. In the presence of PTU, the sulfate-dependent increment of T_3 conjugates represents accumulation of T_3S. Symbols: ●, iodide; ▼, 3,3'-T_2; ○, T_2S; ▲, T_3; □, T_3 conjugates.

former process will inevitably result in a reduction of deiodination.

Sulfation in hepatocytes may be influenced by (i) reduction of the SO_4^{2-} concentration in the medium and (ii) inhibition by compounds such as salicylamide, 2,6-dichloro-4-nitrophenol (DCNP), or pentachlorophenol (PCP) (*5*). We tested both possibilities. Figure 1 shows that I^- production in the absence of PTU and T_2S formation in the presence of this inhibitor are remarkably similar functions of the SO_4^{2-} concentration in the medium. The disappearance of 3,3′-T_2, with or without PTU, also depends on medium SO_4^{2-}. Addition of 10 μ*M* salicylamide, 100 μ*M* DCNP, or 100 μ*M* PCP reduced outer ring deiodination by 50, 48, and 70 percent, respectively. In the presence of PTU, T_2S formation was inhibited by these compounds to similar extents, that is, 60, 57, and 80 percent, respectively. These reductions were identical to the decrease of 3,3′-T_2 clearance.

After incubation of hepatocytes with [3′-^{125}I]T_3, metabolites were separated on Sephadex LH-20 into three peaks, that is, I^-, T_3 conjugates, and unreacted T_3 (Fig. 1). The conjugate peak contained both T_3 sulfate (T_3S) and T_3 glucuronide as revealed by enzymatic hydrolysis. Glucuronidation was not affected by PTU or SO_4^{2-}. At low SO_4^{2-} levels little T_3S formation was observed. The increase of the conjugate peak with increasing SO_4^{2-}, especially in the presence of PTU, reflects the accumulation of T_3S. A similar SO_4^{2-} dependence of T_3 deiodination was observed in the absence of PTU.

Deiodination of T_3 without PTU and T_3S formation with PTU were inhibited to similar extents by 25 μ*M* salicylamide, 10 μ*M* DCNP, and 1 μ*M* PCP, that is, 80, 85, and 75 percent, respectively. The greater effectiveness of DCNP and PCP in this case is explained by the use of albumin-free medium. No effect of these compounds on glucuronidation was noted.

The second part of this study consisted of the determination of the enzymatic characteristics of outer ring deiodination of 3,3′-T_2, T_2S, and rT_3 by rat liver microsomes. This subcellular fraction is the principal site of deiodinase activity in rat liver (*4*). Outer ring deiodination of the three ^{125}I-labeled substrates was estimated from the iodide production in incubations with microsomes. Figure 2 shows the rate of deiodination as a function of the substrate concentration. In accordance with earlier work (*4*) rT_3, with a Michaelis constant (K_M) of 0.1 μ*M*, is the best substrate for outer ring deiodination. The K_M of T_2S (0.3 μ*M*) is close to that of rT_3, making it a much better substrate for microsomal deiodinase than 3,3′-T_2 (K_M 9 μ*M*). Several compounds were tested as potential inhibitors of the deiodination of 0.01 μ*M* rT_3 or T_2S. A close correlation (r = .99, P < .01) was observed between the degrees of inhibition of both reactions by these compounds at 1 μ*M* (*6*). This observation is compatible with deiodination of rT_3 and T_2S by a single enzyme.

Our observations demonstrate that although 3,3′-T_2 and T_3 are poor substrates for deiodination by the microsomal fraction of rat liver, this process readily takes place in rat hepatocytes. Three lines of evidence support the view that sulfation of 3,3′-T_2 and T_3 in hepatocytes yields conjugates which are highly prone to deiodination. First, significant amounts of the sulfate conjugates accumulate only if deiodination is blocked with PTU. Second, deiodination varies with the sulfotransferase activity of the cells, either by restriction of SO_4^{2-} in the medium or by the addition of inhibitors. Of these, salicylamide is the most specific competitive inhibitor of phenolsulfotransferases (*7*). The metabolic inhibitors PCP and DCNP may, in addition, interfere with the synthesis of adenosine 3′-phosphate-5′-phosphosulfate (*7*), while PCP may also inhibit deiodination directly (*6*). The similar influence of the above conditions on I^- production and on sulfate conjugation in the presence of PTU strongly suggests that sulfation is the rate-limiting step preceding deiodination. Third, T_2S and T_3S are preferred substrates for microsomal deiodinase activity. This is illustrated by the low K_M of T_2S (0.3 μ*M*) in comparison with 3,3′-T_2 (9 μ*M*). For T_3S we observed an enhanced inner ring deiodination by rat liver microsomes [K_M 4.6 μ*M*, maximum velocity (V_{max}) 1050 pmole per minute per milligram of protein] compared with T_3 (K_M 10.7 μ*M*, V_{max} 33 pmole/min-mg protein). The T_2S generated is then rapidly deiodinated in the outer ring (*8*).

It is not surprising that 3,3′-T_2 and T_3 are sulfated in rat hepatocytes. They both are substrates for rat liver cytosolic sulfotransferases (*9*). However, the preferential deiodination of iodothyronine sulfates casts a new light on the processes involved with the peripheral metabolism of thyroid hormone. It is interesting that monkey hepatoma cells showed no reduction of T_3 inner ring deiodination when sulfation was inhibited (*10*). The very different characteristics of inner ring deiodination in hepatoma homogenates (K_M 0.034 μ*M*, V_{max} 223 fmole/min-mg protein) and the lack of inhibition by PTU (*10*) resemble the PTU-insensitive deiodination of T_3 in rat cerebral cortex (K_M 0.021 μ*M*, V_{max} 320 fmole/min-mg protein) (*11*). For this type

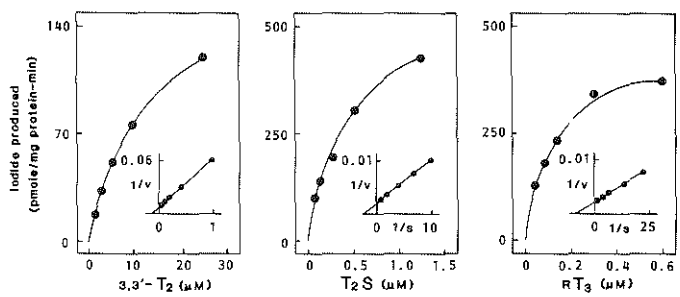

Fig. 2. Linear and double reciprocal (inset) plots of the rate of outer ring deiodination of 3,3′-T_2, T_2S, and rT_3 by rat liver microsomes as a function of substrate concentration. Deiodination was estimated according to the method of Leonard and Rosenberg (*14*) by measuring the production of $^{125}I^-$ in 200-μl reaction mixtures. The mixtures contained diluted rat liver microsomes and adequate concentrations of outer ring ^{125}I-labeled 3,3′-T_2, T_2S, or rT_3 in buffer (0.15*M* sodium phosphate (*p*H 7.2), 3 m*M* EDTA, and 5 m*M* dithiothreitol, the cofactor for deiodination). Microsomal protein concentrations were 8.4 μg/ml for rT_3 and T_2S and 336 μg/ml for 3,3′-T_2. After incubation for 10 minutes (rT_3 and T_2S) or 30 minutes (3,3′-T_2) at 37°C the reaction was stopped with 50 μl of human serum containing 5 m*M* PTU. Protein-bound substrate was precipitated by 350 μl of 10 percent trichloroacetic acid. Iodide was separated from other products in the supernatant by ion-exchange chromatography (Dowex 50W-X_2). Deiodination rates were calculated from the increase of radioactivity in the iodide fractions and expressed as picomoles per minute per milligram of protein (U). The K_M and V_{max} values are given as mean ± standard deviation: 3,3′-T_2, K_M 8.9 ± 3.9 μ*M*, V_{max} 188 ± 94 U (N = 4); T_2S, K_M 0.34 ± 0.07 μ*M*, V_{max} 353 ± 137 U (N = 3); rT_3, K_M 0.10 ± 0.02 μ*M*, V_{max} 445 ± 88 U (N = 4). T_2S was prepared by biosynthesis: 1 μ*M* 3,3′-T_2, 10 μCi of 3,[3′-^{125}I]T_2, and 100 μ*M* PTU were incubated in 4 ml of culture medium with rat hepatocytes at 37°C. After 2 hours approximately 80 percent of both labeled and unlabeled 3,3′-T_2 was sulfated. T_2S was purified by Sephadex LH-20 chromatography, and its yield was calculated from the recovery of added radioactivity.

of deiodinase sulfation does not appear to enhance deiodination. It remains to be established to what extent sulfation determines the hepatic deiodination and clearance of iodothyronines in vivo. Our results support the concept that sulfation is not merely a means to facilitate biliary and urinary excretion of hydrophobic aglycons (*12*).

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6. The respective percentages of inhibition of outer ring deiodination of rT_3 and T-S (0.01 μM) as induced by the following compounds (1.0 μM) were, respectively, T_4, 34 and 22 percent; T_3, 10 and 3 percent; rT_3, 90 and 86 percent; diiodotyrosine, 4 and 0 percent; PTU (10 μM), 80 and 90 percent; PTU (100 μM), 98 and 100 percent; iopanoic acid, 39 and 29 percent; salicylamide, 2 and 0 percent; dichloronitrophenol, 6 and 2 percent; pentachlorophenol, 68 and 70 percent. Linear regression yielded: $r = .99$, $P < .01$.
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PAPER 4

METABOLISM OF 3,3'-DIIODOTHYRONINE IN RAT HEPATOCYTES
Interaction of sulfation with deiodination

M.H. Otten, G. Hennemann, R. Docter, T.J. Visser

Abstract

Production of 3,3'-diiodothyronine (3,3'-T_2) is an important step in the peripheral metabolism of thyroid hormone in man. The rapid clearance of 3,3'-T_2 is accomplished to a large extent in the liver. We have studied in detail the mechanisms of this process using monolayers of freshly isolated rat hepatocytes. After incubation with 3,[3'-^{125}I]-T_2, chromatographic analysis of the medium revealed two major metabolic routes: outer ring deiodination and sulfation. We recently demonstrated that sulfate conjugation precedes and in effect accelerates deiodination of 3,3'-T_2. In media containing different serum concentrations the cellular clearance rate was determined by the non-protein-bound fraction of 3,3'-T_2. Below 10^{-8} M substrate concentration [^{125}I] iodide was the main product observed. At higher concentrations deiodination became saturated, while the sulfate ester of 3,3'-T_2 (T_2S) accumulated in the medium. Saturation of overall 3,3'-T_2 metabolism was found to occur only at very high ($\geq 10^{-6}$ M) substrate concentrations. The sulfating capacity of the cells exceeded that of deiodination by at least 20-fold. Deiodination was completely inhibited by 10^{-4} M PTU or thiouracil resulting in the accumulation of T_2S whereas clearance of 3,3'-T_2 was little affected. No effect was seen with methimazole. Hepatocytes from 72 h fasted rats showed a significant reduction of deiodination but unimpaired sulfation. Other iodothyronines interfered with 3,3'-T_2 metabolism. Deiodination was strongly inhibited by 2 μM T_4 and rT_3 (80%) but little by T_3 (15%), whereas overall clearance was reduced by 25% (T_4 and rT_3) and 12% (T_3).

In the presence of PTU or at high substrate concentrations ($>10^{-7}$ M) a third metabolite was generated, most likely the acetic acid analogue of 3,3'-T_2. It is concluded that the rapid hepatic clearance of 3,3'-T_2 is determined by the sulfate-transferring capacity of the liver cells. Subsequent outer ring deiodination of the intermediate T_2S is inhibited by PTU and by fasting essentially without affecting overall 3,3'-T_2 clearance.

Introduction

The study of the peripheral metabolism of thyroid hormone in vivo is complicated by the large number of successive metabolic reactions, and by the different metabolic properties of the tissues. Best documented are the conversion of the prohormone thyroxine (T_4) to the biologically active 3,3',5-triiodothyronine (T_3) and the biologically inactive 3,3',5'-triiodothyronine (rT_3). A thorough knowledge of the metabolic degradation of these and other iodothyronines is likely to contribute to a better understanding of the changes in their serum levels as induced by illness, drugs or fasting (1).

3,3'-Diiodothyronine (3,3'-T_2) is a common deiodination product of T_3 and rT_3. The production of 3,3'-T_2 in euthyroid subjects has been estimated between 38 and 76 nmol/day (2,3), compared with 115 nmol/day for T_4 (4). Production of 3,3'-T_2 is, therefore, an important step in the sequential deiodination of T_4. Nevertheless, serum levels of 3,3'-T_2 are low, between 34 and 114 pM (2,3,5,6). This is explained by its high metabolic clearance rate, with 560 to 1116 l/day the fastest of all iodothyronines (2,7). About 50% of this clearance is attributed to hepatic extraction of plasma 3,3'-T_2 (6), underlining the important role of the liver in iodothyronine metabolism.

We have studied the mechanisms of 3,3'-T_2 metabolism in monolayers of freshly isolated rat hepatocytes. The advantages of this model for the study of thyroid hormone metabolism have been outlined by Sato and Robbins (8). Apart from practical aspects, the importance of cellular integrity is illustrated by the striking differences in 3,3'-T_2 metabolism observed with hepatocytes or with liver homogenates (9).

In the early stages of this study it appeared that 3,3'-T_2 is metabolized in liver cells mainly by outer ring or 3'-deiodination and by conjugation with sulfate (10). These pathways were believed to be independent and to have different metabolic functions. However, we recently demonstrated that in rat hepatocytes sulfation is an essential step preceding and accelerating the deiodination of 3,3'-T_2 (9). Similarly, sulfation facilitates the inner ring deiodination of T_3

(9,11). Sulfation is, therefore, an important factor in the peripheral metabolism of thyroid hormone.

We now present in detail the quantitative aspects of hepatic 3,3'-T_2 metabolism and the crucial role of sulfate conjugation. In particular, conditions were tested that are known to inhibit deiodinase activity, i.e. in vivo fasting and in vitro addition of iodothyronine analogues or thiouracil derivatives.

Materials and methods

3,3'-Diiodo-L-thyronine (3,3'-T_2), 3,3',5'-triiodo-L-thyronine (rT_3), 3,3',5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) were purchased from Henning Berlin GmbH, FRG. 3,[3'-^{125}I]-diiodothyronine with a specific activity of 3300 Ci/g was prepared as described earlier (5). Before every experiment the tracer was purified on Sephadex LH-20. Contamination with $^{125}I^-$ was less than 1.0%. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The following materials were from Sigma Chemical Co., St. Louis, MO: 2-thiouracil, 6-propyl-2-thiouracil, methimazole, D-saccharic acid-1,4-lactone, sulfatase type VIII, β-glucuronidase type IX, collagenase type I, L-aminoacid oxidase, bovine pancreas insulin, Pipes, Hepes and Bes. Fetal calf serum, newborn calf serum (NBCS), penicillin and streptomycine sulfate were obtained from Flow Laboratories, Irvine, UK. Ham's F_{10} nutrient mixture was purchased from Gibco Europe, Hoofddorp, The Netherlands.

