THYROID HORMONE TRANSPORT INTO LIVER CELLS: ITS (PATHO)PHYSIOLOGICAL SIGNIFICANCE.

DE (PATHO)FYSIOLOGISCHE BETEKENIS VAN SCHILDKLIERHORMOONTRANSPORT OVER HET LEVERCELMEMBRAAN.

PROEFSCHRIFT

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door
MARION DE JONG
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PROMOTIECOMMISSIE

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

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voor Jenny en Bertus

voor Jan, Daan-Jan en Bertram

Ook een weg van duizend mijl
vangt aan bij de eerste stap.

Japans spreekwoord

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VOORWOORD

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LIST OF ABBREVIATIONS

α-AIB	α-amino-iso-butyrate
ATP	adenosine 5'-triphosphate
ATP-ase	adenosine triphosphatase
BES	N,N-bis[2-hydroxy-ethyl]-2-amino ethane sulfonic acid
BSA	bovine serum albumin
BW	body weight
CAA	cell associated radioactivity
cAMP	cyclic adenosine-monophosphate
CMPF	3-carboxy-4-methyl-5-propyl-2-furan propanoic acid
Da	dalton
db	dibutyryl
DNA	deoxyribonucleic acid
F	free (not protein bound)
FC	fast component
G	glucuronide
GOT	alanine transaminase
GPT	aspartate transaminase
HEPES	N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid
IRD	inner ring deiodination
k	kilo
K_m	Michaelis constant
LDH	lactate dehydrogenase
MCR	metabolic clearance rate
Me-	methyl-
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NTI	non thyroidal illness
ORD	outer ring deiodination
PIPES	piperazine-N,N'-bis[2-ethane sulfonic acid]
PR	production rate
PTU	6-propyl-2-thiouracil
RIA	radio-immuno-assay
REP	rapidly equilibrating pool

rT_3	reverse triiodothyronine (3,3',5'-triiodothyronine)
S	sulfate
SC	slow component
SD	standard deviation
SEM	standard error of the mean
SEP	slowly equilibrating pool
T_0	thyronine
T_1	monoiodothyronine
T_2	diiodothyronine
T_3	3,3',5-triiodothyronine
T_4	thyroxine (3,3',5,5'-tetraiodothyronine)
TBG	thyroxine binding globulin
TBPA	thyroxine binding prealbumin
TH	thyroid hormone
TMT	total mass transfer
TNF-α	tumor necrosis factor-α
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
UDP	uridine diphosphate
UDPGT	UDP-glucuronyltransferase
V_{max}	maximum velocity

parameters compartmental models:

($i=1,2,3$; $j=1,2,3$; $i \neq j$)

t	time (min)
y(t)	plasma activity at time t (% dose/L)
A_i	coefficient of the i^{th} exponential component (% dose)
D	dose
λ_i	exponent of the i^{th} exponential component (min^{-1})
Q_1	size of pool 1 (plasma pool; % dose)
Q_2	size of pool 2 (fast or rapidly equilibrating pool; % dose)
Q_3	size of pool 3 (slow or slowly equilibrating pool; % dose)
k_{ij}	fractional transport rate from pool j to pool i (min^{-1})
k_{0i}	fractional disposal rate in pool i (min^{-1})
V_p	plasma volume (L)

CHAPTER 1

INTRODUCTION

In the experiments described in this thesis transmembrane transport of thyroid hormones into liver cells is investigated, in particular the regulatory role of this transport process in the bioavailability of thyroid hormones in (patho)fysiological conditions.

This first chapter is an introduction to the studies described in Chapters 3-9. It opens with a short description of different aspects regarding thyroid hormones, i.e. synthesis, transport in blood to the different organs, transport into cells, nuclear binding and the different routes of intracellular metabolism. Only the main issues will be discussed here. The isolated rat liver perfusion system was used to study the transport process in most of our studies. Therefore, this perfusion system is also described in this chapter and compared to isolated rat hepatocytes in primary culture, in which many studies on thyroid hormone transport have been performed. Furthermore, a two-compartment model, which describes thyroid hormone kinetics in the liver perfusion system, is dealt with here. At the end of the chapter the scope of the thesis and a short overview of the questions that were addressed are presented.

1.1 SYNTHESIS OF THYROID HORMONE

Thyroid hormones are small, relatively simple molecules that are formed in a giant prohormone molecule, thyroglobulin. In the process of thyroid hormone synthesis (1), iodide is taken up by the thyroid follicular cells. It is oxidized and bound to tyrosyl residues of thyroglobulin at the apical membrane, whereby mono- and diiodotyrosyl residues are produced. This iodination step is followed by a coupling reaction, in which two iodotyrosyl residues form a hormone residue. The reactions producing the iodotyrosines and iodothyronines are both oxidative, catalyzed by thyroperoxidase and using hydrogen peroxide as electron acceptor. The hormones, part of the thyroglobulin molecules in which they are formed, are stored in the lumen of the thyroid follicles. Thyroglobulin is subsequently taken up by the follicular cells and hydrolyzed by lysosomal hydrolases, liberating thyroid hormones from their peptide linkage within thyroglobulin. Iodothyronines are thereupon secreted into the blood stream. The thyroid hormone 3,3',5,5'-tetra-

iodothyronine (thyroxine, T_4) is the main product of the thyroid gland (Figure 1); in healthy humans on average 115 nmol T_4 is secreted per day per 70 kg body weight (2).

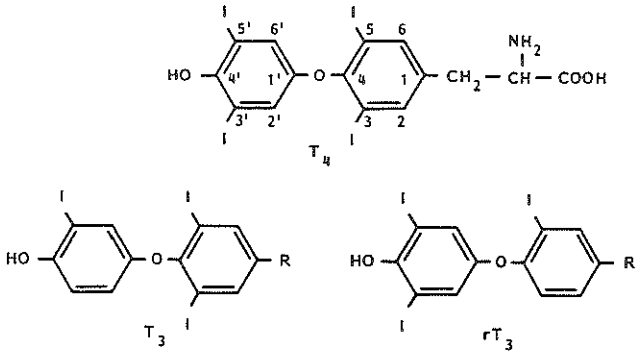

Figure 1
Structures of T_4, T_3 and rT_3

T_4 has little or no intrinsic bioactivity. The thyroid gland also produces 3,5,3'-triiodothyronine (T_3) (Figure 1), the most important bioactive thyroid hormone. However, this production amounts to only 20 % (= 9 nmol T_3 per day per 70 kg body weight) of the total daily production (2); the remaining 80 % is derived from the deiodination of T_4 to T_3 outside the thyroid. T_3 stimulates growth and development of the organism and plays an important role in the regulation of metabolic processes and energy consumption in different tissues (3-6). Also 3,3',5'-triiodothyronine (rT_3) (Figure 1), which is an inactive metabolite, is produced in the thyroid gland; this production amounts to only 5 % of daily rT_3 production in the body (= 2 nmol per day per 70 kg body weight (2)). Contribution of the thyroid to the circulating diiodothyronines in plasma is negligible (7,8).

The major factor regulating the function of the thyroid gland with regard to production and secretion of thyroid hormones is thyroid-stimulating hormone (TSH), a glycoprotein released by the thyrotropic cells of the anterior pituitary gland. TSH binds to specific plasma membrane receptors on the surface of the

follicular cells in the thyroid and exerts its action mainly via the second messenger cAMP (9). Determinants of TSH secretion are inhibition by T_4 and T_3 (10-12), dopamine (13), glucocorticoids and somatostatin (14), and stimulation by thyrotropin-releasing hormone (TRH) (15). The latter two are hypothalamic factors.

1.2 TRANSPORT IN BLOOD

In plasma, the thyroid hormones circulate largely bound to three plasma proteins. These are thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) and albumin, which carry about 75, 15 and 10 % of plasma T_4 in humans, respectively (16). Values for T_3 protein binding range between 38-80 % for TBG, 9-27 % for TBPA and 11-35 % for albumin (16,17). In normal human serum free T_4 (FT_4) and FT_3 average approximately 0.02 % and 0.2 % of total T_4 and T_3 levels, respectively. Except for the neonatal period, the rat lacks TBG as binding protein in the plasma; thyroid hormones are mostly bound to TBPA and to a lesser extent to albumin (18). FT_4 and FT_3 in rats are about 0.025 % and 0.35 %, of total hormone levels, respectively (19,20).