Isolation of rat hepatocytes and in incubation procedures

Isolated rat hepatocytes were prepared essentially as previously described (12). After successive perfusion of the liver with Ca^{2+} free Hanks' salt solution for 10 min and with the same medium containing 0.05% collagenase for 20 min at pH 7.4 under 5% CO_2 and 95% O_2, the liver was extirpated and minced. Cells were separated from tissue debris by repeated centrifugation (50 x g) and resuspending until a clear supernatant was obtained. Immediately after the isolation procedure cell viability, as estimated by trypan blue exclusion, was over 90%. Culture medium (4 ml) containing $\sim$ 2 x 10^6 suspended cells was slowly infused in sterile, plastic culture dishes, 5 cm in diameter (Costan, Cambridge, MA, USA). The dishes were placed in a culture stove at 37C for 4 h under atmospheric conditions. During this period the culture medium was composed of unmodified Ham's F_{10}, 10.6 mM Pipes, 11.2 mM Bes, 8.9 mM Hepes (pH 7.4), 2 mM $CaCl_2$, 12

mU/l insulin, 10 U/ml penicillin, 10 µg/ml streptomycin sulfate and 10% fetal calf serum. After this preincubation the viable cells (> 85%) were firmly attached to the culture dish. Subsequently, incubations were performed at 37C under atmospheric conditions. The dishes were placed on a slightly angled, slowly rotating plate to ascertain optimal cell-fluid interaction. In these incubations the general composition of the culture medium was identical, except that 10% newborn calf serum (NBCS) instead of fetal calf serum was used, and no penicillin or streptomycin were added. This medium contains 0.6 mM $MgSO_4$ which ensures optimal 3,3'-T_2 sulfation (9).

Analytical procedures

Incubation media with unlabeled 3,3'-T_2 were analysed by a specific RIA (5). Incubation media with labeled 3,3'-T_2 were assayed by column chromatography. For this purpose samples (100 µl) of the culture medium were extracted with 1.0 ml ethanol containing 10^{-4} M PTU, and evaporated at 50C under a stream of N_2. The residue was dissolved in 1 ml 0.1 N HCl and applied to small (0.75 ml bed volume) Sephadex LH-20 columns. Good separation of 3,3'-T_2 and its metabolites was obtained by subsequent elution with 1 ml fractions of 0.1 N HCl, 0.1 N NaOH and ethanol. The free fraction of the various iodothyronines in the culture medium was determined by equilibrium dialysis. In some experiments, the free fraction was modified by changes of the NBCS content of the culture medium. The number of cells in every experiment was estimated from the average DNA content of 8 to 16 culture dishes and expressed as µg DNA per dish. DNA content was determined according to the method of Burton (13).

Analysis of 3,3'-T_2 conjugates

The chromatographic fractions, expected to contain 3,3'-T_2 conjugates, were collected, neutralized and evaporated. The remaining material was dissolved in the appropriate buffer for incubation with sulfatase or β-glucuronidase. Approximately 10 nmol of 3,3'-T_2 conjugate was incubated at 37C overnight in 500 µl buffer with either enzyme at various concentrations (25 to 500 µg/ml) with or without 5 mM D-saccharic acid-1,4-lactone, an inhibitor of β-glucuronidase. The buffers used were 0.05 M sodium acetate (pH 5.0) for sulfatase and 0.05 M sodium phosphate (pH 6.8) for β-glucuronidase. The mixtures, after extraction with ethanol, were again chromatographed on Sephadex LH-20. Since 250 µg/l sulfatase

yielded complete hydrolysis of T_2S, this concentration was routinely used.

3,3'-T_2 metabolism by isolated rat hepatocytes

During the initial stages of this study various concentrations (10^{-10} to 10^{-7} M) of unlabeld 3,3'-T_2 were incubated with the cells. Samples of the culture medium in 8 parallel incubations were taken from 0 to 60 min and analysed for 3,3'-T_2 content by RIA. Similar experiments were performed in the presence of 10^{-5} and 10^{-4} M PTU. In later experiments 0.5 µCi 3,[3'-^{125}I]-T_2 was added to enable chromatographic analysis of the culture medium on Sephadex LH-20. The concentration range was extended from 10^{-11} to 10^{-5} M unlabeled 3,3'-T_2. Initially, time curves were produced; later on, samples were taken at 0 and 30 or 60 min. The influence of thiouracil, PTU and methimazole was investigated by addition of 10^{-5} and 10^{-4} M of these substances to the incubations. Similarly, interference of other iodothyronines with 3,3'-T_2 metabolism was studied by co-

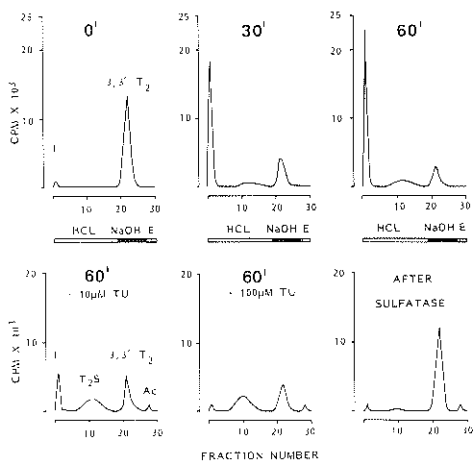

Fig.1. Chromatographic patterns of ethanol extracted culture media. Culture medium (4 ml) containing 10% NBCS, 10^{-8} M 3,3'-T_2, 3,[3'-^{125}I]-T_2, and 0, 10^{-5} or 10^{-4} M thiouracil was incubated with monolayers of isolated rat hepatocytes. Samples (100 µl) of the culture medium at 0, 30 or 60 min were extracted with ethanol and evaporated. The residue was dissolved in 1 ml 0.1 N HCl and applied to small HCl equilibrated Sephadex LH-20 columns. Elution was performed by 1 ml fractions with 0.1 N HCl, 0.1 N NaOH and pure ethanol (E) respectively, as indicated by the horizontal bar. The material eluting between iodide (I) and 3,3'-T_2 was identified as the sulfate ester of 3,3'-T_2 (T_2S) by hydrolysis with sulfatase. The elution profile after hydrolysis is depicted in the lower right panel. A metabolite that was tentatively identified as 3,3'-diiodothyroacetic acid (diac, Ac) eluted in the ethanol fractions.

incubation with 0.1-2 μM T_4, T_3 or rT_3 after 1 h preincubation period with these hormones. To assess the influence of varying numbers of cells in the culture dishes, monolayers were prepared with 25, 50, 100 and 200% of the usual number of cells. Cell number was verified by measuring the DNA content of the various culture dishes (12,13). Other experiments were conducted with cells isolated from 72 h fasted rats. Cell isolation, plating and experimental procedures were otherwise unmodified. Conversion rates were corrected for DNA content when cells from fed and fasted rats were compared.

Determination of substrate in the cellular compartment

The amount of 3,3'-T_2 in the cellular compartment during incubation was estimated by the decrease of total radioactivity in sequential 50 μl samples of the culture medium. The decrease measured was corrected for changes in radioac-

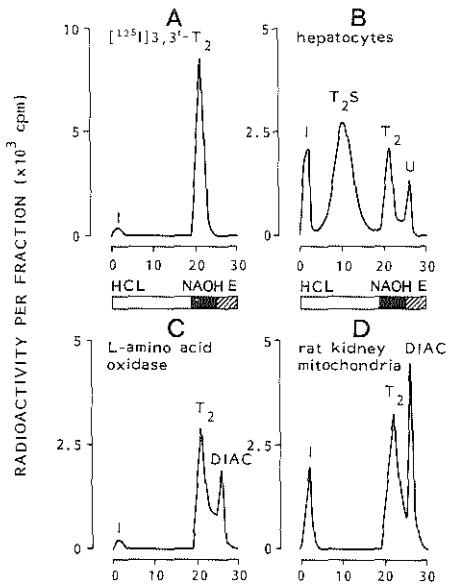

Fig.2. Elution profile of 3,3'-diiodothyroacetic acid (diac) on Sephadex LH-20. Diac was synthetized by incubation of 10^{-6} M 3,3'-T_2 and 3,[3'-^{125}I]-T_2 with L-aminoacid oxidase (C) and sonicated rat kidney mitochondria (D) (for conditions see Materials and Methods). The mixtures were extracted with ethanol, evaporated and dissolved in 1 ml 0.1 N HCl. This sample was applied to HCl equilibrated Sephadex LH-20 columns and eluted with 1 ml fractions of 0.1 N HCl, 0.1 N NaOH and ethanol (E) respectively, as indicated by the horizontal bar. Profiles are shown under C and D. Diac eluted in the same fractions as the unidentified material (U) found after incubation of 10^{-6} M 3,3'-T_2 with isolated rat hepatocytes (B). The elution profile of 3,[3'-^{125}I]-T_2 with a small iodide (I) peak is shown in panel A.

tivity in cell free culture dishes. The result, expressed as percentage of total radioactivity, was assumed to represent the amount of 3,3'-T_2 in the cellular compartment. At the end of the incubation, cells were dissolved in 1 ml 0.1 N NaOH and counted for radioactivity.

Oxidative deamination of 3,3'-T_2

In order to identify the elution pattern of 3,3'-diiodothyroacetic acid (diac) on Sephadex LH-20, this substance was synthetized by incubation of 3,3'-T_2 with both sonicated rat kidney mitochondria (14) or L-aminoacid oxidase (15). Preparation of rat kidney mitochondria was done according to the method of Nakano and Danowski (15). Sonicated mitochondria (1 mg protein/ml) were incubated for 3 h at 37C with 10^{-6} M 3,3'-T_2, 0.02 µCi 3,[3'-^{125}I]-T_2, and 10^{-4} M PTU in a 0.15 M sodium phosphate and 3 mM EDTA (pH 7.4) under 100% oxygen. Similarly, L-aminoacid oxidase (0.1 mg/ml) was incubated for 1 h at 37C with identical amounts of 3,3'-T_2 and PTU in 0.1 M sodium phosphate and 2 mM EDTA (pH 6.5). Both mixtures were extracted with ethanol and chromatographed on Sephadex LH-20.

Results

As shown before (9,10), monolayers of freshly isolated rat hepatocytes are very active in metabolizing 3,3'-T_2. Fig.1 shows that radioiodide was the main product formed in incubations with 10^{-8} M outer ring labeled 3,3'-T_2 in the absence of thiouracil. The second metabolite eluting in our chromatographic procedure was observed especially when deiodination was inhibited with thiouracil. This metabolite was identified as 3,3'-T_2 sulfate (T_2S) by the complete hydrolysis with 0.25 mg/ml sulfatase, independent of the presence of saccharic acid lactone. No substantial hydrolysis was observed with β-glucuronidase. Next to iodide and T_2S a third metabolite was generated in small amounts in the presence of thiouracil but also at high substrate concentrations (see below). This compound was tentatively identified as 3,3'-diiodothyroacetic acid (diac) by comparison with the elution profile in the two diac preparations obtained as described under "Materials and Methods" (Fig.2). The greater adsorption of diac onto Sephadex LH-20 compared with 3,3'-T_2 is in keeping with its reduced polarity due to the loss of the NH_2-group. Increased lipophilicity has also been observed for the acetic acid analogues of T_4 and T_3 in reversed-phase liquid chromatography (16).

Table 1. Effect of thiouracil on the metabolism of 3,3'-T_2 as measured by RIA and Sephadex LH-20 chromatography. Hepatocytes were incubated for 30 min with 10^{-8} M 3,3'-T_2[a].

Thiouracil (M)	% 3,3'-T_2 metabolized[b]				Proportion of products formed (%)[b]		
	n	RIA	n	LH-20	I^-	T_2S	"Diac"
--	15	58.0 ± 2.3	18	60.3 ± 1.7	92 ± 2	8 ± 2	-
10^{-5}	6	55.6 ± 2.7	7	50.9 ± 3.0[c]	32 ± 5	66 ± 6	3 ± 1
10^{-4}	3	50.7 ± 9.2	11	50.6 ± 2.4[d]	5 ± 1	93 ± 1	3 ± 2

[a] Per experimental point chromatography was done in duplicate and RIA in 8-fold; n = number of experiments

[b] Mean ± SE

[c] Significant difference with controls by Student's t test, $p<0.05$;

[d] $p<0.01$

Table 1 compares the rate of 3,3'-T_2 metabolism as measured by RIA or by Sephadex chromatography. No significant difference between the two methods was observed. Since T_2S is the main product in the presence of thiouracil, there is apparently no important crossreactivity of the conjugate in the 3,3'-T_2 RIA. This agress with the observation that 3,3'-T_2 is undetectable by RIA in unhydrolyzed assay mixtures with T_3 sulfate (T_3S) or T_2S (11). Furthermore, it is shown in Table 1 that deiodination of 3,3'-T_2 is inhibited by thiouracil in a dose-dependent manner, being virtually undetectable at 10^{-4} M of the inhibitor. In contrast, overall clearance of 3,3'-T_2 was only reduced by 10%. Identical results were obtained with PTU, but 10^{-4} M methimazole had no effect. The recovery of radioactivity in the chromatographic analysis was 95-97% for the ethanol extraction of the medium, and 99.7 $\pm$ 2.3% (n=100) for the column chromatography.

Influence of substrate concentration on 3,3'-T_2 metabolism

In a previous study (9) the metabolism of 3,3'-T_2 was tested at a single substrate concentration (10^{-8} M). Fig.3 shows the production of medium iodide and T_2S from varying concentrations of added 3,3'-T_2. At low ($< 10^{-8}$ M) 3,3'-T_2

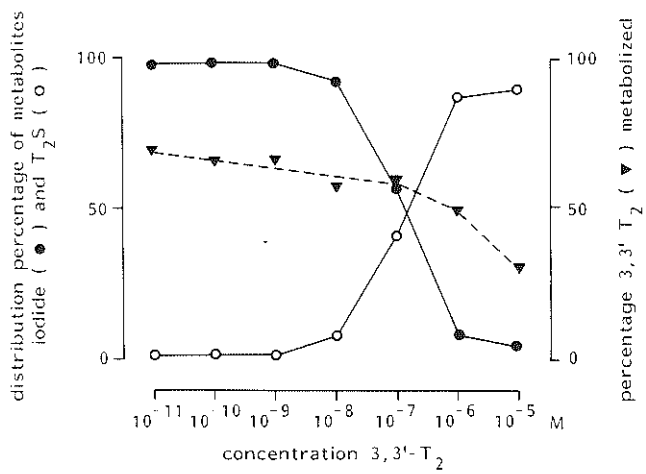

Fig.3. Influence of substrate concentration on 3,3'-T_2 metabolism by isolated rat hepatocytes. Culture medium (4 ml) containing 10^{-11} to 10^{-5} M 3,3'-T_2, 3,[3'-^{125}I]-T_2 and 10% NBCS was incubated for 30 min at 37C with the cells. After incubation analysis of the composition of the culture medium was made by Sephadex LH-20 column chromatography. The radioactivity in the iodide and T_2S fractions was expressed as percentage of the converted amount of 3,3'-T_2 (left hand scale). The decrease of 3,3'-T_2 in the culture medium is depicted as percentage of the original amount present before incubation (right hand scale).

concentrations little T_2S formation was detected. However, if deiodination was saturated at increasing 3,3'-T_2 concentrations, accumulation of T_2S was observed. The results indicate a higher sulfating than deiodinating capacity of the cells. This is further illustrated in Fig.4 that gives the molar amounts of the metabolites produced during incubation of hepatocytes for 60 min with different substrate concentrations. Especially at high 3,3'-T_2 concentrations substantial quantities of "diac" were produced. A fourth possible metabolite, 3'-iodothyronine (3'-T_1) whether free or in the conjugate form, was never detected in the culture medium either by chromatography or by RIA (17).