As for the function of these plasma proteins, several have been considered (16). It has been assumed that they serve as an extrathyroidal storage of thyroid hormones and contribute to thyroid hormone homeostasis in the body by providing a readily available supply of thyroid hormones to cells. Also, a buffering function can be considered, since the binding proteins maintain a low free hormone concentration in the pool of extrathyroidal hormone, serving to protect the cells from excessive hormone entry. This is in agreement with rat liver perfusion studies that showed that the presence of albumin in the perfusion medium was necessary to obtain a uniform distribution of T_4 over the liver cells; in the case of perfusion without any binding protein in the medium virtually all T_4 was taken up by the periportal cells (21). It was also shown that the same effect could be obtained by addition of TBG or TBPA to the perfusion medium. Finally, the binding proteins may have a hormone-releasing function. It can be calculated that in the human liver the rate of dissociation of T_4 from TBG, TBPA and albumin is more than one order of magnitude larger than the unidirectional removal of T_4 from the

plasma during a single pass (22). Because in equilibrium efflux from the cells is almost as large as influx, net removal of T_4 is three orders of magnitude smaller than the dissociation rates of T_4 from its binding proteins, making a role of these proteins in hormone delivery to the cells less probable. Similar calculations show that TBPA, the major carrier protein in rats (18,23) liberates T_4 at a faster rate than required for tissue entry during a single capillary pass. However, the actual situation may be more complicated by events occurring in the microcirculation, such as intracapillary diffusion and capillary wall perfusion (24,25).

1.3 TRANSPORT INTO TISSUES

1.3.1. Transport: general

Biological membranes are essentially bilayers of phospholipids into which molecules of cholesterol and proteins are inserted. Such bilayers form a continuous sheet around the cell and around the individual cellular organelles. A molecule that has to enter the cell from the plasma must cross this sheet. This is possible in several ways, according to the nature of the compound. Simple diffusion may be possible for hydrophobic substances, but uptake of more hydrophilic substances requires the presence of transmembrane or transport proteins through which carrier mediated (facilitated or active) transport can occur.

The intracellular concentration of a certain transported hormone may then be determined by 1) the plasma flow rate, 2) the plasma concentration of hormone and hormone-binding proteins, 3) the rate constant for hormone dissociation from its binding proteins, 4) rebinding to its binding proteins, 5) influx rate constant into the cell, 6) efflux rate constant, 7) intracellular binding, and 8) metabolic rate constant (26), depending on which step is the rate limiting one.

1.3.2. Free hormone hypothesis

In this hypothesis, that was first formulated by Recant and Riggs in 1952 (27) and more fully explored by Robbins and Rall (28), it is proposed that the "free hormone concentration in serum governs hormone delivery to the cell

and ultimately regulates hormonal action". This hypothesis is based on the assumptions that 1) free, but not protein-bound hormone is taken up by tissues; 2) the rate of tissue uptake and, hence, the availability of intracellular hormone is determined by the free hormone concentration in plasma; 3) the biological effect of a hormone is positively correlated with its intracellular concentration.

As the unidirectional removal of thyroid hormone greatly exceeds the free hormone pool in plasma, Pardridge challenged the free hormone hypothesis and stated that besides the free hormone pool, also hormone bound to albumin was available for transport via protein-mediated transport or enhanced dissociation from albumin (29). However, others showed that in analbuminemic rats (rats that lack albumin) transport of T_4 into the liver was the same as in normal rats (30). Furthermore, it was recently shown (31) that in perfused rat livers transport of thyroid hormones into the metabolizing compartment of the liver is only dependent on the free hormone concentration in the perfusion medium, and not on the albumin concentration. These and other (32) studies make a special role for albumin in thyroid hormone transport very unlikely. Therefore, it can be concluded that the transport process is only governed by the free hormone concentration, *under the condition that the membrane transport process itself is unchanged.*

1.3.3. Active transport of iodothyronines

Originally, it was postulated that thyroid hormones, by way of their lipophilic nature, would pass the lipid bilayer of the cell membrane by passive diffusion to become intracellularly handled (33,34). However, later studies indicated that this appeared not to be the case as will be outlined below.

1.3.3.1 In vitro studies

In the last decade the transport process of thyroid hormones over the liver cell membrane has been subject of many studies, and evidence has accumulated that cellular uptake of T_4 and T_3 into several organs is carrier-mediated, saturable and energy dependent (35-54). These studies have been performed in human, rat, and amphibian erythrocytes, human lymphocytes, rat and mouse thymocytes, human and mouse fibroblasts, rabbit and rat adipocytes, rat myoblasts, human hepatoma cells, rat pituitary (tumor) cells,

rat chondrosarcoma cells, human and mouse neuroblastoma cells, and human glioma cells.

Direct evidence for the presence of a carrier-mediated transport system for thyroid hormones was obtained with the use of a monoclonal antibody (ER-22), directed against the rat hepatocyte plasma membrane (55), that inhibited thyroid hormone transport into the cells. In isolated rat hepatocytes in primary culture, two saturable binding sites, one with high affinity and low capacity (K_m in the nmolar range) and the second with a low affinity and a high capacity (K_m in the μmolar range) are present for T_4, T_3 and rT_3 (38-41). Similar findings were reported by other authors for a number of different cell types, such as human cultured fibroblasts (42,46), cultured GH_3 cells (56), human HepG2 hepatocarcinoma cells (48), human cultured lymphocytes (37), mouse thymocytes (50) and mouse neuroblastoma cells (57). The high affinity system can be partially blocked when cells are preincubated with metabolic inhibitors such as KCN, dinitrophenol or oligomycin (40), indicating the energy dependence of the system. It is also strongly influenced by changes in temperature (38). This dependence on both temperature and energy strongly suggest that the high affinity system of thyroid hormones represents an active transport process. Furthermore, this system is inhibited by ouabain, a specific inhibitor of Na^+,K^+-ATP-ase, which could imply that a sodium gradient over the plasma membrane is of importance in the transport process, which was also found in rat skeletal muscle (58).

On the other hand, the low affinity system of thyroid hormones does not show energy or temperature dependence; therefore, it has been suggested that this system represents binding of thyroid hormones to the outer surface of the cell (38,59).

From the work of Cheng et al., using a fluorescence microscopic procedure, it was suggested that T_3 uptake into mouse fibroblasts occurred via receptor-mediated endocytosis (42). This process was stereospecific and inhibited by cooling, oligomycin and monodansylcadavarine. Endocytosis inhibitors were later shown to interfere with T_3 and T_4 uptake in many other types of cells (43,44,57). Although caution is needed when interpreting indirect evidence based on potentially toxic compounds, these data suggest that endocytosis of T_3 and T_4 may be a general phenomenon.

Recently, Docter et al., using a recirculating rat liver perfusion system and a two-compartment model of thyroid hormone distribution and metabolism (see below), were able to divide transport of T_3 into the liver pool into transport into a non-metabolizing liver compartment and transport into the intracellular liver compartment, where metabolism takes place. Transport to the former liver compartment of T_3 was dependent on the medium albumin concentration, whereas the amount of T_3 transported into the cells for further metabolism was determined by the medium free T_3 concentration and independent of the albumin concentration in the medium (31).

From the studies of Krenning et al. with isolated rat hepatocytes, it appeared that T_4 and rT_3 share a common pathway to enter the cell, that is different from that of T_3 (40). Both systems have the characteristics of an active transport system, but there are, however, considerable differences, e.g. concerning K_m and V_{max} for the substrates. Furthermore, mild alterations in ATP concentration have much more profound effects on T_4- and rT_3- than on T_3-transport (38-40). However, the studies of Blondeau et al. (47) were not in accordance with these findings. They suggested that T_4 and T_3 share a single facilitated transport system.

So, in order to become deiodinated to bioactive T_3, T_4 has to be transported from the plasma over the liver cell membrane into the intracellular compartment, as the deiodinating enzyme is located in the endoplasmic reticulum, both in the liver and in the pituitary gland (60). Therefore, if the active transport system for thyroid hormones has physiological significance, it is to be expected that this process plays a role in the regulation of the availability of T_4 to the deiodinating enzyme, thereby influencing the amount of bioactive T_3 formed. Indeed, it was shown in isolated rat hepatocytes that inhibition of thyroid hormone transport was rate-limiting on intracellular conversion (61). Also from other studies in rat hepatocytes and GH_3 cells (43,62), it is apparent that changes in the uptake of iodothyronines into cells have an important effect on their bioavailability and metabolism. Organs like the brain, the pituitary gland and brown adipose tissue, produce T_3 from T_4 locally to meet (part of) their needs of active hormone (63). In contrast, the majority of the T_3 target tissues, such as the liver and muscles, largely depend on plasma T_3 as the major source of their intranuclear T_3 (10,63). In the latter

organs, therefore, T_3 is derived from plasma and not directly from intracellular T_4 conversion, making the plasma membrane transport process the first and important step before the cascade of intracellular events that follow.