Another aspect of interest was the almost constant fractional clearance of 3,3'-T_2 from the culture medium over a wide range of concentrations (Fig.3). In spite of a 10^4-fold increase in concentrations, the fraction of 3,3'-T_2 metabolized only decreased from 69 $\pm$ 5% (10^{-11} M) to 59 $\pm$ 3% (10^{-7} M). At still higher concentrations this percentage more rapidly declined to 49 $\pm$ 4% (10^{-6} M) and 30 $\pm$ 4% (10^{-5} M) indicating saturation of the cellular 3,3'-T_2 metabolizing capacity. These results are not biased by difference in the non-protein-bound substrate fraction since this percentage, as assessed by equilibrium dialysis, was identical (12.9 $\pm$ 2.2%) for all concentrations used. The relevance of this free fraction for hepatic 3,3'-T_2 clearance was demonstrated in experiments where the

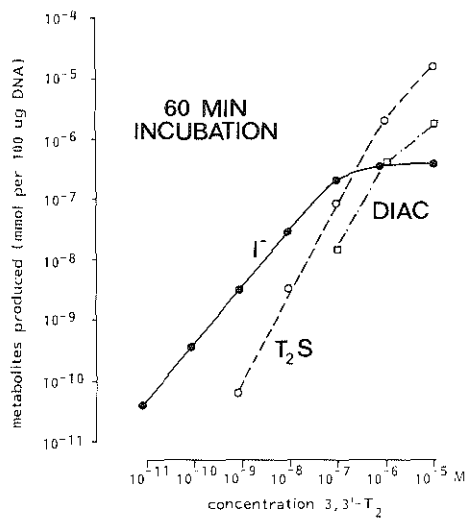

Fig.4. Influence of substrate concentration on production of iodide (I^-), T_2S and a metabolite that was tentatively identified as 3,3'-diiodothyroacetic acid (diac) by isolated rat hepatocytes. Culture medium containing 10^{-11} to 10^{-5} M 3,3'-T_2, 3,[3'-^{125}I]-T_2 and 10% NBCS was incubated at 37C for 60 min with the cells. The molar production of the three metabolites, expressed as mmol per 100 µg DNA, was calculated from the radioactivity in the respective fractions after column chromatography of extracted culture media.

protein content of the culture medium was varied. With increasing concentrations of NBCS (10, 20 and 30%) the non-protein-bound fraction decreased from 12.1 to 10.7 and 9.7%, respectively, which correlated strongly with the diminishing 30 min fractional clearance of 3,3'-T_2: 74.9, 56.3 and 40.3% ($r=0.99$, $p<0.01$). However, the decrease in 3,3'-T_2 clearance is greater than that in the free fractions, indicating that in this model the relation between the two parameters is not a simple one.

Substrate content of the cellular compartment

When corrected for non-specific binding to the culture dish, a maximum of 4.4% of added 3,3'-T_2 (10^{-8} M) was estimated to be in the cellular compartment (Fig.5). After 15 min this cell bound fraction declined as radioactivity gradually appeared in the iodide fraction. Apparently the storage capacity of hepatocytes for 3,3'-T_2 is rather low. The amount of radioactivity recovered in the cells at the end of the incubation corresponded well with that disappeared from the medium.

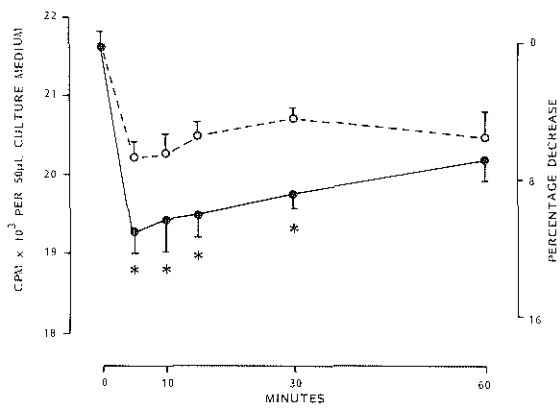

Fig.5. Total radioactivity in culture medium during incubation of 3,3'-T_2 with 3,[3'-^{125}I]-T_2 in culture dishes with (●——●) and without (o- - -o) isolated rat hepatocytes. Culture medium (4 ml) containing 10^{-8} M 3,3'-T_2, 3,[3'-^{125}I]-T_2 (~0.5 µCi/ml) and 10% NBCS was incubated for 60 min in culture dishes containing either no or approximately 2 x 10^6 cells. Sequential 50 µl samples of the culture medium were taken at 0, 5, 10, 15, 30 and 60 min from 6 different culture dishes. Radioactivity of these samples is given as mean ± SD. A significant difference ($p<0.01$) was noted for the 5 to 30 min samples as indicated by the asterisk. The right hand scale gives the percentage decrease from the original amount of radioactivity in the culture medium.

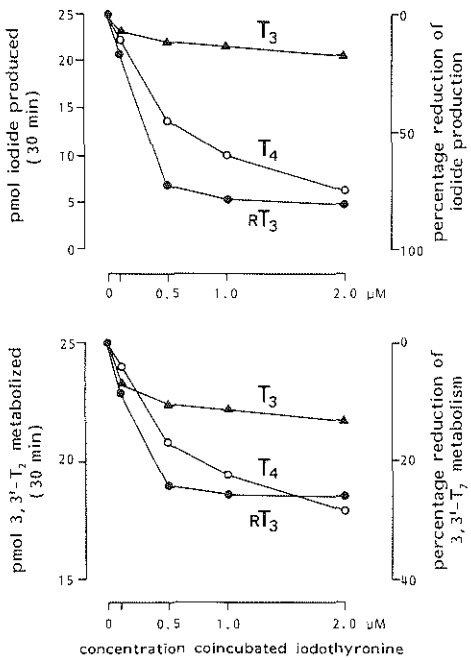

Fig.6. Influence of other iodothyronines on 3,3'-T_2 metabolism by rat hepatocytes. Cells were preincubated at 37C for 60 min with the indicated concentrations of unlabeled T_4 (o), T_3 (▲) or rT_3 (●) in the presence of 10% NBCS. After this preincubation the culture medium contained, in addition to the same amount of the respective iodothyronines, 10^{-8} M 3,3'-T_2, 3,[3'-^{125}I]-T_2 and 10% NBCS. After 30 min of incubation 100 µl of the culture medium was extracted with ethanol and chromatographed on Sephadex LH-20. The production of iodide (upper panel) and disappearance of 3,3'-T_2 (lower panel), expressed as pmol/30 min, were calculated from the radioactivity in the respective fractions (left hand scale). The decrease of iodide production and 3,3'-T_2 clearance, induced by T_4, T_3 and rT_3, is expressed as percentage of control experiments on the right hand scale.

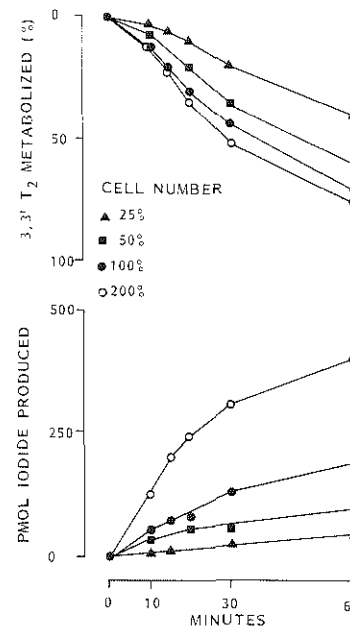

Fig.7. Influence of varying cell number on 3,3'-T_2 metabolism by rat hepatocytes. Cell isolation was performed as described in Materials and Methods. Before plating separate cell suspensions were prepared containing 25, 50, 100 or 200% of the usual 2 x 10^6 cells. The actual number of cells was verified by determination of the DNA content in the various culture dishes. All other procedures were unaltered. Hereafter, culture medium (4 ml) containing 10^{-6} M 3,3'-T_2, 3,[3'-^{125}I]-T_2 and 10% NBCS was incubated for 60 min at 37C with the various cell concentrations in duplicate. Sequential 100 µl samples were drawn at the indicated times, extracted with ethanol and chromatographed on Sephadex LH-20. 3,3'-T_2 decrease, expressed as percentage of original amount, as well as iodide production (pmoles) were calculated from the radioactivity in the respective fractions.

Effects of other iodothyronines on 3,3'-T_2 metabolism

The effects of higher iodothyronines on 3,3'-T_2 metabolism were twofold. Both deiodination and overall clearance were affected but not to the same extent. Fig.6 demonstrates that the reduction of deiodination was much more pronounced than the reduction of overall clearance of 10^{-8} M 3,3'-T_2. Reverse T_3 proved to be the most powerful inhibitor of deiodination, affording 80% inhibition at 2 μM, whereas T_3 only had a weak inhibitory potency, i.e. 15% inhibition at 2 μM. Inhibition of deiodination by rT_3 and T_4 was accompanied by an accumulation of T_2S. The clearance of 3,3'-T_2 was decreased by 25% with 2 μM T_4 or rT_3, and by 15% with 2 μM T_3. These differences were not caused by changes in the non-protein-bound fraction of 3,3'-T_2 since it was not affected by the other iodothyronines.

Effects of in vivo fasting on in vivo 3,3'-T_2 metabolism

The DNA corrected metabolic properties of hepatocytes of 72 h fasted rats and of control animals are given in Table 2. Two observations can be made from these experiments. First, the total amount of 3,3'-T_2 metabolized by the cells in 30 min did not significantly differ in both groups. Second, deiodination was reduced in cells from fasted animals resulting in the accumulation of T_2S. This reduction varied from 20 to 40% over the range of concentrations tested. Judged from the deiodination rates at saturating substrate levels, the total deiodinative capacity of the fasted cells was reduced. Since overall clearance was not diminished this suggested a decrease in the activity of the deiodinating enzyme. The lack of significance at 10^{-6} M 3,3'-T_2 may be attributed to the small fraction of substrate deiodinated at high concentrations, increasing the variation between experiments. Mean values, however, corresponded well with those found in the incubations containing 10^{-7} M 3,3'-T_2.

Influence of cell number

The dependence of 3,3'-T_2 clearance and deiodination on the number of hepatocytes in the culture dishes is given in Fig.7. Cell number amounted to 0.5, 1, 2 and 4 x 10^6 cells per dish. The DNA content varied accordingly: 9 ± 1, 19 ± 2, 44 ± 9 and 92 ± 6 μg DNA. In this experiment a concentration of 10^{-6} M 3,3'-T_2 was used in order to study the maximal deiodinative capacity of the

Table 2. METABOLISM OF $3,3'-T_2$ BY RAT HEPATOCYTES. INFLUENCES OF 72 h IN VIVO FASTING BEFORE CELL ISOLATION ON $3,3'-T_2$ METABOLIC RATE AND PRODUCTION OF IODIDE.

$3,3'-T_2$ (M)	$3,3'-T_2$ metabolized (pmol/100 µg DNA ± SD)				iodide produced (pmol/100 µg DNA ± SD)			decrease of iodide production %
	control*	fasted*	P		control	fasted	P	
10^{-11}	0.056 ± 0.006 (2)	0.040 ± 0.011 (2)	NS		0.056 ± 0.006	0.037 ± 0.008	<0.05	34
10^{-10}	0.51 ± 0.05 (5)	0.43 ± 0.17 (3)	NS		0.51 ± 0.04	0.41 ± 0.08	<0.05	20
10^{-9}	5.1 ± 0.4 (5)	4.2 ± 0.9 (3)	NS		5.0 ± 0.4	3.8 ± 0.6	<0.05	24
10^{-8}	46 ± 15 (18)	43 ± 15 (8)	NS		43 ± 13	27 ± 9	<0.05	37
10^{-7}	439 ± 96 (5)	399 ± 91 (3)	NS		254 ± 73	153 ± 41	<0.05	40
10^{-6}	3624 ± 1191 (6)	3614 ± 902 (3)	NS		238 ± 146	183 ± 162	NS	24

* Number of experiment in parentheses. Statistical analysis was performed using the Student t test.

cells. Production of iodide appeared to be linear with the number of cells but the disappearance of 3,3'-T_2 was not.

Discussion

Obviously, isolated hepatocytes provide a more physiological system to study iodothyronine metabolism than liver homogenates or subcellular fractions. Deiodination of 3,3'-T_2 by hepatocytes is much faster than deiodination by microsomes (10,18). This has been explained by the initial sulfation of 3,3'-T_2 in hepatocytes that facilitates subsequent deiodination (9). It was demonstrated that in rat liver cells both 3,3'-T_2 and T_3 have to be conjugated with sulfate before any substantial deiodination occurs (9). Both T_2S and T_3S appeared to be much better substrates for deiodination by rat liver microsomes than the non-sulfated compounds as indicated by the V_{max}/K_m ratio's. This ratio amounted to 1038 for T_2S and 21 for 3,3'-T_2 (9), and 228 for T_3S and 3 for T_3 (11).

It was demonstrated in the present study that the total capacity of 3,3'-T_2 sulfation in hepatocytes is at least 20 times higher than their deiodinating potential. On the other hand, there is litle accumulation of T_2S at low ($< 10^{-7}$ M) substrate concentrations, indicating that under these conditions sulfation is the rate-limiting step. This notion is supported by the fact that inhibition of sulfation reduces overall 3,3'-T_2 clearance (9), whereas inhibition of deiodination by, for instance, thiouracil does not affect 3,3'-T_2 clearance (Table 1). The observed rapid clearance of 3,3'-T_2 by our hepatocytes agrees with studies using isolated, perfused rat livers (19,20). In one of these, Flock et al (19) identified iodide as the principal product at a perfusate concentration of 8×10^{-8} M 3,3'-T_2.

Sulfation is the crucial step in the hepatic metabolism of both 3,3'-T_2 and T_3 (9). The latter is not as good a substrate for phenol sulfotransferases as 3,3'-T_2 (21), explaining at least in part the slower hepatic T_3 clearance (9,19,20).

Another factor influencing 3,3'-T_2 metabolism appeared to be the non-protein-bound fraction of this compound in the medium. Similar observations have been made concerning the metabolism of T_3 by hepatocytes (22). However, also in our case there is not a simple 1:1 relationship between the free 3,3'-T_2 fraction and the extent of its degradation with increasing serum concentrations in the medium. Cellular storage, on the other hand, does not contribute significantly to the 3,3'-T_2 disappearance, since no more than 5% of added radioactivity was at any time associated with the cells. A similar low percentage up-

take of 3,3'-T_2 was observed by Flock et al (19) in the perfused rat liver.

Finally, the disappearance of medium 3,3'-T_2 depended on the number of cells. The clearance of 3,3'-T_2 was not linearly related to the number of cells in contrast to iodide production. A likely explanation for this phenomenon may be found in the different capacities of the sulfating and deiodinating systems of the hepatocyte. At the high 3,3'-T_2 concentrations used (10^{-6} M) deiodination is saturated, whereas sulfation, which determines the 3,3'-T_2 clearance, is not (Fig.4). Interestingly, Faber et al (23) noted in euthyroid and hyperthyroid human subjects an identical 40% hepatic 3,3'-T_2 clearance in spite of the greatly elevated levels in the latter group. This may reflect the constant 3,3'-T_2 clearance over a wide range of concentrations observed by us.

Oxidative deamination is a minor pathway for the elimination of iodothyronines (24), Roche et al observed generation of diac from 3,3'-T_2 in rat kidneys (25). Especially at high substrate concentrations we found that the hepatocytes produced a compound with chromatographic properties that are compatible with diac. However, definitive identification would require the demonstration of co-elution in more refined chromatographic systems such as HPLC. It is of interest that tetrac is only detected in rat bile after administration of a high dose of T_4 (16).

The occurence of inner ring deiodination of 3,3'-T_2 or T_2S in hepatocytes is unlikely since no formation of 3'-T_1 or its sulfate was observed. This is not due to the subsequent metabolism of these compounds by rapid outer ring deiodination since both 3'-T_1 and its sulfate proved to be poor substrates for microsomal deiodination (Otten and Visser, unpublished observations).