1.3.3.2. In vivo studies

Most of the first kinetic studies in vivo have been performed with the non-compartmental analysis as first applied to thyroid hormones by Oppenheimer et al. (64). This calculation technique yields values for total body hormone pool sizes, fractional clearance rates and total body production rates. However, it is not possible to estimate tissue pool sizes or fluxes into tissues (65). These data can be obtained using a three-compartment model of thyroid hormone distribution and metabolism, consisting of a central plasma pool in equilibruim with a rapidly equilibrating tissue pool (REP) and a slowly equilibrating tissue pool (SEP). It is assumed that disposal of hormone only occurs in the tissue pools. Since the original publications of DiStefano et al. on the use of this three-compartment model of T_3 and T_4 metabolism (66,67), a large number of reports has appeared using this model. It can be argued that the REP in this model is mainly composed of liver and kidney (66), the size of the liver pool being quantitatively the most important. Therefore, mass transport to this REP can be equated to hormone transport to the liver.

Some reports showed evidence of active transport of thyroid hormones in vivo, as this transport process was reduced to about one-half of the normal value during caloric restriction (68). There are indications that a similar diminution occurs in non-thyroidal illness (69), and with acute amiodarone administration (70) (see below). This decrease of the transport process is not related to the serum levels of total or free thyroid hormone.

Thus, the transport process over the cell membrane may also play a role in vivo in the regulation of the intracellular availability of thyroid hormones.

However, not all findings on the transport processes of thyroid hormones in vitro and in vivo are uniform. Some workers did not demonstrate saturability of T_3 transport in isolated rat hepatocytes (71) or perfused rat liver (72) or they did not show a dependence on cellular energy (47). Weisiger et al. (73) did not find a significant difference between the free clearances for influx and efflux of T_3 in the perfused rat liver. Therefore, they concluded that

in this organ T_3 uptake is equilibrative rather than actively driven by cellular energy metabolism. Furthermore, some studies did not show energy-dependent uptake of T_4 in the same systems in which energy-dependent T_3 uptake was demonstrated (45,58,74-76). There are several explanations for these discrepancies on transport of thyroid hormones, which will be described in the General Discussion of this thesis.

1.3.4 Thyroid hormone efflux from cells

The intracellular bioavailability of thyroid hormone is determined by the intracellular free concentration, which is the net result of uptake, intracellular production, intracellular breakdown, binding and efflux from the cells. Particularly in the liver, this bioavailability is mainly governed by uptake and efflux rates, because intracellular production and degradation are comperatively small (23,68). In contrast to the influx process, it has been shown in isolated rat hepatocytes that efflux of thyroid hormones is not energy dependent and not saturable with unlabeled free hormone in the nanomolar range (62), in agreement with a passive process.

1.3.5 Thyroid hormone transmembrane transport in (patho)physiological conditions

Non-thyroidal illness (NTI), caloric deprivation, surgery and several medications (PTU, propranolol, dexamethasone, and X-ray contrast agents) induce a "low T_3 syndrome" (77,78), characterized by high total and free serum rT_3, and low total and free T_3. A continuum of changes in serum thyroid hormone levels relative to the severity of NTI has been observed (79,80). Total T_4 levels correlate fairly well with clinical outcome; the highest mortality occurring in the patients with the lowest total T_4 levels. Total T_4 levels may be increased (in mild disease) or decreased (in more severe disease), but a consistent decrease in serum T_3 and an increase in rT_3 levels are observed. These effects are due to decreased production with unaltered clearance of T_3 and a decreased metabolic clearance rate with unaltered production of rT_3 (3,77). TSH is mostly in the normal range, in spite of the low plasma T_3 levels (81,82).

The cause of these changes in plasma iodothyronine production or

clearance during the low T_3 state has been subject of many studies. As T_4 to T_3 deiodination and rT_3 clearance are both catalyzed by type I deiodinase in the liver (and kidney) (see below), this enzyme has been implied as an important factor in the genesis of the syndrome. In humans, d-propranolol-induced changes in thyroid hormone metabolism, resulting in a low T_3 syndrome, were ascribed to inhibition of thyroid hormone type I deiodination (83). This enzyme inhibition by propranolol was also shown in isolated rat renal tubulus (84). Other drugs that inhibit T_4 to T_3 conversion, thereby eliciting a low T_3 syndrome, are 6-propyl-thiouracil (PTU) (85-87), dexamethasone (88) and X-ray cholecystographic agents (89-91). Studies using rat liver homogenates from two days fasted rats, which have low serum T_4 and T_3, also revealed that the deiodinating activity was decreased (92). This decreased activity during early and late starvation has been accounted for by either a diminished activity of the enzyme itself or a deficiency of cytosolic cofactors, such as NADPH or glutathione (93-96). However, some reports suggested that tissue deiodinase activity is, at least in vitro, not consistently decreased in experimental models of NTI (97,98).

Another mechanism for the NTI-induced changes in thyroid hormone metabolism might be a diminished transport of thyroid hormones into the cells, which will also lead to a reduced production of T_3 from T_4 and reduced breakdown of rT_3 (52,53). This diminished transport may be caused by a) decreased intracellular ATP concentrations (shock, food deprivation); b) a circulating inhibitor, that inhibits the uptake of iodothyronines into hepatocytes. Evidence for a) was found in a study on caloric deprivation in humans. During caloric deprivation, transport of both T_4 and T_3 into tissues was diminished. It was concluded that, regardless of any possible change in deiodinase activity, inhibition of T_4 transport per se contributed to low T_3 production and therefore low T_3 serum levels, due to a lesser substrate (i.e. T_4) availability for T_3 production (68). Also, it has been described that after fasting intracellular ATP is decreased (99); therefore, transport inhibition after caloric deprivation could be caused by a decrease of intracellular ATP. As for b): Vos et al. (100) showed that sera of patients with NTI inhibit thyroid hormone uptake in hepatocytes in primary culture and in the perfused rat liver without a direct effect on deiodination. Lim et al. (101) showed that in NTI caused by uremia,

3-carboxy-4-methyl-5-propyl-2-furan propanoic acid (CMPF) and indoxyl sulfate, both present in serum during uremia, caused T_4 transport inhibition into isolated rat hepatocytes. Therefore, they suggested that these substances may account for the low T_3 in uremic patients by inhibiting T_4 transport into the T_3-producing tissues. Interesting in this respect are also the recent studies of Hennemann et al. (102) on the effects of CMPF and indoxyl sulfate on T_3 uptake and TSH secretion into isolated rat pituitary cells. They tested whether these substances influenced T_3 uptake and/or affected the basal and TRH-stimulated TSH release, to explain serum TSH levels in uremia, which are not increased in spite of low serum T_3. In contrast to the findings of Lim et al. in rat hepatocytes, no effects were found on T_3 uptake and also not on basal or TRH-stimulated TSH release, indicating that these compounds cannot explain the absence of TSH increase in the low T_3 syndrome in uremia.

Lim et al. suggested that in non-uremic NTI, non-esterified fatty acids and bilirubin may be the putative inhibitors of thyroid hormone transport (103), as these substances caused transport inhibition into rat hepatocytes, that was significantly correlated with the molar ratios of bilirubin:albumin and non-esterified fatty acid:albumin. No evidence was found for an inhibition of the deiodinase activity as well. There is also a possible indirect effect of non-esterified fatty acids, i.e. causing a displacement of other inhibiting compounds from albumin, due to the very high affinity of non-esterified fatty acids for albumin, as was recently shown to occur with a number of drugs (104,105). It was therefore concluded that in combination with the inhibiting effects of low intracellular ATP on T_3 neogenesis, elevated bilirubin and non-esterified fatty acids together with the low albumin concentration in non-uremic criticall illness may be (at least partly) responsible for the T_4 transport inhibition in T_3-producing tissues (103).

In many diseases, cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 1 and 6, may be involved as metabolic mediators (106). Studies in laboratory animals indicated that these cytokines also induce a low T_3 syndrome (107,108). Injection of TNF in humans resulted in changes in thyroid hormone parameters consistent with the low T_3 syndrome, i.e. a significant lowering of serum T_3 concentration and elevated rT_3 concentration, without a significant change in serum T_4 (109). However, studies in rat hepatocytes

showed no inhibition by either TNF-α or interleukin-1ß on transmembrane T_4 transport (103). Therefore, there is no evidence at present that these substances are directly responsible for the T_4 transport inhibition in T_3-producing tissues during NTI.

1.4 NUCLEAR BINDING

Most effects of thyroid hormone appear to be initiated by the interaction of T_3 with specific nuclear receptors, whereafter the receptor-hormone complex interacts with specific regulatory DNA sequences in the promotor region of T_3-responsive genes, initiating transcription or processing of specific mRNAs. The effects of T_3 on the level of specific mRNAs appear to be tissue-dependent, correlating with the presence of nuclear high-affinity binding sites for T_3 in thyroid hormone-responsive tissues (110). These specific T_3-receptors have been found in several tissues of rat and man (111-113).

The mechanism by which T_3 is transferred from the cytoplasm to the nucleus is not clear at the moment, but Oppenheimer et al. suggested that a stereospecific, energy-dependent transport system is also involved in this translocation (114).