It is important to realize that the conditions used in our experiments do not reflect the actual in vivo situation. The concentrations 3,3'-T_2 are very high in comparison with those found in sera of rat and man (5), while the protein content of the culture medium is considerably lower. However, this experimental design, by its simplicity, provides a suitable model for studying the multiple processes involved in hepatic iodothyronine handling. We have investigated apart from substrate concentrations, the influences of thiourea derivatives, fasting and other iodothyronines on 3,3'-T_2 metabolism in these cells. PTU and thiouracil proved to be potent inhibitors of T_2S deiodination in cells as well as with rat liver microsomes (9). A rapid cellular uptake and metabolic availability of these compounds is suggested by the immediate onset of inhibition without preincubation. With the highest concentration tested (10^{-4} M PTU) no deiodination could be demonstrated. A similar inhibition was noted for outer ring deiodination of T_4 in hepatocytes (8). Since this concentration is in the range of therapeutic serum levels of PTU-treated patients (8,26) it is remark-

able that peripheral T_3 production from T_4 still occurs in PTU-treated and T_4-substituted hypothyroid patients (27) or thyroidectomized rats (28). This is compatible with an extra-hepatic deiodination by a less PTU-sensitive deiodinase (28).

The experiments with hepatocytes from 72 h fasted rats are in agreement with observations by others, that in the fasted state the hepatic deiodinase activity is reduced. The reduction in our study (20-40%) was somewhat lower than generally found in the literature (29-31). This may be explained by partial restoration of the fasted state during the 4 h preincubation with fully supplemented culture medium. Clearance of 3,3'-T_2 in these cells was not reduced. This accords with a recent report on uninhibited sulfation in starved rats (32).

In contrast to fasting, other iodothyronines did interfere with 3,3'-T_2 clearance. Disappearance of 3,3'-T_2 from the medium was affected to a lesser extent than deiodination. Whereas deiodination is probably inhibited by competition for deiodinase activity, the reduced clearance might theoretically be caused by competition either for cellular uptake or for intracellular conjugation. At present, it is not possible to distinguish between these alternatives. It is also a matter of future interest whether the iodothyronines themselves or their respecitve conjugates caused the inhibition of deiodination. The fact that the inhibition was enhanced by preincubation with the respective iodothyronines does not answer this question.

The demonstration of the importance of sulfation in the hepatic metabolism of iodothyronines (9,11, this paper) revives the interest in earlier work on this subject. Following the initial reports by Roche et al (33,34) on the in vivo generation of T_3S in rats, conjugation of iodothyronines with sulfate has received little attention. This is intelligible in the light of the small quantities of iodothyronine sulfates in human plasma (35), urine (36) and rat bile (16), giving the impression that sulfation is a minor metabolic pathway. The low levels of sulfated iodothyronines in bile can now be explained by the finding that these conjugates are highly susceptible to deiodination before they are released. Significant secretion of sulfates is only expected when deiodination is inhibited. Indeed, increased amounts of T_2S have been observed in the bile of thiouracil-treated rats (37). The augmented biliary excretion of radioactivity after PTU administration to rats equilibrated with labeled T_3 or T_4 (38) may be based on the same phenomenon.

In conclusion, the present study shows that the clearance of 3,3'-T_2 by rat hepatocytes is determined by the large sulfate-transferring capacity of the cells. This ensures a constant fractional clearance of 3,3'-T_2 by the liver, independent of factors that affect deiodinase activity such as PTU or fasting.

The T_2S generated normally undergoes rapid outer ring deiodination before it is released. This process is inhibited by thiouracil, PTU, fasting and other iodothyronines, but not by methimazole. Only when deiodination is inhibited, accumulation of T_2S is observed.

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PAPER 5

RAPID DEIODINATION OF TRIIODOTHYRONINE SULFATE BY RAT LIVER MICROSOMAL FRACTION

T.J. Visser, J.A. Mol, M.H. Otten

Transmitted by Michael M. Kaplan on January 7, 1983

ABSTRACT: Triiodothyronine sulfate (T_3S) was synthetized chemically, and reacted at 37 C and pH 7.2 with rat liver microsomes and 5 mM dithiothreitol (DTT). The production of 3,3'-diiodothyronine sulfate (3,3'-T_2S) from unlabeled T_3S was measured after hydrolysis with a specific 3,3'-T_2 radioimmunoassay, and the production of radioactive I^- from outer ring-labeled T_3S by ion-exchange chromatography. Production of both 3,3'-T_2S and I^- was DTT dependent, strongly inhibited by 6-propyl-2-thiouracil (PTU), and abolished after heat-inactivation of the microsomes. The time sequence and enzyme dependence of product formation suggested that most if not all I^- was produced via 3,3'-T_2S. Inner ring deiodination of T_3S was two orders of magnitude faster than that of T_3 as the result of a lower apparent K_m (4.6 vs 10.7 μM) and a markedly increased V_{max} (1050 vs 33 pmol/mg protein·min). It is postulated that T_3S is a key intermediate in the metabolism of T_3.

Deiodination and conjugation with sulfate or glucuronic acid are major pathways in the metabolism of iodothyronines. In rats equilibrated with outer ring radioiodine-labeled T_3 or T_4, roughly half of the radioactivity appears in the urine as I^-. The other half, which is excreted with the feces, presumably reflects biliary excretion of conjugates (1). If in these rats deiodination is blocked by PTU treatment, urinary excretion decreases and fecal excretion increases before plasma radioactivity changes (1). Similarly, during incubation of iodothyronines with rat kidney slices (2) or isolated hepatocytes (3,4) conjugates accumulate if deiodination is inhibited (e.g. by PTU) or saturated.

A possible explanation is that a large fraction of iodothyronines is conjugated first and deiodinated subsequently, implying that the conjugates are more prone to deiodination than the parent compounds. We recently obtained evidence in support of this hypothesis, as it was observed that outer ring deiodination of 3,3'-T_2 in rat hepatocytes was strictly correlated with the sulfotransferase activity of these cells[1]. This indicated that sulfate conjugation was the rate-limiting step preceding deiodination. Consonantly, 3,3'-T_2S was rapidly deiodinated by rat liver microsomes[1].

We now report that the inner ring deiodination of T_3S by rat liver microsomes is extremely rapid compared with that of T_3.

MATERIALS AND METHODS

T_3 was obtained from Henning, and [3'-^{125}I] T_3 (> 1200 μCi/μg) from Amersham. T_3S was synthetized by a modification of a previous method (5). In short, mixtures of either 0.5 μmol T_3 and 0.1 μCi ^{125}I-T_3, or 5 nmol T_3 and 10 μCi ^{125}I-T_3, were prepared, the solvent was evaporated, and the residues were reacted for 1 h at -5 C with 100 μl concentrated H_2SO_4. After dilution with water of 0 C, the reaction products I^-, T_3S and T_3 were separated on Sephadex LH-20 by successive elution with 0.1 N HCl, water and 0.1 N NaOH-ethanol (1:1). The yield of both labeled (2 mCi/μmol) and "unlabeled" (0.2 μCi/μmol) T_3S was 40%. The identity of T_3S was demonstrated by acid and sulfatase hydrolysis, resulting in the quantitative liberation of T_3 as analyzed by chromatography and radioimmunoassay.

Rat liver microsomes were prepared as described (6). Deiodination was studied by measurement of A) the release of radioactive I^- from outer ring-labeled T_3S, and B) the production of "unlabeled" 3,3'-T_2S from T_3S of low specific radioactivity. Reaction mixtures contained microsomes and T_3S at the indicated concentrations in 0.1-0.2 ml 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA and 5 mM DTT. Incubation was done at 37 C under air. In assay A the reaction was stopped by the addition of 50 μl 50% human serum containing 5 mM PTU. After protein precipitation with trichloroacetic acid, I^- was measured in the supernatant by ion-exchange chromatography (7). To assay B was added 1 ml 1 N HCl followed by reaction for 1 h at 80 C to hydrolyze 3,3'-T_2S. The pH was neutralized with 1 ml 1 N NaOH, and 3,3'-T_2 was measured in duplicate in 50 μl aliquots by RIA (6).

Each experimental point was determined in triplicate and corrected for non-enzymatic deiodination as estimated in control incubations without microsomes. In the controls less than 1% of added radioactivity was recovered as I^-, and 3,3'-T_2S formation was less than 10% of that in complete mixtures. After hydrolysis recovery of 3,3'-T_2 from added 3,3'-T_2S, prepared similarly as T_3S, was 93%. 3,3'-T_2 was undetectable if hydrolysis of assay mixtures with added T_3S or 3,3'-T_2S was omitted.

RESULTS AND DISCUSSION

Fig. 1 depicts the production of 3,3'-T_2S and I^- from 0.5 μM T_3S as a function of reaction time and microsomal protein concentration. In both instances there is an initial increase in the accumulation of 3,3'-T_2S with little production of I^-. In a second phase the concentration of 3,3'-T_2S formed reaches a plateau, while I^- formation becomes more prominent and roughly linear with time and protein concentration. This suggests that T_3S first undergoes inner ring deiodination, and that I^- arises mainly from the subsequent outer ring deiodination of 3,3'-T_2S. The

1) M.H. Otten, J.A. Mol, and T.J. Visser, submitted for publication

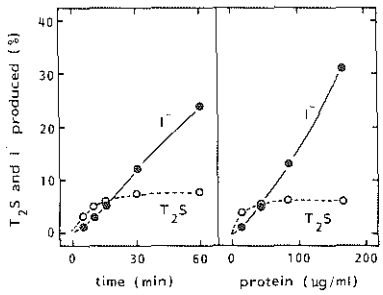

Fig. 1. Production of 3,3'-T_2S (o) or radioactive I^- (●) during incubations of 0.5 µM "unlabeled" or 3'-^{125}I-T_3S, respectively, for 5-60 min with 42 µg microsomal protein per ml (left), or for 15 min with 16.8-168 µg protein per ml (right). The amounts of 3,3'-T_2S and I^- produced were estimated as described under "Materials and Methods", and expressed as percentage of added T_3S. The results are the means of two closely agreeing experiments, each performed with two different preparations of labeled and unlabeled T_3S.

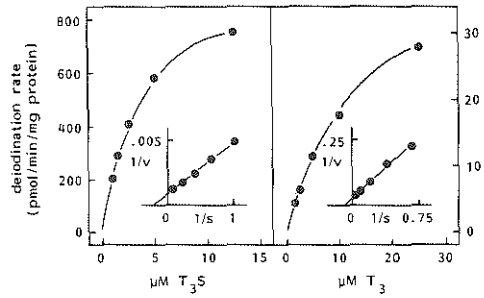

Fig. 2. Linear and double-reciprocal (inset) plots of the production of 3,3'-T_2S during incubation of varying concentrations of "unlabeled" T_3S for 5 min with 8.4 µg microsomal protein per ml (left), or the production of 3,3'-T_2 during incubation of T_3 for 15 min with 84 µg protein per ml (right). In both cases 3,3'-T_2 immunoreactivity was measured after acid treatment, as described under "Materials and Methods".

high rate of 3,3'-T_2S deiodination this implies was confirmed by the measurement of I^- generation from added 3,3'-T_2S [1]. The intermediate formation of 3'-T_1S appears unlikely, as this compound is a poor substrate for microsomal deiodinase activity [1].

Production of 3,3'-T_2S and I^- was not detectable when microsomes were preheated for 30 min at 80 C, or when incubations were done in the absence of DTT. The effects of potential inhibitors on 3,3'-T_2S production were tested in incubations of 0.5 µM T_3S for 5 min with 8.4 µg microsomal protein per ml. I^- formation was assayed in 15 min incubations with 42 µg protein per ml. One µM T_4, T_3, iopanoic acid and DIT inhibited 3,3'-T_2S production by 58, 14, 25 and 0%, and I^- production by 56, 10, 31 and 0%, respectively. As rT_3 is rapidly converted to 3,3'-T_2, its effect on 3,3'-T_2S formation could not be assessed, but 1 µM rT_3 was found to decrease I^- generation by 90%. The order of potency of these compounds resembles their effects on the deiodination of nonconjugated iodothyronines (6,8) [1]. This analogy also holds for PTU, which at 10 µM lowered 3,3'-T2S production by 82%, and I^- production by 97%.

The kinetics of T_3S inner ring deiodination were measured under conditions of initial reaction rates, i.e. short reaction time (5 min) and low protein concentration (8.4 µg/ml). Parallel experiments with outer ring-labeled T_3S demonstrated that I^- production was less than 10% of 3,3'-T_2S production. This indicates not only that further deiodination of 3,3'-T_2S is negligible under these circumstances but also that direct outer ring deiodination of T_3S is, if anything, a minor pathway. The results are shown in Fig. 2, and compared with the inner ring deiodination of T_3. At low substrate concentrations ($\leqslant$ 1 µM) deiodination of T_3S is two orders of magnitude faster than the deiodination of T_3. Lineweaver-Burk plots reveal for T_3S an apparent K_m of 4.6 ± 1.3 µM and V_{max} of 1050 ± 190 pmol/mg protein·min (mean ± SD, n = 4), and for T_3 10.7 µM and 33 pmol/mg protein·min, respectively. The latter values are very similar to those previously reported (6). The enhancement of the inner ring deiodination of T_3 by sulfation, therefore, appears to result primarily from an increase in V_{max} rather than a decrease in K_m. It is likely, however, that lower K_m and V_{max} values will be measured at reduced DTT levels (7).

Recent studies have suggested that deiodination of both rings of iodothyronines is catalyzed by a single deiodinase in rat liver microsomes (9,10). The similar effects of inhibitors on the inner ring deiodination of T_3S and outer ring deiodination of 3,3'-T_2S [1] as on the deiodination of nonconjugated iodothyronines (6,8) are compatible with this notion, and suggest that the sulfate conjugates are also substrates for this enzyme.

The facilitated deiodination of sulfates is unexpected, considering that the deiodinase is in a lipid environment in the membrane, whereas such conjugates are less lipid soluble than the nonconjugated iodothyronines. Neither would sulfation of the phenolic hydroxyl group be expected to affect the binding strength of the iodines to the inner ring. The mechanism of this facilitated deiodination, therefore, remains obscure but may be related to the deiodinase being a basic protein (11).

The physiological relevance of our observations depends on the extent to which this mechanism operates in vivo. At least they indicate that T_3S is not an end stage in the metabolism of T_3. The demonstration of a rapid increase in the biliary excretion of T_3 conjugates in rats treated with PTU (1) would tend to support the hypothesis that little T_3 deiodination takes place without prior sulfate conjugation. Preliminary experiments in our laboratory indicate that deiodination of T_3 by rat hepatocytes depends on the sulfate concentration in the medium, but much further work is needed to test the above hypothesis. The question is equally intriguing whether deiodination of T_4 is similarly facilitated by sulfation of glucuronidation.

ACKNOWLEDGEMENTS

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PAPER 6

3,3',5-TRIIODOTHYRONINE METABOLISM IN RAT HEPATOCYTES

The significance of sulfation for metabolic clearance and deiodination

M.H. Otten, G. Hennemann, R. Docter, T.J. Visser

Abstract

It was recently demonstrated by us that deiodination of the biological active thyroid hormone 3,3',5-triiodothyronine (T_3) and of 3,3'-diiodothyronine (3,3'-T_2) in rat hepatocytes only occurs after conjugation with sulfate. Sulfation of these iodothyronines accelerates their deiodination by rat liver microsomes. In the light of the increasing interest in tissue-specific iodothyronine handling, we have investigated in detail the multiple processes involved in hepatic T_3 metabolism with monolayers of freshly isolated rat hepatocytes. After incubation of the cells with 10^{-8} M T_3 and [3'-^{125}I]T_3 for 3 h at 37C column chromatography of the medium revealed besides unprocessed T_3 three labeled metabolites: 60% iodide (I), 30% T_3 glucuronide (T_3G) and 10% T_3 sulfate (T_3S). Propylthiouracil (PTU) inhibited deiodination effectively in a dose-dependent manner. This inhibition resulted in a reciprocal increase of the intermediate T_3S. Glucuronidation was affected only at high concentrations PTU. Interference with sulfotransferase activity by medium SO_4^{2-} depletion lowered T_3 clearance and iodide production but also T_3S generation when deiodination was blocked by PTU. Inhibitors of sulfation, e.g. salicylamide, dichloronitrophenol and pentachlorophenol, induced an inhibition of iodide formation which correlated strongly with the effect on T_3S production in the presence of PTU. Under the conditions used, overall T_3 metabolism showed no saturation at concentrations up to 10 μM while the apparent K_m for glucuronidation appeared lower than for sulfation.