Other thyroid hormone effects might be initiated through binding to mitochondrial receptors (115) or by direct interaction with the plasma membrane (116)

1.5 INTRACELLULAR METABOLISM

In principle, iodothyronines may undergo four metabolic processes. These are: deiodination, conjugation, ether-link cleavage, and oxidative deamination and decarboxylation. As the last 2 pathways are quantitatively of minor importance, they will not be further described.

1.5.1 Deiodination

Reviews on this subject have been published recently (3-6,60). Deiodination of iodothyronines is, at least in man, quantitatively the most important metabolic route. Two distinct deiodination reactions are recognized.

The process through which T_4 is converted to T_3 is termed outer ring deiodination (ORD) or 5'-deiodination, while rT_3 is formed by inner ring deiodination (IRD) or 5-deiodination of T_4. Further IRD of T_3 or ORD of rT_3 results in the formation of 3,3'-diiodothyronine (3,3'-T_2). ORD of T_3 and IRD of rT_3, thus forming 3,5-diiodothyronine (3,5-T_2) and 3',5'-diiodothyronine (3',5'-T_2), respectively, are considered minor reactions. As T_3 is the most important bioactive iodothyronine, 5'-deiodination can be regarded as an activating pathway and 5-deiodination as an inactivating pathway. In healthy humans, roughly 80 % of the circulating T_3 and 95 % of rT_3 are derived from conversion of T_4 in peripheral tissues.

Until now, 3 different iodothyronine deiodinases have been identified. Type I deiodinase is a selenocysteine containing, non-selective enzyme capable of both ORD and IRD. High activities have been found in liver, kidney and thyroid. In the rat liver, it is an integral membrane protein located in the endoplasmic reticulum, but in the rat kidney it is associated with the plasma membrane. PTU non-competitively inactivates the enzyme, by forming an enzyme-PTU selenosulfide. The preferred substrate for the type I deiodinase is rT_3. Its ORD to 3,3'-T_2 is at least 500-fold more efficient than the deiodination of T_4 and T_3. Type I enzyme activity is decreased in the hypothyroid and increased in the hyperthyroid state. Facilitation of type I deiodination has been observed after sulfation of 3,3'-T_2, T_3, and T_4 in incubations with isolated rat hepatocytes and microsomes.

The type II enzyme catalyzes ORD, converting T_4 to T_3 and rT_3 to 3,3'-T_2. Its activity has been localized in the central nervous system, pituitary, and brown adipose tissue. The enzyme is not inhibited by PTU. T_4 is preferred over rT_3 as substrate. Type II enzyme activity is increased during hypothyroidism and decreased during hyperthyroidism.

The type III enzyme catalyzes IRD, thus catalyzing the production of rT_3 from T_4 and producing 3,3'-T_2 from T_3. It has been detected in the rat central nervous system, human and rat placenta, rat skin, and in chicken embryo liver. Like the type II deiodinase, the enzyme is not inhibited by PTU. T_3 is preferred over T_4 as substrate. Like the type I enzyme, the activity of type III is decreased in hypothyroidism and increased in hyperthyroidism.

In man monodeiodination of T_4 accounts for about 80 % of its disposal

(2,3). Approximately equal proportions of T_4 are used for T_3 and rT_3 generation. rT_3 is produced by type I and type III IRD of T_4. However, as the efficiency of ORD of rT_3 by the type I enzyme is > 900-fold higher than that of the production of rT_3 by type I IRD of T_4, it follows that rT_3 once produced is rapidly further deiodinated by this enzyme (60) and does not contribute to plasma rT_3. It is therefore logical to assume that plasma rT_3 is predominantly derived from type III IRD of T_4, while it is mainly cleared by type I ORD in liver and kidney (117). T_3 is produced by type I and II ORD of T_4 and metabolized by type III IRD. In euthyroid rats 60-70 % of peripheral T_3 production is derived from the former pathway (118).

1.5.2 Conjugation

Conjugation is a phase II reaction, which transforms lipophilic endogenous compounds and xenobiotics into hydrophilic derivatives, thus facilitating their excretion in the bile and urine (119,120). For thyroid hormones, like T_3, the most important conjugation reactions are glucuronidation and

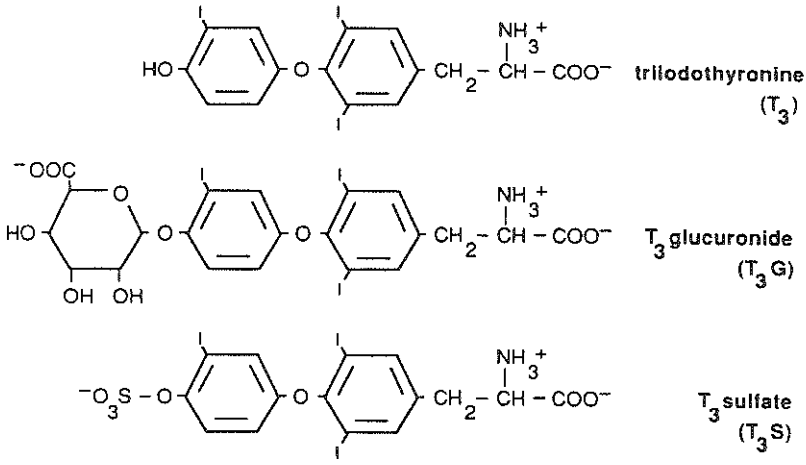

Figure 2
Conjugation of T_3

sulfation (Figure 2).

Glucuronidation followed by excretion is one of the most important pathways of detoxification of various compounds in man and most other mammals. It is performed by uridine diphosphate (UDP)-glucuronyltransferases (UDPGT), a group of enzymes located in the endoplasmic reticulum of predominantly liver, kidney, and intestine, but also in other tissues (119). Recently, it has been shown that glucuronidation of T_3, but not T_4, is closely related with androsterone UDPGT, while glucuronidation of T_4 seems to be catalyzed by both phenol and bilirubin UDPGTs (121-123).

The phenol sulfotransferases have been identified in the cytosolic fraction of especially liver, but also kidney, small intestine and brain, and catalyze the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate to a phenolic acceptor molecule (120). Otten and Visser (124) showed that sulfation of 3,3'-T_2, T_3, and T_4 facilitated subsequent deiodination.

Under normal conditions, glucuronidated iodothyronines are not found in the blood and urine, but are excreted in the bile. However, biliary excretion does not always result in the irreversible elimination of the substance. After passage to the intestinal lumen, glucuronides may become substrates for the ß-glucuronidases produced by the intestinal microflora. The liberated compound may then be reabsorbed. Evidence for such an enterohepatic cycle has been shown for thyroid hormone glucuronides (125).

As for sulfated thyroid hormones, only small amounts of T_3S appear in the plasma of rats, dogs and humans, and insignificant amounts of the iodothyronine sulfates and glucuronides are excreted in the urine.

1.6 THE LIVER: A THYROID HORMONE TRANSPORTING AND METABOLIZING ORGAN

As the liver plays quantitatively an important role with regard to transport of T_4 and subsequent production of bioactive T_3, this organ will be described in more detail in this paragraph.

1.6.1 Liver: general

The mammalian liver is a highly vascularized organ, which receives its

blood from the portal vein (ca 75 %) and the hepatic artery (ca 25 %). All compounds ingested orally and absorbed in the gastrointestinal tract must pass the liver via the portal vein before reaching the general circulation. The terminal branches of the portal vein and the hepatic artery empty their contents into the liver capillaries called the sinusoids, which are in turn drained by the hepatic vein. The wall of the sinusoids is formed by endothelial cells. No basal membrane is present, and fenestrae (± 0.1 μm) are present in the capillary wall (126); therefore, there is no barrier between the sinusoidal blood and the perisinusoidal space (space of Disse).

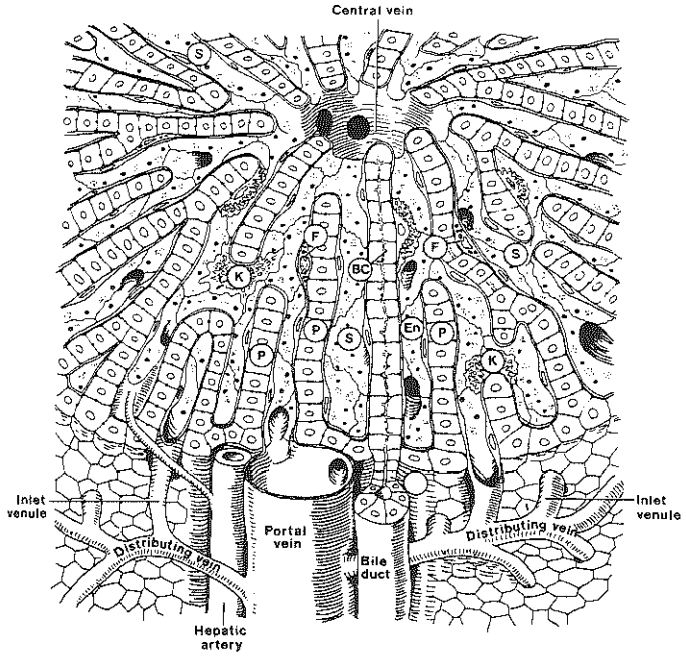

Figure 3

Structure of the normal liver. In the upper center, the central vein (vena hepatica); in the lower center, the portal vein (vena porta). BC, bile canaliculi; P, liver plates; K, Kupffer cells; S, sinusoids; F, fat-storing cell, En, sinusoid endothelial cell.

The hepatocytes or parenchymal cells, which represent 83 % of the liver volume and 60 % of the total number of liver cells (127) are arranged along the

sinusoids in plates, which are interconnected to form a three dimensional lattice (Figure 3). The hepatocytes can exchange compounds with the blood plasma via the space of Disse, which is in open contact with the sinusoidal blood. The Kupffer cells (phagocytic cells) are situated in the sinusoidal lumen and the fat storing cells (vitamin A storage) are situated in the space of Disse (128).

In 1833 Kiernan (129) proposed the hexagonal lobule as the structural unit of the liver. This liver lobule is surrounded by six terminal branches of the portal vein and of the hepatic artery. The hepatic venule is considered as the central vein and the region around the terminal portal venules forms the periphery of the lobule. The cells around the central veins are called the centrolobular cells, the cells in the periphery the periportal cells.

The hepatocytes are involved in the formation of primary bile, which is secreted into the bile canaliculi. These are formed by a specialization of the plasma membrane of two adjacent cells. The bile canalicular space is separated from the intercellular space and the space of Disse by junctional complexes (tight junctions) between the plasma membranes of adjacent hepatocytes. These tight junctions are impermeable even for small molecules. The bile canaliculi empty their contents into the bile ductuli, that terminate in the bile duct, which runs in parallel with the branches of the portal vein and the hepatic artery (128). In addition, the hepatocytes are involved in the maintenance of glucose homeostasis, in the uptake, metabolism and secretion into plasma and bile of endogenous and exogenous compounds and in the synthesis of, among other things, plasma proteins, cholesterol and bile salts.

1.6.2 Isolated perfused rat liver

The function of the liver in transport and metabolic studies can be studied with a spectrum of liver preparations ranging from the intact organ in vivo, perfusion systems, liver slices, isolated hepatocytes, homogenates, and subcellular fractions, to purified enzymes. Each preparation has its special advantages; some of the in vitro techniques are helpful in elucidating processes that cannot be studied well in vivo, but, on the other hand, the use of less integrated liver preparations has the potential of introducing artefacts that may cause misunderstanding of what is really going on in the intact organ.

The isolated perfused rat liver permits studies of liver transport and metabolism in a system that approaches normal physiology, but with much less interference of alterations in hemodynamic or hormonal parameters than in vivo, such as blood flow, blood pressure, or hormonal changes. Advantages furthermore include the large number of the perfusate samples that may be collected and the fact that the composition of the perfusion medium can easily be manipulated. Compared to the isolated hepatocytes preparation, in which most of the studies on thyroid hormone transport were done in the past, the perfusion system does not require damaging treatment with Ca^{2+}-free solutions and digestive enzymes, and the normal functional polarity of the cells and their localization in the liver lobule is maintained.

There are of course also disadvantages to the use of the isolated perfused rat liver. It is not possible to obtain many identical liver samples at the same time; this in contrast to e.g. the isolated hepatocytes preparation. Furthermore, with the perfusion technique only relatively short-term experiments are possible, generally not more than of 6 h duration. However, in spite of these disadvantages, the isolated rat liver perfusion system may permit study of specific experimental questions which could not be adequately evaluated by other methodologies.

The system that we used in most of our studies described in this thesis is shown and described in Chapter 2.

1.6.3 Two-compartment model of thyroid hormone distribution and metabolism in the isolated perfused rat liver

We recently showed that, equivalent to the three-compartment model as used to describe thyroid hormone kinetics in vivo, a two-compartment (or two-pool) model describes the kinetics of T_3 in a recirculating liver perfusion system (31). The two pools are the medium pool and one tissue (liver) pool, equivalent to the rapidly equilibrating pool of the three-pool model. From the thyroid hormone disappearance curve from the medium, it is possible to calculate transport of hormone into and out of the liver, besides metabolism (disposal) in the liver. A very good correlation was found between thyroid hormone parameters calculated by the model and those actually measured (medium pool, liver pool, and total metabolism), indicating that the model correctly

described the fate of thyroid hormones in the perfusion system (31). The two-pool model is shown and further described in Chapter 2.

1.7 SCOPE OF THE THESIS

In our recirculating rat liver perfusion model, in which thyroid hormone disappearance from the medium can be described by a two-pool model, it is possible to investigate both thyroid hormone transport and subsequent intracellular metabolism (31). Using this liver system, we studied the following questions regarding the transport process of thyroid hormones:

- Is thyroid hormone transport into the rat liver affected after 48-h fasting?
  There exists controversy in the literature about the effects of fasting on transmembrane transport and deiodination of thyroid hormones (see above). During fasting intracellular liver ATP is decreased. A concomittant decrease in transport of thyroid hormones during fasting could play an energy-saving role, as less bioactive thyroid hormone can reach its receptors in the nucleus when transport is decreased. We have investigated if such an effect exists and also investigated the effects of "refeeding", by addition of insulin, cortisol and glucose to the medium (Chapter 3).
- Is thyroid hormone transport into the rat liver affected during hypo- and hyperthyroidism?
  In this study we investigated the role of the transport process in altered thyroid states (Chapter 4).
- Is thyroid hormone transport into the liver affected after amiodarone treatment?
  Amiodarone, 2-n-butyl-3-[4-(2-diethylaminoethoxy)3,5-diiodobenzoyl]-benzofuran, is used as an anti-arrhythmic drug. It contains 39.4 % iodine on a weight basis and has structural resemblance to thyroid hormones. Patients receiving short-term treatment with amiodarone develop a low T_3 syndrome. It is, however, not clear whether this syndrome is due to a decreased T_4 to T_3 conversion per se, or to a decreased transport of T_4 into extra-thyroidal (T_3 producing) tissues (e.g. the liver). This study is described in Chapter 5.
- Is thyroid hormone transport into the liver of rats and humans affected after fructose administration, which causes a (transient) decrease in intracellular

ATP?

In this study we investigated energy dependence of thyroid hormone transport *in vivo* (Chapter 6).

- Are the thyroid hormone transport systems also present in isolated *human* hepatocytes and to what extent do they resemble the transport systems as described in rat hepatocytes?

  We were able to perform these studies in liver cells isolated from human donor livers, partly used for auxiliary liver transplantation (Chapter 7).
- Are the thyroid hormone systems in rat hepatocytes the same as the transport system A for amino acids?

  Amino acid transport processes in animal cells have been extensively studied. The transport systems of thyroid hormones (which are in fact derivatives of the amino acid tyrosine) in rat hepatocytes have several characteristics in common with one of these, i.e. transport system A (a neutral amino acid transport carrier), e.g. temperature-, Na^+- and ATP-dependence and stimulability by insulin after fasting. Therefore, the question was raised if amino acid transport system A and the thyroid hormone transport pathway in rat liver could be identical (Chapter 8).
- Is thyroid hormone availability in the rat liver dependent on perfusate flow rate? (Chapter 9).
- Is it possible to measure a gradient of free thyroid hormone over the rat liver cell plasma membrane?

  The finding of such a gradient for thyroid hormones would be strongly suggestive for active transport, i.e. acting against a concentration gradient (Chapter 9).