In conjunction with previous findings, it is concluded that hepatic deiodination of T_3 involves 3 consecutive metabolic steps: sulfation, inner ring deiodination and finally outer ring deiodination of the 3,3'-T_2 sulfate generated. PTU (10 μM) effectively blocks both deiodinating reactions leaving T_3 disappearance essentially intact. Hepatic T_3 metabolism and clearance capacity is predominantly determined by sulfation and glucuronidation. Deiodination of T_3 appears not to be enhanced by conjugation with glucuronic acid.

Introduction

In euthyroid subjects 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) is the predominant secretion product of the thyroid gland. To exert the required biological action T_4 has to be converted to 3,3',5-triiodothyronine (T_3). This process takes place by outer ring or 5'-deiodination of T_4 in many extrathyroidal tissues. However, the contribution of plasma T_3 or local T_3 generation to the nuclear T_3 occupancy varies per tissue, indicating tissue specific thyroid hormone metabolism (1,2,3). The metabolic kinetics of multiple iodothyronines have been extensively studied in vivo in both man and rat (4,5). However, the use of these serum sampling techniques do not allow the estimate of the actual contribution of the respective tissues to overall metabolism. Moreover the rapid succession of intracellular metabolic processes may remain undetected. Therefore, detailed studies of thyroid hormone metabolism in isolated tissues may contribute to a better integrated understanding.

We have studied the metabolism of both 3,3'-diiodothyronine (3,3'-T_2) and T_3 in monolayers of freshly isolated rat hepatocytes. By these experiments a hitherto unrecognized metabolic pathway of iodothyronines was disclosed. Whereas formerly deiodination and conjugation with either glucuronic acid or sulfate were considered to have different metabolic functions it was demonstrated by us that sulfation of 3,3'-T_2 and T_3 considerably facilitates their rate of deiodination in hepatocytes (6). Moreover, sulfation with its large capacity, appeared to be a major rate-determing factor in the overall hepatic clearance of these iodothyronines. In comparison with 3,3'-T_2, which is mainly subjected to sulfation and subsequent outer ring deiodination (7), T_3 metabolism is more complex. Next to sulfation, this hormone is also conjugated with glucuronic acid. Furthermore, T_3S is metabolized through primary deiodination in the inner ring (5-deiodination) with subsequent outer ring or 3'-deiodination of the 3,3'-T_2 sulfate (T_2S) generated (8). In this paper we describe the quantitative contributions of these pathways to overall T_3 metabolism in rat hepatocytes as well as

the effects of several compounds that interfere with sulfation, glucuronidation and deiodination.

Materials and methods

3,3',5-Triiodo-L-thyronine was obtained from Henning Berlin GmbH, Berlin, FRG. [3'-^{125}I]T_3 with a specific activity of 1200 Ci/g was purchased from Amersham, UK. The following materials were from Sigma Chemical Co., St. Louis, MO.: 6-propyl-2-thiouracil, L-ascorbic acid, d-saccharic acid-1,4-lactone, bovine pancreas insulin, collagenase type I, sulfatase type VIII, β-glucuronidase type IX, Hepes, piperazine-N,N'-bis (2-ethanesulfonic acid) (Pipes), N,N-bis (2-hydroxylethyl)-2-aminoethane sulfonic acid (Bes). Sephadex LH-20 came from Pharmacia Fine Chemicals, Uppsala, Sweden. Salicylamide, 2,6-dichloronitrophenol and pentachlorophenol were purchased from Riedel-de Haen A.G., Hannover, FRG. Fetal calf serum, penicillin and streptomycine sulfate were obtained from Flow Laboratories, Irvine, UK. Hams' F_{10} nutrient mixture was provided by Gibco Europe, The Netherlands. Hanks balanced salt solution (9) and Dulbecco phosphate buffered saline (10) were prepared in our own laboratory.

Hepatocyte monolayer preparation and incubation procedures

Hepatocytes were isolated according to the method of Berry and Friend (11) with minor modifications as described earlier (12). Livers of male Wistar rats, weighing 300 g, were first perfused for 10 min with Ca^{2+} free Hanks' salt solution and additionally for 20 min with the same medium containing 0.05% collagenase. The perfusion medium was kept at 37C and pH 7.4 under 5% CO_2 and 95% O_2. After mincing of the liver the cells were washed by repeated centrifugation and resuspending. Thereupon the cells were suspended in culture medium to yield 10^6 cells per 2 ml. This suspension was slowly infused in plastic culture dishes (1.8 ∅, Greiner, Nürtingen, FRG) and placed for 4 h at 37C in a culture stove under atmospheric conditions. At this stage the culture medium was composed of unmodified Ham's F_{10}, 10.6 mM Pipes, 11.2 mM Bes, 8.9 mM Hepes, 2 mM $CaCl_2$, 12 mU/l insulin, 10 U/l penicillin, 10 µg/ml streptomycin sulfate and 10% fetal bovine serum at pH 7.4. Cell viability as estimated by trypan blue exclusion at the end of the 4 h incubation period as well as after the experiments, was over 85%. Subsequently, the incubations were initiated and performed at 37C under atmospheric conditions. For the incubations 2 ml Dulbecco balanced salt

medium with 1.0 mM $MgSO_4$ was used, containing 10^{-10} to 10^{-5} M T_3 and 0.2 µCi [3'-^{125}I]T_3. To test the influence of inhibited sulfation on T_3 metabolism, essentially two methods have been used: a) reduction of the sulfate availability in the culture medium (13) and b) addition of compounds known to interfere with sulfotransferase activity. To reduce the intracellular sulfate concentration hepatocytes were preincubated for one hour in SO_4^{2-} free Dulbecco medium, prior to incubation with T_3 and variable amounts of $MgSO_4$. As inhibitors of sulfotransferase activity have been tested the competitive inhibitor salicylamide (14) and the selective inhibitors 2,6-dichloro-4-nitrophenol (DCNP) and pentachlorophenol (PCP) (15). In other experiments the influence of 6-propyl-2-thiouracil (PTU), as inhibitor of deiodination, has been investigated. In all incubations 1 mM L-ascorbic acid was added to prevent non-specific deiodination. All experiments were performed in triplicate. The data presented in the figures are expressed as the triplicate mean and representative of at least three different experiments.

Analysis of culture medium

After incubation, 0.5 ml samples of the culture medium were prepared for chromatographic analysis by acidification with an equal volume of 1 N HCl. The mixure was applied to small (1 ml) Sephadex LH-20 columns equilibrated in 0.1 N HCl. The columns were eluted subsequently with serial 1 ml fractions of 0.1 N HCl, 0.1 M sodium acetate at pH 4.0, H_2O and ethanol. This new chromatographic method provided a good separation of iodide, T_3 glucuronide (T_3G), T_3S and unreacted T_3, respectively.

T_3 clearance and metabolite production were calculated from the radioactivity in the respective fractions. Due to the inconsistent adsorption of T_3 to the culture dishes and uptake in the cellular compartment this amount was omitted from the calculations. Analysis of the nature of the conjugates was accomplished by hydrolysis with β-glucuronidase and sulfatase with or without saccharic acid lactone and subsequent chromatography on Sephadex LH-20 as described above. Furthermore, the composition of iodothyronine conjugates in the culture medium was verified by a newly developed high performance liquid chromatography (HPLC) technique using a reversed phase C18 Bondapak column (Waters) and elution with a 1.5 ml/min flow of a 70% 0.01 M KH_2PO_4 and 30% acetonitrile (v/v) mixture at pH 2.1. Good separation was obtained for respectively eluting iodide, T_2S, T_3S and T_3G.

Analysis of radioactivity in the cellular compartment

The amount of total ^{125}I radioactivity with the cellular compartment was estimated by the decrease of radioactivity in sequential 100 ul samples from the medium in the same culture dish with or without hepatocytes. These experiments were carried out in the presence of 10^{-10}, 10^{-8} or 10^{-6} M unlabeled T_3 and 0.4 µCi [3'-^{125}I]T_3. The composition of the radioactivity in the cells was determined in ethanol extracts by chromatographic analysis on Sephadex LH-20 as described above.

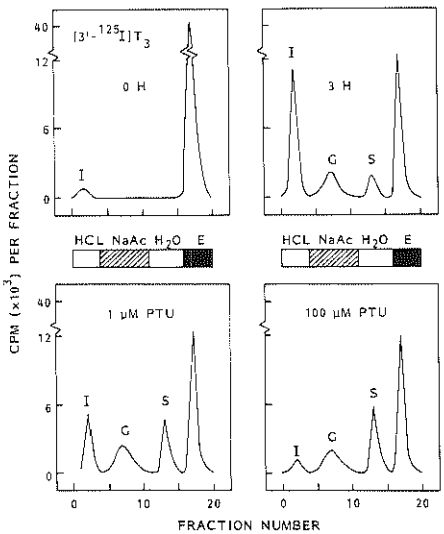

Fig.1. Chromatography of culture medium before and 3 h after incubation of 10^{-8} M T_3 and [3'-^{125}I]T_3 with isolated rat hepatocytes (upper panel). Parallel incubates contained 1 or 100 µM PTU (lower panel). Culture medium (500 µl) was acidified by addition of 500 µl 1 N·HCl and directly applied to small (1 ml bed volume), 0.1 N HCl equilibrated Sephadex LH-20 columns. Serial elution was performed with 1 ml fractions of 0.1 N HCl, 0.1 M sodium acetate at pH 4.0 (NaAc), H_2O and ethanol (E) as indicated by the horizontal bars. The respective fractions contained: iodide (I), T_3 glucuronide (G), T_3 sulfate (S) and unreacted T_3. Identification of the radioactive material G and S was obtained by hydrolysis with β-glucoronidase and sulfatase. Chromatography of the hydrolysates yielded exclusively T_3 as liberated compound.

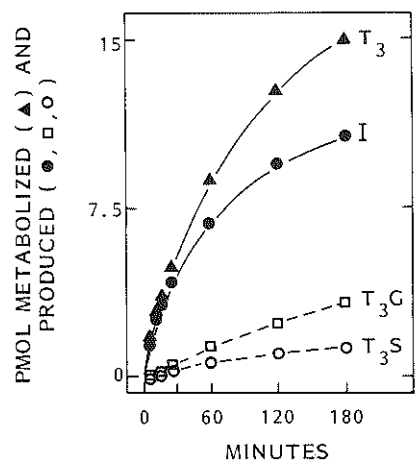

Fig.2. Disappearance of T_3 and simultaneous production of T_3 metabolites during 3 h incubation of 10^{-8} M T_3 with [3'-^{125}I]T_3. Isolated rat hepatocytes (2 x 10^6 cells) were incubated for 3 h at 37C in larger than usual culture dishes (5 cm ∅), Costan, Cambridge, MA, USA) with 4 ml instead of 2 ml protein-free Dulbecco medium. This was done in order to reduce the influence of serial (100 µl) sampling on total T_3 content of the culture medium. By this fashion only 12.5% of the incubation medium was removed. The samples were chromatographed as described in Fig.1 and the conversion rates calculated from the radioactivity in the various fractions. No other products but iodide (I), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) were detected.

Results

T_3 metabolism in rat hepatocytes

Column chromatography of the culture medium on Sephadex LH-20 before and after 3 h incubation of rat hepatocytes with 10^{-8} M T_3 in the presence of [3'-^{125}I]T_3 revealed 3 distinct radioactive products. The $^{125}I^-$ produced by outer ring deiodination eluted in the HCl fractions. The sodium acetate and H_2O fractions contained the respective T_3 conjugates with glucuronic acid (T_3G) and sulfate (T_3S) (Fig.1). Identification of T_3G and T_3S was accomplished by hydro-

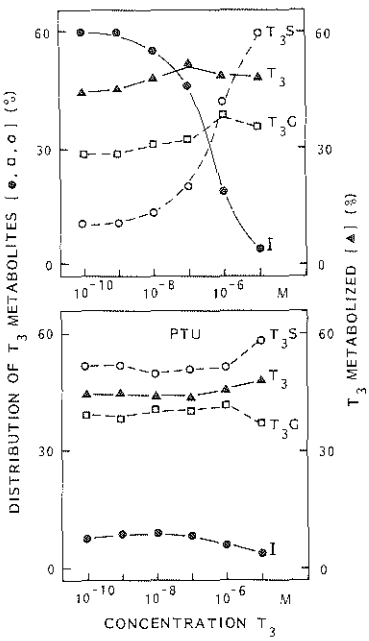

Fig.3. Influence of substrate concentration and PTU on T_3 metabolism by rat hepatocytes. Monolayers of isolated hepatocytes were incubated for 3 h at 37C with protein-free Dulbecco medium containing 10^{-10} to 10^{-5} M T_3 and [3'-^{125}I]T_3 with or without 10 μM PTU. Culture medium was analysed by column chromatography on Sephadex LH-20 as described in Fig.1. T_3 metabolism, expressed as fractional clearance, is shown on the right hand scale. The distribution of T_3 metabolites, i.e. iodide (I), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) expressed as percentage of total metabolite production, is depicted on the left hand scale.

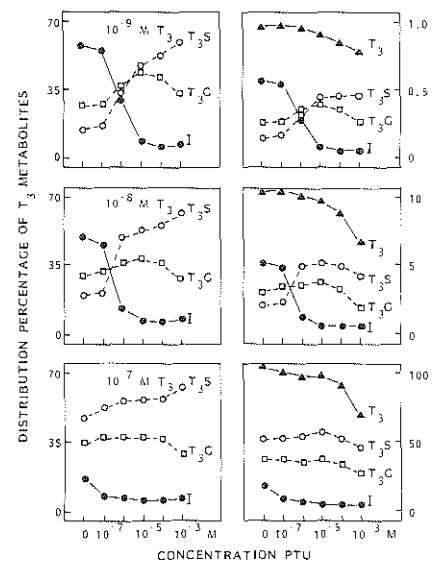

Fig.4. Influence of PTU on hepatic T_3 metabolism. Isolated rat hepatocytes were incubated for 3 h at 37C with Dulbecco medium containing 10^{-9}, 10^{-8} or 10^{-7} M T_3 with [3'-^{125}I]T_3 and either no or 10^{-7} to 10^{-3} M PTU. The left hand panels depict the distribution of the T_3 metabolites, e.g. iodide (I), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) expressed as percentage of total metabolite production. The right hand panels show the calculated amounts in pmol/dish of T_3 metabolized in relation to the production of its metabolites.

lysis of the radioactive material in the respective fractions with either -glucuronidase or sulfatase. T_3G was completely hydrolysed by β-glucuronidase, but not in the presence of its inhibitor, saccharic acid lactone. T_3S was hydrolysed by sulfatase, irrespective of the presence of 5 mM saccharic acid lactone. Chromatography of these incubates revealed no other liberated radioactive material but T_3. Fig.1 also shows the chromatograms of T_3 incubations in the presence of 1.0 and 100 µM PTU. The decreased iodide production and increased formation of T_3S is presented in greater detail below. Analysis of 50 µl unprocessed culture medium by HPLC demonstrated an identical composition of iodide, T_3S and T_3G as obtained by the Sephadex chromatography. No T_2S or T_2G were detected irrespective of the presence of PTU during the incubations.

The disappearance of 10^{-8} M T_3, as well as the production of T_3 metabolites over a 3 h period, are depicted in Fig.2. Iodide constituted the principal metabolite (60%), but T_3G formation (30%) predominated over the appearance of T_3S (10%) in the culture medium.

The influence of increasing T_3 concentrations with or without 10 µM PTU is given in Fig.3. Over a concentration range from 10^{-10} to 10^{-5} M the fraction of T_3 cleared in 3 h remained fairly constant, i.e. 45 to 55 percent of medium T_3 (right hand scale). This was somewhat less in the presence of PTU (lower panel). Similarly, the relative T_3G formation with 30-40% of total metabolite production (left hand scale) was little affected by either substrate concentration or PTU. However, with increasing T_3 concentrations the proportion of iodide formation decreased while T_3S production showed a reciprocal rise. In the

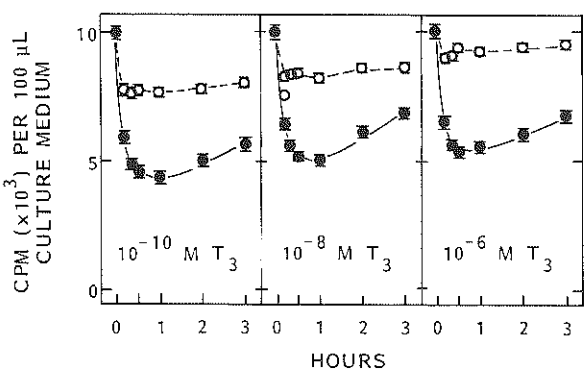

Fig.5. Total ^{125}I radioactivity in culture medium during incubation of [3'-^{125}I]T_3 and unlabeled T_3 with (●----●) or without (o----o) hepatocytes. Dulbecco medium (4 ml) containing 0.5 µCi [3'-^{125}I]T_3 with 10^{-10}, 10^{-8} or 10^{-6} M unlabeled T_3 was incubated for 3 h at 37C in the large culture dishes described in Fig.2, with or without 2x 10^6 isolated rat hepatocytes. Serial 100 µl samples were taken from the medium up to 3 h. The amount of radioactivity (cpm per 100 µl) is given as mean ± SD of 6 parallel incubates.

presence of 10 μM PTU little iodide was generated. Instead, T_3S appeared as the major metabolite.

The dose-response curves of PTU influence on T_3 metabolism are given in Fig.4. The left hand panels show the distribution of T_3 metabolites, expressed as percentages of total metabolite production for the 3 concentrations tested, i.e. 10^{-9}, 10^{-8} and 10^{-7} M T_3. The right hand panels depict the production rates of the three metabolites in relation to the amount of T_3 metabolized. Deiodination was maximally blocked by 10 μM PTU whereas T_3 clearance was not yet affected to the extent that occured at higher PTU concentrations. Therefore, 10 μM PTU was used for inhibition of deiodination in other experiments. T_3G formation from 10^{-9} M T_3 was slightly increased at moderate PTU concentrations. However, at the highest PTU concentrations tested T_3G production invariably declined in all experiments, thus contributing to the observed reduction of T_3 clearance. The generation of T_3S was again inversly proportional to that of iodide, but tended to decline at higher PTU concentrations. During initial experiments no complete inhibition of iodide production could be achieved by PTU. This PTU-insensitive deiodination, which was not saturable at 10 μM T_3, was ascribed to oxidizing compounds in the protein-free culture medium, possibly liberated from lysosomes of degraded cells. Non-specific deiodination was almost completely prevented by addition of 1 mM L-ascorbic acid which per se, did not affect hepatic T_3 metabolism.

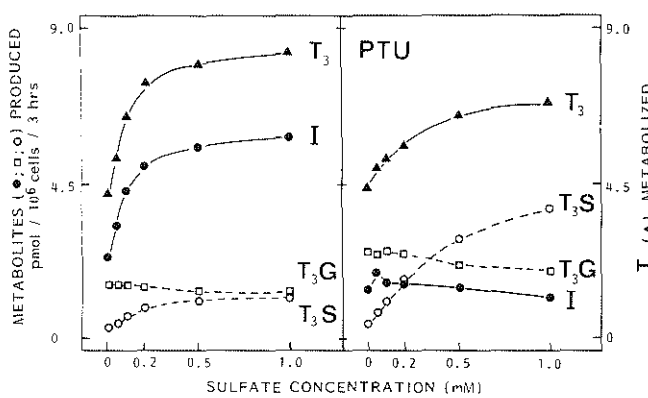

Fig.6. Influence of medium SO_4^{2-} concentration on hepatic T_3 metabolism. Rat hepatocytes were preincubated at 37C for 1 h with SO_4^{2-} depleted Dulbecco medium to reduce the cellular SO_4^{2-} content. Subsequently 2 ml Dulbecco containing 10^{-8} M T_3, [3'-^{125}I]T_3 with or without 10 μM PTU and increasing amounts upto 1.0 mM $MgSO_4$ was incubated for 3 h at 37C with 10^6 isolated cells. The calculated amounts in pmol/10^6 cells/3 h of T_3 metabolized are depicted on the right hand scale as a function of the medium SO_4^{2-} concentration. The production of the T_3 metabolites e.g. iodide (I), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) is shown on the left hand scale.

T_3 in the cellular compartment

The amount of T_3 associated with the cellular compartment and the surface of the plastic culture dish was approximated by measuring the decrease of radioactivity in the culture medium with or without hepatocytes. Equilibrium between medium and the cells was reached within 30 to 60 min incubation. The medium radioactivity in the dishes with hepatocytes showed a remarkably similar decrease of approximately 50% for the three concentrations tested e.g. 10^{-10}, 10^{-8} and 10^{-6} M T_3 (Fig.5). On the other hand, the decline in culture medium radioactivity without hepatocytes tended to diminish at higher T_3 concentrations, suggesting saturated adsorption to the culture dish. The radioactivity in the cellular compartment as determined by chromatography, consisted for over 85% of T_3, the remaining being distributed over iodide, T_3G and T_3S.

Influence of inhibited sulfation on T_3 metabolism

The effects of culture medium SO_4^{2-} concentration on T_3 metabolism are depicted in Fig.6. Without SO_4^{2-} the rate of deiodination as well as T_3 clearance was low but increased rapidly when small amounts of $MgSO_4$ were added. In the presence of 10 µM PTU deiodination remained low but instead a progressive SO_4^{2-} dependent accumulation of T_3S was observed. The production curves of iodide in the absence and T_3S in the presence of PTU, showed a distinct resemblance in

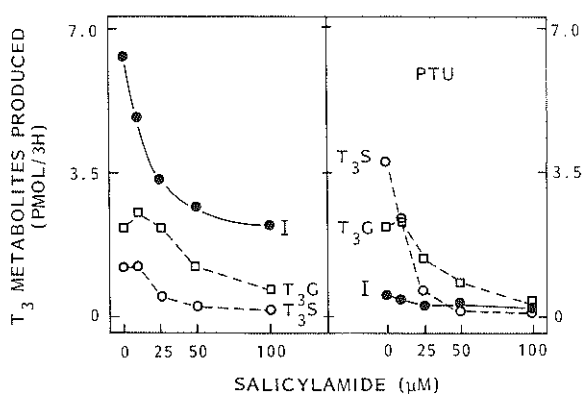

Fig.7. Influence of salicylamide on T_3 metabolism in isolated rat hepatocytes. Monolayers (10^6 cells per dish) were incubated for 3 h at 37C with 2 ml Dulbecco medium containing 10^{-8} M T_3, [3'-^{125}I]T_3 with or without 10 µM PTU and various amounts, upto 100 µM, salicylamide. Production of T_3 metabolites , iodide (I), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) is given in pmol/3 h as a function of the salicylamide concentration.

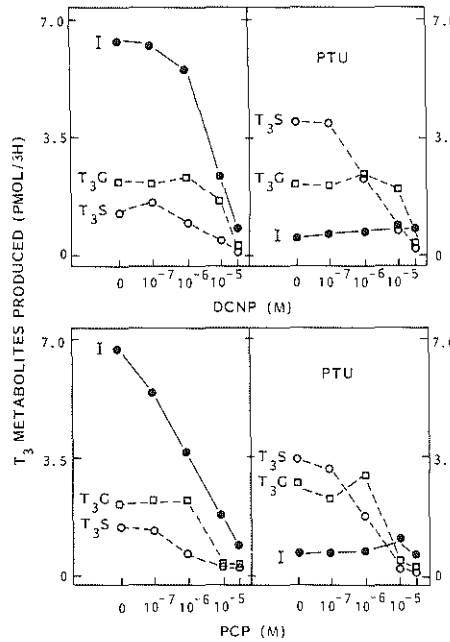

Fig.8. DCNP and PCP influence on hepatic T_3 metabolism. Isolated rat hepatocytes were incubated for 3 h at 37C with 2 ml Dulbecco medium containing 10^{-8} M T_3, [3'-^{125}I]T_3 with or without 10 µM PTU and variable amounts of 2,6-dichloro-4-nitrophenol (DCNP) or pentachlorophenol (PCP). The production of T_3 metabolites e.g. iodide (I), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) is expressed in pmol/3 h as a function of the DCNP or PCP concentration.

their sulfate dependence. The generation of T_3G tended to decline at higher SO_4^{2-} concentrations. As depicted in Fig.7, the competitive inhibitor of sulfotransferase activity, salicylamide greatly reduced deiodination as well as the accumulation of T_3S in the presence of 10 µM PTU. The inhibition of the former proved to be somewhat less pronounced than that of the latter i.e. 70 and 94% at 100 µM salicylamide (Table I). However, a strong correlation between the degrees of inhibition was found ($r=0.99$, $p<0.01$). The observed reduction of T_3G production is in agreement with competitive inhibition of glucuronidation at high concentrations of salicylamide (16). The influences of DCNP and PCP on T_3 metabolism are depicted in Fig.8. PCP proved to be the more powerful inhibitor of T_3 sulfation, as judged by the respective percentages inhibition of deiodination and that of T_3S production in the presence of 10 µM PTU (Table I). Similar to salicylamide a strong correlation was found for the inhibition of deiodination and T_3 sulfation by DCNP and PCP. T_3 glucuronidation was only affected at high levels of these compounds.

Table I. Reduction of T_3 deiodination and T_3S production in the presence of 10 µM PTU in rat hepatocytes by salicylamide, DCNP and PCP.*

Compound	Concentration µM	Production inhibition (%) Iodide	T_3S	correlation coefficient
Salicylamide	5	7	17	
	10	25	36	
	25	52	82	0.99, $p<0.01$
	50	62	92	
	100	70	94	
DCNP	0.1	2	0.3	
	1.0	15	40	0.96, $p<0.01$
	10	68	79	
	50	96	94	
PCP	0.1	22	9	
	1.0	51	49	0.99, $p<0.01$
	10	81	94	
	50	96	98	

* DCNP: 2,6-dichloro-4-nitrophenol; PCP: pentachlorophenol

Discussion

When our monolayers of isolated rat hepatocytes were incubated with serum-free medium, containing $[^{125}I]T_3$ and unlabeled T_3, we noted in 30 to 60 min a 50% decrease of medium radioactivity. A similar percentage has been found for T_3 after 60 min incubation with cultured monkey hepatocarcinoma cells (17). The 50% decrease occured irrespective of a 10.000 fold difference in T_3 concentrations, illustrating the large cytosolic and probably non-specific binding capacity for T_3 of rat liver cells (18-21). The radioactivity in the cellular compartment consisted for over 85% of unchanged T_3. As T_3 metabolism progressed with time, giving rise to hydrophylic metabolites, the radioactivity gradually reappeared in the culture medium in a similar fashion for the 3 concentrations tested. This is in close agreement with earlier observations in monkey hepatoma cells (17) and T_3-perfused rat liver (22). At the same time however, we noted an important non-specific but saturable T_3 binding to cell-free culture dishes, underlining the complexities of metabolic studies with this model (17,23). T_3 absorption by the cellular compartment and the culture dishes, varied per experiment and in the time, and was further influenced by added compounds as PTU and salicylamide. This sequestration of T_3 reduced the actual T_3 medium concentration by an inconsistent factor. Therefore, the various metabolic conversion rates calculated from the original T_3 concentration, are somewhat overestimated.

This, however, does not affect the conclusion made from this study.

Indications for the crucial role of sulfation in hepatic iodothyronine metabolism have recently been obtained in this laboratory. Although both 3,3'-T_2 and T_3 are poor substrates for rat liver microsomal deiodinase activity (24), deiodination of these substrates occured with surprising rapidity in isolated hepatocytes. It appeared that both iodothyronines had to be conjugated first with sulfate before any substantial deiodination occured (6). This was explained by the observation that the sulfate esters of 3,3'-T_2 and T_3 (T_2S and T_3S) are very good substrates for rat liver microsomal deiodinase. For outer ring deiodination of T_2S the K_m is 0.34 μM with a V_{max} of 353 pmol/mg protein.min (U), whereas the respective values for 3,3'-T_2 are 8.9 μM and 188 U. For T_3S the primary step of degradation is inner ring deiodination (8). Interestingly, this reaction is predominantly accelerated by an increase in V_{max} (30 vs 1050 U), whereas the K_m was only moderately lowered (10.7 vs 4.6 μM). The production of iodide from T_3 in our model therefore is a reflection of 3 successive metabolic steps: sulfation, inner ring or 5-deiodination and finally the removal of $^{125}I^-$ from the 3'-position.

According to this concept, hepatic T_3 metabolism and clearance capacity is mainly determined by the combined rate of sulfation and glucuronidation. This notion is further supported by the findings in the present study. Reduction of sulfation by either SO_4^{2-} depletion or sulfotransferase inhibitors invariably led to a decrease of T_3 clearance from the medium. On the other hand, inhibition of deiodination by increasing PTU concentrations gradually decreased iodide formation but increased T_3S accumulation. No T_2S was detected. Therefore in accordance with microsomal incubations (8), PTU apparently also in cells effectively inhibits inner ring deiodination of T_3S. This contrasts with the less PTU-sensitive T_3 and T_4 5-deiodinase activity observed by Sato and Robbins in cultured rat hepatocytes (25). Furthermore, both overall T_3 clearance and the formation of T_3 glucuronide were reduced at high (10^{-3} M) PTU concentrations. Since PTU is known to be a substrate for UDP-glucuronyltransferase (26), competition with T_3 for glucuronidation may well contribute to the observed reduction of T_3 metabolism. However, sulfation appears to be the major determinant of the T_3 clearance rate. The experiments with decreasing amounts of SO_4^{2-} in the medium show a rapid decline in T_3 disappearance and iodide production below 0.5 mM SO_4^{2-}. The lack of complete inhibition of deiodination without added SO_4^{2-} may reflect incomplete depletion of the cellular SO_4^{2-} content. Incomplete inhibition in the absence of SO_4^{2-} has also been observed for sulfation of salicylamide in isolated rat hepatocytes (14). Since in these cells the cysteine-oxidizing pathway is well preserved (27,28) sulfate neogenesis from

sulfur-containing protein residues is also feasible (14,27).

Glucuronidation, on the other hand, appears not to be of major significance for deiodination of iodothyronines. Whereas deiodination of T_3 is clearly inversely proportional to the production of T_3S with increasing PTU concentrations (Fig.4), no such relation was found for T_3G. Also contrasting with T_3S, no rise in T_3G formation was observed with increasing and deiodination saturating T_3 concentrations (Fig.3). Furthermore, after preincubation of the cells for 30 min with 25 mM galactosamine, a compound that inhibits glucuronidation by cell depletion of UDP-glucuronic acid (29), iodide production remained unchanged while T_3G generation was abolished (Otten and Visser, unpublished).