REFERENCES

1 Björkman U, Ekholm R 1990 Biochemistry of thyroid hormone formation and secretion. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 83-125

2 Hennemann G 1986 Thyroid hormone deiodination in healthy man. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 277-296

3 Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocrine Rev 5:151-184

4 Hesch RD, Köhrle J 1986 Intracellular pathways of iodothyronine metabolism. In: Ingbar

SH, Braverman LE (eds) The Thyroid. Lippincott, Philadelphia. pp 154-200
5 Visser TJ, Docter R, Krenning EP, Hennemann G 1986 Regulation of thyroid hormone bioavailability. J Endocrinol Invest 9 (suppl 4):17-26
6 Visser TJ 1988 Metabolism of thyroid hormone. In: Cooke BA, King RJB, Van der Molen HJ (eds) Hormones and their actions, part I. Elsevier Science Publishers, Amsterdam. pp 81-103
7 Chopra IJ, Geola F, Solomon DH, Maciel RMB 1978 3',5'-Diiodothyronine in health and disease. Studies by a radioimmunoassay. J Clin Endocrinol Metab 47:1198-1207
8 Geola F, Chopra IJ, Solomon DH, Maciel RMB 1979 Metabolic clearance and production rates of 3',5'-diiodothyronine and 3,3'-diiodothyronine in man. J Clin Endocrinol Metab 48:297-301
9 Van Sande J, Mockel J, Boeynaemas JM, Dor P, Andry G, Dumont JE 1980 Regulation of cyclic nucleotide and prostaglandin formation in normal thyroid tissue and in autonomous nodules. J Clin Endocrinol Metab 50:776-785
10 Larsen PR 1982 Thyroid-pituitary interaction. Feedback regulation of thyrotropin secretion by thyroid hormones. N Engl J Med 306:23-32
11 Larsen PR, Silva JE, Kaplan MM 1981 Relationship between circulating and intracellular thyroid hormones: physiological and clinical implications. Endocr Rev 2:87-102
12 Kaplan MM 1984 The role of thyroid hormone deiodination in the regulation of hypothalamo-pituitary function. Neuroendocrinol 38:254-260
13 Peters JR, Foord SM, Diegues C, Scanlon MF 1983 TSH neuroregulation and alterations in disease states. Clinics Endocrinol Metab 12:669-694
14 Reichlin S 1980 Somatostatin. N Engl J Med 309:1495-1501, 1556-1563
15 Wilber JF, Yamada M 1990 Thyrotropin-releasing hormone: current concepts. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 127-145
16 Robbins J, Bartalena L 1986 Plasma transport of thyroid hormones. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 3-38
17 Hennemann G, Docter R 1990 Plasma transport proteins and their role in tissue delivery of thyroid hormone. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 221-231
18 Sutherland RL, Simpson-Morgan MW 1975 The thyroxine binding properties of serum proteins. A competitive binding technique employing Sephadex G-25. J Endocrinol 65:319-332
19 Rondeel JMM, Heide R, De Greef WJ, Van Toor H, Van Haasteren GAC, Klootwijk W, Visser TJ 1992 Effect of starvation and subsequent refeeding on thyroid function and release of hypothalamic thyrotropin-releasing hormone. Neuroendocrinol 56:348-353
20 Rutgers M, Bonthuis F, De Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758-762
21 Mendel CM, Weisiger RA, Jones AL, Cavalieri RR 1987 Thyroid hormone-binding proteins

in plasma facilitate uniform distribution of thyroxine within tissues: a perfused rat liver study. Endocrinology 120:1742-1749

22 Docter R, Bos G, Krenning EP, Fekkes D, Visser TJ, Hennemann G 1981 Inherited thyroxine excess: a serum abnormality due to an increased affinity for modified albumin. Clin Endocrinol 15:363-371

23 Davis PJ, Spaulding SW, Gregerman RI 1970 The three thyroxine-binding proteins in rat serum: binding capacities and effects of binding inhibitors. Endocrinology 87:978-986

24 Ekins R, Edwards P, Newman B 1982 The role of binding proteins in hormone delivery. In: Albertini A, Ekins RP (eds) Free hormones in blood. Elsevier Science Publishers, Amsterdam. pp 3-45

25 Ekins R 1986 The free hormone concept. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekkers, New York. pp 77-106

26 Mendel CM, Cavalieri RR, Weisiger RA 1988 Uptake of thyroxine by the perfused rat liver: implications for the free hormone hypothesis. Am J Physiol 255:E110-E119

27 Recant L, Riggs DS 1952 Thyroid function in necrosis. J Clin Invest 31:789-797

28 Robbins J, Rall JE 1957 The interaction of thyroid hormones and protein in biological fluids. Recent Prog Horm Res 13:161-202

29 Pardridge WM 1987 Plasma protein-mediated transport of steroid and thyroid hormones. Am J Physiol 252:E157-E164

30 Mendel CM, Cavalieri RR, Gavin LA, Petterson T, Inoue M 1989 Thyroxine transport and distribution in Nagase analbuminemic rats. J Clin Invest 83:143-148

31 Docter R, De Jong M, Van der Hoek JH, Krenning EP, Hennemann G 1990 Development and use of a mathematical two-pool model of distribution and metabolism of T_3 in a recirculating rat liver perfusion system: albumin does not play a role in cellular transport. Endocrinology 126:451-459

32 Sarne DH, Refetoff S 1988 Normal cellular uptake of thyroxine from serum of patients with familial dysalbuminemic hyperthyroxinemia or elevated thyroxine-binding globulin. J Clin Endocrinol Metab 67:1166-1170

33 Freinkel N, Ingbar SH, Dowling JT 1957 The influence of extracellular thyroxine-binding proteins upon the accumulation of thyroxine by tissue slices. J Clin Invest 36:25-38

34 Lein A, Dowben RM 1961 Uptake and binding of thyroxine and triiodothyronine by the rat diaphragm in vitro. Am J Physiol 200:1029-1032

35 Rao GS, Eckel J, Rao ML, Breuer H 1976 Uptake of thyroid hormone by isolated rat liver cells. Biochem Biophys Res Commun 73:98-104

36 Parl F, Korcek L, Siegel JS, Tabachnick M 1977 Uptake of triiodothyronine and thyroxine by isolated rabbit adipocytes. FEBS Lett 83:145-147

37 Holm AC, Wong KY, Pliam NB, Jorgensen EC, Goldfine ID 1980 Uptake of L-triiodothyronine into human cultured lympfocytes. Acta Endocrinol (Copenh) 95:350-358

38 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of

triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett 91:113-116

39 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314-320

40 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1982 Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229-233

41 Krenning EP, Docter R, Visser TJ, Hennemann G 1983 Plasma membrane transport of thyroid hormone: its possible pathophysiological significance. J Endocrinol Invest 6:59-66

42 Cheng SY, Maxfield FR, Robbins J, Willingham MC, Pastan IH 1980 Receptor-mediated uptake of 3,3',5-triiodo-L-thyronine by cultured fibroblasts. Proc Natl Acad Sci USA 77:3425-3429

43 Horiuchi R, Cheng SY, Willingham MC, Pastan IH 1982 Inhibition of nuclear entry of 3,3',5-triiodo-L-thyronine by monodansylcadaverine in GH_3 cells. J Biol Chem 257:3139-3144

44 Halpern J, Hinkle PM 1982 Evidence for an active step in thyroid hormone transport to nuclei: drug inhibition of L-^{125}I-triiodothyronine binding to nuclear receptors in rat pituitary tumor cells. Endocrinology 110:1070-1072

45 Pontecorvi A, Robbins J 1986 Energy-dependent uptake of 3,5,3'-triiodo-L-thyronine in rat skeletal muscle. Endocrinology 119:2755-2761

46 Docter R, Krenning EP, Bernard HF, Hennemann G 1987 Active transport of iodothyronines into human cultured fibroblasts. J Clin Endocrinol Metab 65:624-628

47 Blondeau JP, Osty J, Francon J 1988 Characterization of the thyroid hormone transport system of isolated hepatocytes. J Biol Chem 263:2685-2692

48 Movius EG, Phyillaier MM, Robbins J 1989 Phloretin inhibits cellular uptake and nuclear receptor binding of triiodothyronine in human HEPG2 hepatocarcinoma cells. Endocrinology 124:1988-1997

49 Topliss DJ, Kolliniatis E, Barlow JW, Lim CF, Stockigt JR 1989 Uptake of 3,5,3'-triiodothyronine by cultured rat hepatoma cells is inhibitable by nonbile acid cholephils, diphenylhydantoin, and nonsteroidal antiinflammatory drugs. Endocrinology 124:980-986

50 Centanni M, Mancini G, Andreoli M 1989 Carrier-mediated [^{125}I]-T_3 uptake by mouse thymocytes. Endocrinology 124:2443-2448

51 Everts ME, Docter R, Van Buuren JCJ, Van Koetsveld PM, Hofland LJ, De Jong M, Krenning EP, Hennemann G 1993 Evidence for carrier-mediated uptake of triiodothyronine in cultured anterior pituitary cells of euthyroid rats. Endocrinology 132:1278-1285

52 Krenning EP, Docter R 1986 Plasma membrane transport of thyroid hormone. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 107-131

53 Docter R, Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York.

pp 233-254

54 Pontecorvi A, Robbins J 1989 The plasma membrane and thyroid hormone entry into cells. TEM sept/oct pp 36-40

55 Mol JA, Krenning EP, Docter R, Rozing J, Bernard HF, Hennemann G 1986 Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. J Biol Chem 261:7640-7643

56 Horiuchi R, Johnson ML, Willingham MC, Pastan IH, Cheng SY 1982 Affinity labeling of the plasma membrane 3,3',5-triiodo-L-thyronine receptor in GH_3 cells. Proc Natl Acad Sci USA 79:5527-5531

57 Gonçalves E, Lakshmanan M, Robbins J 1989 Triiodothyronine transport into differentiated and undifferentiated mouse neuroblastoma cells (NB41A3). Endocrinology 124:293-300