In recent years multiple reports mentioned elevations of T_3 serum levels in rats fed a diet deficient in protein or essential aminoacids (30-32). In these studies, quite opposite to the fasted state (33-37), a normal T_4 5'-deiodinase activity and T_3 production rate appeared to exist. Instead, a reduced metabolic clearance rate and distribution volume of T_3 was found and tentatively explained by a decrease in cellular T_3 uptake (31). The data on free T_3 concentration in these studies are controversial: either low, normal (32) or high (31). In rats fed a comparable low protein diet (8% casein), serum SO_4^{2-} was lowered from 0.9 to 0.15 mM, resulting in decreased sulfation of the xenobiotic compound harmol (38). Identical low sulfate concentrations resulted in a marked reduction of T_3 clearance and deiodination in our hepatocytes. Therefore, it is tempting to speculate that the reduced T_3 clearance in the protein malnourished rats, at least in part, might be due to diminished hepatic sulfation of T_3. In a similar way, the much better substrate properties of 3,3'-T_2 for rat hepatic sulfotransferases in comparison with T_3 (39) might, in part, explain the differences in their respective metabolic clearance rates (40-43).

The sequential metabolism of T_3 with sulfation preceding deiodination, is further illustrated by the effects of DCNP, PCP and salicylamide. DCNP and PCP selectively inhibit sulfation with respect to glucuronidation (13,15,44). Moreover, a close correlation was found between the dose-dependent inhibition of deiodination and that of sulfation in the presence of PTU (Table I). Glucuronidation of T_3, however, is also affected at very high concentrations of these compounds. Which is somewhat surprising since 100 μM DCNP did not influence in vitro glucuronidation of harmol (44). This may be due to the heterogeneity of glucuronyl transferases (45). However, DCNP and PCP are also inhibitors of mitochondrial oxydative phosphorylation (46). Thus, by reducing the cellular ATP content these compounds might well inhibit the energy-dependent generation of the respective co-substrates, i.e. activated sulfate (adenosine 3'-phosphate 5'-sulfatophosphate, PAPS) and glucuronic acid (UDP-glucuronate)

(47). Furthermore, PCP was found to inhibit deiodination directly (6). Salicylamide, on the other hand, is known to interfere by substrate competition especially with sulfation but also with glucuronidation (14,15). This is in agreement with our observation that T_3 sulfation was more rapidly reduced than glucuronidation. The lack of complete inhibition of deiodination at the highest concentration salicylamide is not completely understood. Since T_3 is processed by multiple sulfotransferases (39), it may be speculated that these enzymes are not inhibited to the same degree by salicylamide. At the same time PTU, as potential inhibitor of sulfation (Fig.4), might abolish the remaining sulfotransferase activity thus preventing the expected generation of T_3S. However, this inconsistency leaves open the possibility of some sulfation-independent T_3 deiodination.

Of further interest is the remarkable constant disappearance rate of T_3 (40-50%) in spite of a substrate concentration range from 10^{-10} to 10^{-5} M (Fig.3). Due to the long incubation time and absence of medium albumin in these experiments the contribution of the recently demonstrated active uptake system for T_3 in rat hepatocytes (48) will remain undetected. In the presence of PTU the sulfation/glucuronidation ratio of T_3 is constant upto 1 μM and increases at 10 μM, suggesting that the apparent K_m of T_3 for sulfation is higher than for glucuronidation, the latter being in the μM range. A theoretical kinetic model for this type of metabolism has recently been presented (49). The lower K_m of T_3 glucuronidation in comparison with sulfation seems controversial since for phenolic compounds usually the reverse is found (13,15). However, this phenomenon may be understood since iodine substitution of phenol in the para-position, (T_3 has iodine in the ortho-position), appears to lower the K_m for glucuronidation 30-fold (13).

Finally, we were intrigued by the differences in T_3 metabolism found by Sato and Robbins (26) in 2 day-cultured rat hepatocytes in comparison with our results using freshly isolated cells. Apart from the mentioned difference in PTU sensitivity of 5-deiodination another aspect deserves comment. A much higher 3 h proportional clearance rate of T_3 (85%) was observed by Sato and Robbins upto a concentration of 10^{-7} M declining to 35% at 10^{-5} M. The higher clearance rate may be explained by a larger cell number (2 vs 1 x 10^6 cells used by us) but the reduction of this fractional clearance suggests saturation of the metabolic capacity not occuring with the freshly isolated cells. Secondly, virtually no glucuronidation of T_3 was found with the cultured hepatocytes, whereas we noted 30 to 40% T_3 glucuronide. Expression of fetal phenotype has been reported to occur in cultured rat hepatocytes (50). At the same time, hepatic UDP-glucuronyltransferase activity is known to be low at perinatal age whereas

sulfation is present early in fetal development (15). Also a rapid 50% decline in glucuronidation of phenolsulfonphtalein in 2 days cultured hepatocytes has been described (51). Therefore, the lack of T_3 glucuronidation found by Sato and Robbins may be due to dedifferentation of the cultured cells. Since glucuronidation is known to be an active metabolic pathway of T_3 in vivo (52,53) freshly isolated hepatocytes would seem to be the preferred model for studying in vitro hepatic iodothyronine metabolism.

It is concluded from the present study that there are two primary metabolic pathways for T_3 in freshly isolated rat hepatocytes: sulfation and glucuronidation. Whereas T_3G seems not to be subjected to important deiodination, sulfation of T_3 gives rise to a substrate that is rapidly degraded by subsequent inner ring and outer ring deiodination. In spite of some indirect evidence, the physiologic significance of this metabolic property awaits further in vivo investigation of T_3 kinetics in relation to body sulfotransferase activity.

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PAPER 7

IODOTHYRONINE SULFATASE ACTIVITY OF TWO ANAEROBIC BACTERIAL STRAINS FROM RAT INTESTINAL MICROFLORA

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1. INTRODUCTION

In man and animal many endogenous and xenobiotic compounds are conjugated in the liver and other tissues with either glucuronic acid or sulfate. Conjugation, apart from other aspects, increases the molecular polarity of the aglycon thereby facilitating its biliary excretion. However, when biliary conjugates reach the gut lumen they may be hydrolysed by bacterial β-glucuronidase or sulfatase. This deconjugation promotes intestinal reabsorption of the original compound, thus creating an enterohepatic circulation. Bacterial β-glucuronidase activity in the gut contents is known to be very active. Much less information exists about the hydrolytic activity of gut bacteria towards sulfate esters. Thyroid hormones are secreted in the bile both as sulfates and glucuronides. They probably are subjected to an enterohepatic circulation, of which the exact magnitude has never been documented [1].

We have investigated the sulfatase activity of rat gut bacteria for the sulfate ester of 3,3'-diiodothyronine (3,3'-T_2). This is a direct metabolite of the biologically active hormone triiodothyronine [T_3]. Simultaneously a preliminary identification of the nature of two sulfatase containing bacterial strains was obtained.

2. MATERIALS AND METHODS

2.1. Production of the sulfate ester of 3,3'-T_2 (T_2S) and determination of its deconjugation

T_2S is not commercially available. It was prepared by biosynthesis by incubating 1 μM 3,3'-T_2 plus 20 μCi 3,[3'-^{125}I]T_2 with primary cultures of isolated rat hepatocytes. After an incubation period of 2 h at 37°C in the presence of 100 μM propylthiouracil (to inhibit deiodination) over 80% of the original 3,3'-T_2 was sulfated [2]. After extraction of the incubation medium with ethanol, the extract was evaporated and chromatographed on Sephadex LH-20 columns. By this method T_2S could be isolated with > 90% purity. The molar mass was calculated from the amount of radioactivity present in the isolated material.

To determine the amount of T_2S deconjugation by various bacterial strains the same chromatographic procedure was used. After ethanol extraction and evaporation of the bacterial culture medium, the remaining material was chromatographed on Sephadex LH-20 by elution with 0.1 M HCl and 0.1 M NaOH, respectively. A good separation was obtained for iodide, T_2S and 3,3'-T_2.

Sulfatase hydrolysis of T_2S was estimated from the decrease of radioactivity in the T_2S fraction

and its reappearance in the 3,3'-T_2 fraction. Correction was made for nonspecific deiodination.

2.2. Incubation of T_2S with bacteria from rat intestinal flora

Diluted caecal contents from rats were cultured on a nonselective medium (Schaedler Broth, Oxoid, Basingstoke, U.K.) solidified with 2% agar in anaerobic cultures flasks [3]. Isolated bacteria were subcultured on the same medium with 0.3% agar in tubes. Caecal contents, anaerobic bacteria or bacterial strains and 10 nM T_2S with tracer activity were incubated in 10 ml broth at 37°C covered with paraffin for anaerobic conditions. After inoculation 1-ml samples were taken from the incubate at 0, 6, 10, 14, 18, 24 and 34 h and analysed for desulfation of T_2S. Control incubates of T_2S in broth were performed without bacteria. At 30 h samples from the incubate with and without T_2S were subcultured and the number of colony-forming units was determined. To evaluate a possible substrate induction of sulfatase activity, bacteria were preincubated with 10 nM unlabelled T_2S for 30 h prior to the incubation with labelled T_2S. For the incubation of T_2S with *Escherichia coli* and *Streptococcus* aerobic conditions were used.

3. RESULTS

Preliminary anaerobic incubations of rat caecal contents with T_2S resulted in its partial desulfation in 24 h. The subcultured anaerobic part of the intestinal flora also showed hydrolytic activity. Therefore, more detailed studies were performed using strictly anaerobic strains. Two anaerobic, Gram-positive strains from the intestinal flora of rats were found to actively desulfate T_2S. Both strains (A and B) hydrolysed within 18 h 80% of the 10 pmol/ml T_2S originally present in the medium (Fig. 1). Bacterial growth was not markedly influenced by the presence of T_2S. After 30 h of incubation with or without T_2S the numbers of bacteria for strain A amounted to 1.8 and $1.4 \cdot 10^6$ bacteria/ml, respectively. For strain B these numbers were 4.6 and $4.1 \cdot 10^8$ bacteria/ml. The deconjugating potential of strain A was higher since it performed the same rate of T_2S hydrolysis in spite of a hundred fold lower number of bacteria/ml (Fig. 1).

Examples of the chromatographic patterns of T_2S containing incubates with the anaerobic strain A, the aerobic strain C (*E. coli*) and without bacteria, before and after incubation are given in Fig. 2. No desulfation was noted with strain C or

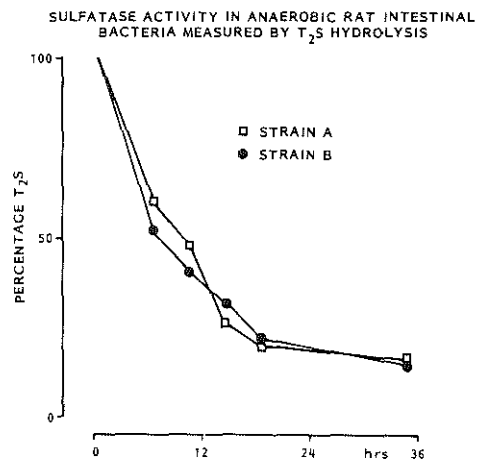

Fig. 1. Hydrolysis of 10 nM sulfate ester of 3,3'-T_2 (T_2S) by two anaerobic Gram-positive strains A and B, isolated from rat intestinal microflora.

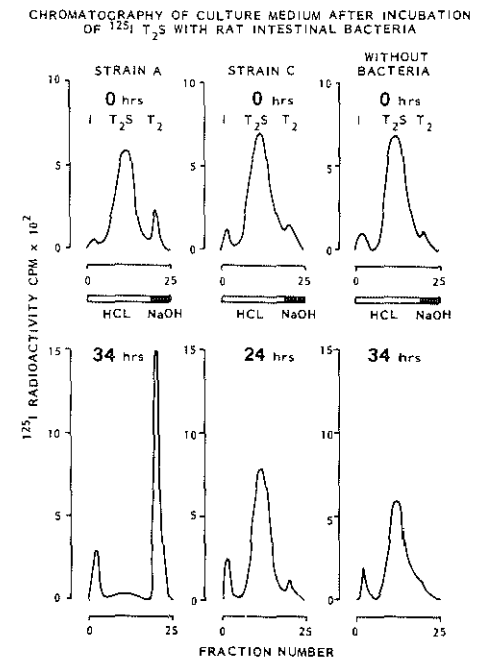

Fig. 2. Chromatography of culture medium containing 10 nM of the sulfate ester of 3,[3'-^{125}I]T_2 before and after incubation with: anaerobic strain A, aerobic strain C (*E. coli*), both from rat intestinal microflora, and without bacteria.

in control incubates, but 3.4% aspecific deiodination occurred. Other strains of bacteria isolated from the intestinal flora of rats (*Bacteroides*, *Escherichia* and *Streptococcus* species) had no deconjugating activity towards T_2S. No substrate induction of sulfatase activity by preincubation with unlabelled T_2S was observed.

4. DISCUSSION

Sulfatase activity of anaerobic rat intestinal flora has been documented in vitro for sulfate esters of bile acids [4] and steroids [5]. The actual presence of sulfatase activity of the intestinal microflora in vivo is suggested by the increased excretion of orally administered oestrone [^{35}S]sulfate in the faeces of rats after treatment with antibiotics [6]. The role of bacterial sulfatase activity in relation to a possible enterohepatic circulation has recently been reviewed [7]. We have isolated from rat intestinal microflora 2 strictly anaerobic, gram positive bacterial strains, which show sulfatase activity for the sulfate ester of 3,3'-T_2 in vitro. Sulfatase activity in mixed cultures of rat faecal preparations has also been demonstrated for the sulfate ester of T_3 [8]. However, these incubations were done under aerobic conditions and therefore are different from the actual in vivo situation. Comparison of hydrolytic activity with our study can not be made since the bacterial concentration in the incubates is not given. The 24 h needed for 80% desulfation of T_3S corresponds well with our study. A similar time has been documented for complete hydrolysis of ^{35}S-labelled lithocholate sulfate by *P. aeruginosa* from human faeces [9]. No sulfatase activity was demonstrated for *E. coli*, *Bacterioides* species, *Streptococcus* species and bifidobacteria. Contrary to other experiments, which showed a substrate induction of glycosulfatase activity in mixed faecal cultures [7], we were unable to demonstrate such induction in our anaerobic strains with a low concentration of T_2S. Since anaerobic conditions prevail in the intestinal lumen and the two anaerobic strains isolated by us from rat intestinal contents are very active in hydrolysing T_2S it is reasonable to assume that desulfation of biliary secreted sulfated iodothyronines does actually take place in the rat gut. As iodothyronines are much better absorbed from the gastro-intestinal tract when they are in the free, that is the unconjugated form [10], this bacterial sulfatase activity therefore may actually enhance the enterohepatic circulation of iodothyronines. Depending on the turnover rate of such an enterohepatic circulation, disturbance of the normal bowel function (e.g., diarrhoea, intestinal operation or malabsorption) may alter thyroid hormone disposal and bioavailability. However, the actual contribution of the gut bacteria to this process awaits further studies.

ACKNOWLEDGEMENTS

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SUMMARY

Peripheral thyroid hormone metabolism is the denomination for the multitude of biochemical reactions and feedback mechanisms involved in maintaining the euthyroid state of the body. The aim of the research presented in this thesis has been to contribute to a better understanding of some aspects of this broad field of investigation. The manuscript consists of two sections. Section I attempts to integrate the current knowledge of peripheral thyroid hormone metabolism with the most relevant observations from our research. In Section II the factual data of this work are presented as separate papers, which have either been published or are submitted for publication.

In essence, three different lines of investigation have been followed. The first line studied the influences of dietary composition on iodothyronine serum levels in four healthy, non-obese subjects. The results suggested an active role of dietary fat in the regulation of these serum levels. A diet composed of 100% fat induced a similar decrease of 3,3',5-triiodothyronine (T_3) and increase of 3,3',5'-triiodothyronine (reverse T_3) as total starvation. Fat also antagonized the T_3-stabilizing action of carbohydrates in hypocaloric nutrition. For the influence of food on thyroid hormone metabolism the dietary composition appears to be more important than the total amount of calories.

Kinetic studies with labeled iodothyronines in human beings and animals give information about the respective production and metabolic clearance rates. However, the individual contribution of the various tissues to overall metabolism remains obscure, and rapid successive intracellular reactions cannot be detected. Since the liver plays a central role in peripheral thyroid hormone metabolism, the second line of research dealt with iodothyronine metabolism at the tissue-level with the use of isolated rat hepatocytes. For this purpose hepatocyte monolayers were incubated with thyroxine, T_3, reverse T_3 and 3,3'-diiodothyronine (3,3'-T_2).

Hepatic processing of reverse T_3 appeared to be quite different from that of 3,3'-T_2 and T_3. Reverse T_3 is rapidly degraded by liver cells through direct

deiodination in the outer ring yielding iodide and 3,3'-T_2. Only little conjugation, presumably with glucuronic acid, occurs. This contrasts with 3,3'-T_2 and T_3, for which conjugation appeared to be the first and principal metabolic process. 3,3'-T_2 is exclusively sulfated, whereas T_3 can also be conjugated with glucuronic acid. Sulfation considerably enhanced the inner ring deiodination of T_3 and the outer ring deiodination of 3,3'-T_2. Inhibition of sulfation virtually abolished deiodination. It was concluded that hepatic clearance and deiodination of 3,3'-T_2 and T_3 largely depends on the sulfate transferring capacity of the liver cells.

Iodothyronines preferentially appear in bile as conjugates. Hydrolysis of these conjugates by the intestinal microflora might enhance enteral reabsorption of the aglycons, thereby promoting an enterohepatic circulation. The third line of research has investigated the hydrolytic potential of rat gut bacteria towards iodothyronine sulfates. From rat cecal contents 2 anaerobic bacterial strains were isolated that actively hydrolysed 3,3'-T_2 sulfate.

In Section I of this thesis special attention is given to the recent recognition of two tissue-specific pathways for iodothyronine deiodination. By this distinction the body tissues can be subdivided in having deiodinase activity which is either inhibited, or unaffected by propylthiouracil (PTU). Instigated by this notion and our own results a tentative model is proposed, postulating a close metabolic interaction of the two tissue types in the modulation of iodothyronine serum levels. It is conceived that the serum levels of a number of iodothyronines are determined by production in the one type of tissue and by degradation in the other. Support for this hypothesis was found in our preliminary observation that human brains (PTU-insensitive) may be an important site of 3,3'-T_2 production, whereas the liver (PTU-sensitive) very efficiently extracts this compound from the blood.

SAMENVATTING

Een allesomvattende definitie van wat er onder het perifere metabolisme van schildklierhormoon verstaan wordt, is niet eenvoudig te geven. De belangrijkste funktie van schildklierhormoon lijkt te zijn de regulatie van een gelijkmatige basale stofwisseling in het lichaam. De basale stofwisseling draagt zorg voor een bepaalde grond-snelheid van veel intracellulaire processen en biochemische reacties. Hierdoor ontstaat er een zekere uitgangs-toestand, waarop alle specifieke funkties van het lichaam, ieder met zijn eigen behoefte en regulatie, uitgevoerd kunnen worden. In grote lijnen kan men stellen, dat hoe meer (minder) schildklierhormoon in het lichaam aanwezig is, hoe hoger (lager) het basaal metabolisme zal zijn afgesteld.

De totale hoeveelheid schildklierhormoon in het lichaam wordt bepaald door twee faktoren: de produktie en de afbraak ervan. Door deze individueel of gelijktijdig te veranderen heeft het lichaam de mogelijkheid het basaal metabolisme te reguleren. De produktie van schildklierhormoon vindt plaats in de schildklier, terwijl de afbraak in buiten de schildklier gelegen of "perifere" weefsels geschiedt.

Het voornaamste produkt van de schildklier, het thyroxine, bestaat uit een moleculair skelet met 4 jodium atomen. Hiervan stamt de voor thyroxine veel gebruikte afkorting "T_4". Deze stof heeft zelf geen biologische aktiviteit, maar moet om deze te verkrijgen een omzetting ondergaan. Bij deze omzetting wordt een van de 4 jodium-atomen van het basis-skelet afgesplitst, waardoor een molecuul ontstaat met 3 jodium-atomen en grote biologische aktiviteit, het zogenaamde trijodothyronine of T_3. Dit omzettingsproces (dejodering) wordt uitgevoerd door verschillende enzymen in de "perifere" weefsels. Ook de overige 3 jodium-atomen kunnen op hun beurt gedejodeerd worden, waardoor een hele reeks metabolieten (stofwisselingsprodukten) ontstaat (zie Fig.1, pagina 18). Een van de metabolieten die de laatste jaren veel aandacht heeft gekregen is het reverse T_3 (rT_3). Dit jodothyronine heeft net als T_3 3 jodium atomen maar met een

andere verdeling op het basis-skelet waardoor het iedere biologische aktitiveit mist.

Naast deze dejoderings-reakties kunnen de metabolieten of jodothyronines ook nog een koppeling ondergaan met glucuronzuur (glucuronidatie) of sulfaat (sulfatering). Hierdoor veranderen de biochemische eigenschappen van de jodothyronines en kunnen ze bijvoorbeeld gemakkelijker via gal of urine uitgescheiden worden. Van alle weefsels die bij deze processen betrokken zijn neemt de lever een centrale plaats in.

Het is al geruime tijd bekend, dat ziekte, geneesmiddelen of verandering in dieet-samenstelling het perifere metabolisme van schildklierhormoon kunnen beinvloeden. De meest opvallende verandering is een verminderde omzetting, in onder andere de lever, van het inaktieve T_4 naar het aktieve T_3. Hierbij daalt de bloedspiegel van T_3 en secundair hieraan het basaal metabolisme. Men speculeert dat dit een energie-besparende aanpassing van het lichaam is in stress-situaties of tijdens voedselschaarste. Een interessante waarneming onder deze omstandigheden is de vrijwel reciproke stijging van reverse T_3 in het bloed. Terwijl de daling van T_3 veroorzaakt wordt door een verminderde produktie, komt de stijging van rT_3 tot stand door een afname van de klaring.

De in dit proefschrift beschreven experimenten hebben tot doel gehad meer inzicht te verkrijgen in deze stofwisselingsprocessen van jodothyronines. In totaal zijn drie onderzoekslijnen te onderscheiden.

1) De bestudering van de invloed van vet in het voedsel op het perifere metabolisme van schildklierhormoon. Uit dieetproeven met gezonde vrijwilligers kon worden opgemaakt dat vet een aktieve rol speelt bij de samenstelling van de jodothyronines in het bloed. Opvallend was hierbij dat een dieet bestaande uit 1500 KCal 100% vet, dezelfde veranderingen induceerde als volledig vasten.

2) T_4 en zijn metabolieten worden voornamelijk uitgescheiden in de gal als conjugaten van glucuronzuur of sulfaat. In de darm worden de conjugaten slecht geresorbeerd terwijl dit voor de ongeconjugeerde of vrije jodothyronines juist gemakkelijk geschiedt. Onderzocht werd in hoeverre darmbacterieën in staat zijn om de jodothyronines los te maken van hun conjugaten. Door incubaties van uit rattefaeces geïsoleerde bacterieën met de conjugaten kon inderdaad aangetoond worden dat bepaalde bacterie-soorten tot deze ontkoppeling in staat zijn. Hiermee is de mogelijkheid van het uit de darm terugwinnen van reeds uitgescheiden schildklierhormoon (de entero-hepatische circulatie) aannemelijker geworden. Dit mechanisme zou vooral van betekenis kunnen zijn in tijden van hormoon-schaarste zoals voorkomt bij een te langzaam werkende schildklier.

3) De derde lijn bestond uit gedetailleerd onderzoek naar het metabolisme van jodothyronines in de lever. Als model werd daarbij gebruik gemaakt van

gekweekte rattelevercellen. Verschillende jodothyronines werden gedurende enige tijd met de celcultures geincubeerd, waarna het kweekmedium onderzocht werd op gevormde produkten. Hierbij bleek dat er voor 3,3'-T_2, een direkte metaboliet van het aktieve T_3 (Fig.1, pagina 18), 2 stofwisselingsprocessen bestaan in de lever: 1) dejodering en 2) sulfatering. Het wekte verbazing dat de dejodering van 3,3'-T_2 zo gemakkelijk verliep in gekweekte levercellen, omdat uit vroeger onderzoek bekend was, dat 3,3'-T_2 zeer slecht gedejodeerd wordt door het uit rattelever geisoleerde enzym dejodase. Op grond hiervan werd gepostuleerd dat 3,3'-T_2 eerst gesulfateerd en pas daarna gedejodeerd zou worden.

Een belangrijk deel van het proefschrift wordt gevormd door de experimenten die de juistheid van deze hypothese aannemelijk gemaakt hebben. De essentie van de bewijsvoering wordt gevormd door drie waarnemingen: a) selektieve remming van de sulfatering in rattelevercellen veroorzaakt ook remming van de dejodering b) daarentegen geeft remming van de dejodering juist ophoping van het met sulfaat geconjugeerde 3,3'-T_2 (T_2S) c) T_2S bleek, in tegenstelling tot het oorspronkelijke 3,3'-T_2 heel goed gedejodeerd te worden door het lever-dejodase.

Op identieke wijze kon aangetoond worden, dat dejodering van het biologisch aktieve T_3 eveneens slechts optreedt na voorafgaande sulfatering van het T_3 molecuul. Vooral deze laatste waarneming trekt de aandacht omdat hiermee sulfatering in de lever een essentiele en snelheids-bepalende stap blijkt te zijn in de afbraak van het enige biologisch aktieve schildklierhormoon. Hierdoor wordt het begrijpelijk waarom het lichaams-T_3 (en dus het basaal metabolisme) tijdens ziekte of vasten kan dalen: de produktie van T_3 uit T_4 in de lever vermindert onder deze omstandigheden, terwijl de klaring (afbraak) van T_3 via sulfatering gelijk blijft.

Daarnaast hebben incubatie experimenten van de levercellen met reverse T_3 aangetoond dat de klaring van deze inaktieve metaboliet wel door rechtstreekse dejodering tot stand komt, d.w.z. zonder dat voorafgaande sulfatering hoeft plaats te vinden. Dit lijkt dan ook de verklaring waarom onder omstandigheden van verminderde dejodase-aktiviteit in de lever (ziekte, vasten) er een verminderde klaring en stijging van het serum rT_3 optreedt.

Terwijl door deze experimenten een beter inzicht verkregen werd in de mechanismen van het jodothyronine metabolisme kan de betekenis van deze waarnemingen voor ziekteprocessen, vermagering of geneesmiddelen-gebruik nog niet exakt gedefinieerd worden. Hiervoor is nader onderzoek vereist.

NAWOORD

De mogelijkheid om aan een promotieonderzoek te werken heb ik als een voorrecht beschouwd. Het is een leerzame periode die naast de incidentele voldoening van wetenschappelijk onderzoek, de gelegenheid geeft om als arts vertrouwd te raken met andere dan medische denk- en onderzoekswijzen. De onontbeerlijke ontwikkeling van een kritische instelling ten opzichte van met name eigen waarnemingen als ook die van anderen, komt de besluitvorming en het klinisch inzicht van de medicus ten goede.

Het totstandkomen van een dissertatie is van veel mensen en een aantal onvoorspelbare faktoren afhankelijk. Op deze plaats wil ik graag mijn dank betuigen aan diegenen die voor mij de onzekere faktoren tot een minimum hebben weten te beperken en mij geinspireerd, gestimuleerd en geholpen hebben om dit proefschrift te voltooien.

Dat betreft in de eerste plaats mijn promotor, Jorg Hennemann, die mij op geheel eigen wijze wist te winnen voor een onbekend onderzoeksgebied en mijn research-belangstelling gewekt heeft. Met veel genoegen denk ik terug aan de altijd stimulerende discussies over ons werk, zijn grote hulpvaardigheid bij moeilijkheden en het inzicht en de ervaring waarvan zijn adviezen getuigden.

Het is Theo Visser geweest die vanaf mijn eerste onwennige begin in het laboratorium steeds op bijzonder plezierige wijze leiding heeft gegeven aan mijn onderzoek. Zijn kennis van zaken, heldere manier van denken en goede ideeën zijn voor mij een bron van inspiratie geweest. Ook van de vrijheid die hij mij liet bij de experimentele uitvoering heb ik veel geleerd.

Roel Docter was als hoofd van het laboratorium altijd behulpzaam met praktische adviezen. Zijn computerprogramma's waren van onschatbare waarde bij het uitwerken van de talloze chromatografieën.

Jannet Blom-Leenheer en Marla van Loon hebben op buitengewoon coöperatieve wijze de vaak gecompliceerde experimenten en massale chromatografieën verzorgd. Hun vrolijkheid en persoonlijke inzet zijn mij zeer tot steun geweest.

Bert Bernard verrichtte de eerste experimenten en ontwikkelde de techniek van de leverperfusie.

De gezamenlijke experimenten en plezierige discussies met Maarten Hazenberg (Medische Microbiologie) en Wouter de Herder hebben een wezenlijke bijdrage aan dit proefschrift geleverd.

Aan de samenwerking met Hans van der Heyden en Eric Krenning, evenals aan de vele onderhoudende en leerzame uurtjes met mijn kamergenoten Jan Mol en Huib Pols, bewaar ik de beste herinneringen.

De grote bereidwilligheid en inzet van Yvonne van Dodewaard bij het accuraat tikken en verzorgen van het manuscript, maakten ook dit onderdeel van het promoveren tot een genoegen.

De medewerkers van het laboratorium bedank ik voor de goede en vriendschappelijke sfeer tijdens de jarenlange samenwerking.

De Audiovisuele Dienst verzorgde op uitstekende wijze de figuren en Henk van Beek van de afdeling Automatische Signaal Verwerking (Centrale Research Werkplaatsen) was zeer behulpzaam bij het gebruik van de tekstverwerker.

CURRICULUM VITAE

The author of this thesis was born in 1947 in The Hague. After completing secondary school (Gymnasium B) he graduated in 1974 at the Medical Faculty of the State University of Utrecht. Following a 6 month training period as general practitioner (practice of Dr. L.W.S. van der Burg) he served as medical officer RNR at the internal ward (head A.D.J. Verburg) of the Royal Navy Hospital. From 1976 till 1981 he was resident at the Department for Internal Medicine III and Clinical Endocrinology (head Prof.Dr. J.C. Birkenhäger) Dijkzigt Hospital, Erasmus University, Rotterdam. In 1979 he participated in an exchange-program of the European Society for Clinical Investigation and spend a year at the Department for Internal Medicine (head Prof. W. Stauffacher) of the Kantonsspital, University of Basle, Switzerland. From 1981 till 1983 he was a staff-member of the Department for Internal Medicine III, during which period this thesis was completed. Since June 1983 he is working at the Department for Gastroenterology (head Dr. J.H.M. van Tongeren) of the St. Radboud Hospital, University of Nijmegen.