58 Centanni M, Robbins J 1987 Role of sodium in thyroid hormone uptake by rat skeletal muscle. J Clin Invest 80:1068-1072

59 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1979 The essential role of albumin in the active transport of thyroid hormone into primary cultured rat hepatocytes. FEBS Lett 107:227-230

60 Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 189-229

61 Hennemann G, Krenning EP, Polhuys M, Mol JA, Bernard HF, Visser TJ, Docter R 1986 Carrier-mediated transport of thyroid hormone into rat hepatocytes is rate-limiting in total cellular uptake and metabolism. Endocrinology 119:1870-1872

62 Hennemann G, Krenning EP, Bernard B, Huvers F, Mol J, Docter R, Visser TJ 1984 Regulation of influx and efflux of thyroid hormones in rat hepatocytes: possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity. Horm Metab Res (suppl) 14:1-6

63 Van Doorn J, Van der Heide D, Roelfsema F 1984 Sources and quantity of 3,5,3'-triiodothyronine in several tissues of the rat. J Clin Invest 72:1778-1792

64 Oppenheimer JH, Schwarz HL, Surks MI 1975 Determination of common parameters of iodothyronine metabolism and distribution in man by noncompartmental analysis. J Clin Endocrinol Metab 41:319-324, and Erratum: J Clin Endocrinol Metab 41:1172-1173

65 DiStephano JJ III 1986 Modeling approaches and models of the distribution and disposal of thyroid hormones. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 39-76

66 DiStefano III JJ, Jang M, Malone TK, Broutman M 1982 Comprehensive kinetics of triiodothyronine production, distribution, and metabolism in blood and tissue pools of the rat using optimized blood-sampling protocols. Endocrinology 110:198-213

67 DiStefano III JJ, Malone TK, Jang M 1982 Comprehensive kinetics of thyroxine distribution and metabolism in blood and tissue pools of the rat from only six blood samples: dominance of large, slowly exchanging tissue pools. Endocrinology 111:108-117

68 Van der Heijden JTM, Docter R, Van Toor H, Wilson JPH, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251:E156-E163

69 Bianchi R, Mariani G, Molea N, Vitek F, Cazzuola F, Carpi A, Mazzuca N, Toni MG 1983 Peripheral metabolism of thyroid hormones in man. I. Direct measurement of the conversion rate of thyroxine to 3,5,3'-triiodothyronine (T_3) and determination of the peripheral and thyroidal production of T_3. J Clin Endocrinol Metab 56:1152-1163

70 Lambert MJ, Burger AG, Galeazzi RL, Engler D 1982 Are selective increases in serum thyroxine (T_4) due to iodinated inhibitors of T_4 monodeiodination indicative of hyperthyroidism? J Clin Endocrinol Metab 55:1058-1065

71 Mooradian AD, Schwartz HL, Mariash CN, Oppenheimer JH 1985 Transcellular and transnuclear transport of 3,5,3'-triiodothyronine in isolated hepatocytes. Endocrinology 117:2449-2456

72 Terasaki T, Pardridge WM 1987 Stereospecifity of triiodothyronine transport into brain, liver and salivary gland: role of carrier- and plasma protein-mediated transport. Endocrinology 121:1185-1191

73 Weisiger RA, Luxon BA, Cavalieri RR 1992 Hepatic uptake of 3,5,3'-triiodothyronine: electrochemical driving forces. Am J Physiol 262:G1104-G1112

74 Rao GS, Rao ML 1983 L-thyroxine enters the rat liver cell by simple diffusion. J Endocrinol 97:277-284

75 Pontecorvi A, Di Cerbo A, Gonçalves E, Antonozzi I, Andreoli M, Robbins J 1988 Thyroxine entry by simple diffusion in rat myoblasts. Presented at the 8th International Congress of Endocrinology, Kyoto, Japan.

76 Galton VA, St. Germain DL, Whittemore S 1986 Cellular uptake of 3,5,3'-triiodothyronine and thyroxine by red blood and thymus cells. Endocrinology 118:1918-1923

77 Vagenakis AG 1981 Non-thyroid diseases affecting the thyroid hormone metabolism. In: Hesch RD (ed) The Low T_3 Syndrome. Academic press, New York. 40:128-139

78 Docter R, Krenning EP, De Jong M, Hennemann G 1993 The sick euthyroid syndrome: changes in thyroid hormone serum parameters and hormone metabolism. Accepted for publication in Clin Endocrinol

79 Kaptein EM, Weiner JM, Robinson WJ, Wheeler WS, Nicoloff JT 1982 Relationship of altered thyroid hormone indices to survival in nonthyroidal illness. Clin Endocrinol 16:565-574

80 Hennemann G, Docter R, Vos RA, Van Toor H, Krenning EP 1987 Vergelijking van gebruikelijke schildklierfunctietests met nieuwere TSH-test bij patienten met ziekten die niet de schildklier betreffen. Ned Tijdschrift Geneeskunde 131:2355-2359

81 Krenning EP, Docter R, Visser TJ, Vos RA, Van Toor H, Hennemann G 1987 "Modern" evaluation of thyroid function. Progr Med Lab 1:237-243

82 Spencer CA, Eigen A, Shen D, Duda M, Qualls S, Weiss S, Nicoloff J (1987) Specificity of

sensitive assays of thyrotropin (TSH) used to screen for thyroid disease in hospitalized patients. Clin Chem 33:1391-1396

83 Van der Heijden JTM, Krenning EP, Van Toor H, Hennemann G, Docter R 1988 Three-compartmental analysis of effects of D-propranolol on thyroid hormone kinetics. Am J Physiol 255:E80-E86

84 Heyma P, Larkins RG, Campbell DG 1980 Inhibition by propranolol of 3,5,3'-triiodothyronine formation from thyroxine in isolated rat renal tubulus: an effect independent of ß-adrenergic blockade. Endocrinology 106:1437-1441

85 Abuid J, Larsen PR 1974 Triiodothyronine and thyroxine in hyperthyroidism - Comparison of the acute changes during therapy with antithyroid drugs. J Clin Invest 54:201-208

86 Geffner DL, Azukizawa M, Hersmann JM 1975 Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin respons in man. J Clin Invest 55:224-229

87 Visser TJ 1978 A tentative review of recent in vivo observations of the deiodination of iodothyronines and its possible physiological implications. Mol Cell Endocrinol 10:241-247

88 Duick DS, Warren DW, Nicoloff JT, Otis CL, Croxson MS 1974 Effect of a single dose of dexamethasone on the concentration of serum triiodothyronine in man. J Clin Endocrinol Metab 39:1151-1154

89 Bürgi H, Wimptheimer C, Burger A, Zaunbauer W, Rösler H, Lemarchand-Béraud T 1976 Changes in the circulating thyroxine, triiodothyronine and reverse triiodothyronine after radiographic contrast agents. J Clin Endocrinol Metab 43:1203-1210

90 Wu SY, Chopra IJ, Solomon DH, Bennet LR 1978 Changes in the circulating iodothyronines in euthyroid and hyperthyroid subjects given ipodate (Orogravin); an agent for oral cholecystography. J Clin Endocrinol Metab 46:691-697

91 Suzuki H, Kadema N, Takeuchi K, Nakagawa S 1979 Effect of three-day oral cholecystography on serum iodothyronines and TSH concentrations. Acta Endocrinol 92:477-488

92 Harris ARC, Fang SL, Vagenakis AE, Braverman LE 1978 Effect of starvation, nutriment replacement, and hypothyroidism on in vitro hepatic T_4 to T_3 conversion in the rat. Metabolism 27:1680-1690

93 Kaplan MM 1979 Subcellular alterations causing reduced hepatic thyroxine 5'-monodeiodinase activity in fasted rats. Endocrinology 104:58-64

94 Harris ARC, Fang SL, Kinerfeld K, Braverman LE, Vagenakis AE 1979 The role of sulfhydryl groups on the impaired hepatic 3,3',5-triiodothyronine generation from thyroxine in the hypothyroid, starved, fetal, and neonatal rodent. J Clin Invest 63:516-524

95 Balsam A, Ingbar SH 1979 Observations on the factors that control the generation of triiodothyronine from thyroxine in rat liver and the nature of the defect induced by fasting. J Clin Invest 63:1145-1151

96 Sawada K, Hummel BCW, Walfish PG 1986 Properties of cytosolic components activating

rat hepatic 5'-deiodination in the presence of NADPH. Biochem J 234:391-398

97 Huang TS, Chopra IJ, Boado R, Wu TC, Tashkin DP, Solomon DH, Chua Teco GN 1988 Alterations in thyroidal economy in a systemic illness induced by turpentine oil injection to the rat. Acta Endocrinol (Copenh) 117:51-58

98 Grussendorf M, Wollensack G, Hüffner M 1988 Iodothyronine deiodination in rats with severe non-thyroidal illness. Acta Endocrinol (Copenh) 118:31-37

99 Cunningham CC, Malloy CR, Radda GK 1986 Effect of fasting and acute ethanol administration on the energy state of in vivo liver as measured by ^{31}P-NMR spectroscopy. Biochim Biophys Acta 885:12-22

100 Vos RA, de Jong M, Docter R, van Toor H, Bernard HF, Krenning EP, Hennemann G 1991 Morbidity-dependent thyroid hormone transport inhibition by serum of patients with non-thyroidal illness (NTI) in rat hepatocytes and in the perfused rat liver. In: Gordon, Gross and Hennemann (eds) Progress in Thyroid Research. Balkema Rotterdam. pp 693-696

101 Lim CF, Bernard HF, De Jong M, Docter R, Krenning EP, Hennemann G 1993 A furan fatty acid and indoxyl sulfate are the putative inhibitors of thyroxine metabolism in uremia. J Clin Endocrinol Metab 76:318-324

102 Hennemann G, Lim CF, Everts ME 1993 Effects of a furan fatty acid and indoxyl sulfate on ^{125}I-T_3 uptake and TSH secretion in cultured rat anterior pituitary cells. J Endocrinol Invest 16, Suppl 2 to no. 6, p10

103 Lim CF, Docter R, Visser TJ, Krenning EP, Bernard HF, Van Toor H, De Jong M, Hennemann G 1993 Inhibitors of thyroxine hepatocytes transport in non-uremic critical illness. Effects of bilirubin and non-esterified fatty acids. J Clin Endocrinol Metab (in press)

104 Lim CF, Bai Y, Topliss J, Barlow JW, Stockigt JR 1988 Drug and fatty acid effects on serum thyroid hormone binding. J Clin Endocrinol Metab 67:682-688

105 Lim CF, Curtis AJ, Barlow JW, Topliss J, Stockigt JR 1991 Interactions between oleic acid and drug competitors influence specific binding of thyroxine in serum. J Clin Endocrinol Metab 73:1106-1110

106 Evans RD, Argiles JM, Williamson DH 1989 Metabolic effects of tumor necrosis factor alpha (cachectin) and interleukin-1. Clin Sci 77:357-364

107 Ozawa M, Sato K, Han CH, Kawakami M, Tsushima T, Shizume K 1988 Effect of tumor necrosis factor/cachectin on thyroid hormone metabolism in mice. Endocrinology 123:1461-1467

108 Dubuis JM, Dayer JM, Siegrist-Kaiser CA, Burger AG 1988 Human recombinant interleukin-1 bèta decreases plasma thyroid hormone and thyroid stimulating hormone levels in rats. Endocrinology 123:2175-2181

109 Van der Poll T, Romijn JA, Wiersinga WM, Sauerwein HP 1990 Tumor necrosis factor: a putative mediator of the euthyroid sick syndrome in man. J Clin Endocrinol Metab 71:1567-1572

110 Nikodem VM, Petty KJ, Mitsuhashi T, Desvergne B 1990 Structure and mechanism of

action of thyroid hormone receptors. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 307-321

111 Oppenheimer JH, Schwartz HL 1986 Thyroid hormone action at the nuclear level. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 383-416

112 Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NCW, Freake HC 1987 Advances in our understanding of thyroid hormone action at the cellular level. Endocr Rev 8:288-308

113 Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889-895

114 Oppenheimer JH, Schwartz HL 1985 Stereospecific transport of triiodothyronine from plasma to cytosol and from cytosol to nucleus in rat liver, kidney, brain and heart. J Clin Invest 75:147-154

115 Sterling K, Brenner MA, Sakurada T 1980 Rapid effect of triiodothyronine on the mitochondrial pathway in rat liver in vivo. Science 210:340-343

116 Goldfine ID, Smith GJ, Simons CG, Ingbar SH, Jorgensen EC 1976 Activities of thyroid hormones and related compounds in an in vitro thymocyte assay. J Biol Chem 251:4233-4238

117 Bauer AGC, Wilson JHP, Lamberts SWJ, Docter R, Hennemann G, Visser TJ 1987 Handling of iodothyronines by liver and kidney in patients with chronic liver disease. Acta Endocrinol (Copenh) 116:339-346

118 Silva JE, Gordon MB, Crantz FR, Leonard JL, Larsen PR 1984 Qualitative and quantitative differences in the pathways of extrathyroidal triiodothyronine generation between euthyroid and hypothyroid rats. J Clin Invest 73:898-907

119 Dutton GJ 1980 Glucuronidation of drugs and other compounds. CRC Press, Boca Raton, Florida

120 Mulder GJ 1981 Sulfation of drugs and related compounds. CRC Press, Boca Raton, Florida

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

122 Kleiman de Pisarev DL, Sancovich HA, Ferramola de Sanchovich AM 1989 Enhanced thyroxine metabolism in hexachlorobenzene-intoxicated rats. J Endocrinol Invest 12:767-772

123 Visser TJ, Kaptein E, Harper ES 1991 Differential expression and ciprofibrate induction of hepatic UDP-glucuronyltransferase for thyroxine and triiodothyronine in Fisher rats. Biochem J Pharmacol 42:444-446

124 Otten MH, Mol JA, Visser TJ 1983 Sulfation preceeding deiodination of iodothyronines in rat hepatocytes. Science 221:81-83

125 De Herder WW, Hazenberg MP, Pennock-Schröder AM, Oosterlaken AC, Rutgers M, Visser TJ 1989 On the enterohepatic cycle of triiodothyronine in rats; importance of the intestinal microflora. Life Sci 45:849-856

126 Wisse E 1970 An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. J Ultrastruct Res 31:125-150

127 Weibel ER, Staubli W, Gragi HR, Hess FA 1969 Correlated morphometric and biochemical studies of the liver cell. I. Morphometric model, stereologic methods and normal morphometric data for rat liver. J Cell Biol 42:68-91

128 Jones AL, Schmucker DL 1977 Current concepts of liver structure as related to function. Gastroenterol 73:833-851

129 Kiernan F 1833 The anatomy and physiology of the liver. Phil Trans Roy Soc London B Biol Sci 123:711-770

CHAPTER 2

MATERIALS AND METHODS

2.1 SUBJECTS, ANIMALS AND MATERIALS

2.1.1 Subjects (Fructose study)

Four (three women) healthy subjects, age 27-36 years (mean 30.8 years) participated in our studies. All had normal thyroid function as measured by serum T_4, T_3-resin uptake and thyrotropin response to an intravenous bolus of TRH. None of the persons had recently (within three months) used any medication.

2.1.2 Animals

Male Wistar rats, weighing 200-250 g, were used in the experiments. They were obtained from IFFA Credo (Brussels, Belgium) and unless otherwise stated, they had free access to food (standard rat chow of Hope Farms, Woerden, The Netherlands (except for the amiodarone-study)) and water.

In the studies on the effects of fasting, food was withdrawn for 48 h, but free access to water was maintained.

In the studies on the effects of hyper- and hypothyroidism rats were made hyperthyroid by daily ip injection of T_4 (10 μg/100 g body weight) for 15 days. Hypothyroidism in the rats was induced by addition of 0.05 % mercapto-methyl-imidazole (inhibitor of the thyroperoxidase) to the drinking water for at least 3 weeks. Hyper- and hypothyroidism was confirmed by estimation of T_4 and TSH levels in plasma by RIA.

In the studies on the effects of amiodarone, male Wistar rats weighing 300-350 g were used. Amiodarone-treated rats received the drug (40 mg/kg BW/day, 22 days) added to their diet. This diet, recommended by the American Institute of Nutrition (1), consisted of a semisynthetic, powdered substance, which was mixed with water to a homogeneous paste. The control rats received potassium iodide in their food, in an amount similar as the amount of iodide released from amiodarone in amiodarone-treated rats (0.3 mg/day), to ensure an iodide intake similar to that of the rats receiving amiodarone.

2.1.3 Materials

^{131}I-T_4 for the human studies was prepared by iodination of 3,5,3'-T_3 with ^{131}I by the chloramine-T method (2). Iodination was performed by mixing 10 μL volumes of Na^{131}I (Amersham International, Aylesbury, UK.), 0.5 M sodium phosphate buffer (pH 7.5), 3,5,3'-T_3 (1 μg/μL in 0.05 M sodium phosphate buffer (pH 7.5)) and chloramine-T (1 μg/μL in 0.05 M sodium phosphate buffer (pH 7.5)). The reaction was terminated after one minute by adding 100 μl sodium bisulfite (1 mg/mL in 0.05 sodium phosphate buffer (pH 7.5)). The reaction mixture was applied on a small Sephadex LH-20 column and eluted with 30 % ethanol in 0.05 M sodium carbonate (pH 11.5) (v/v). T_4 fractions were pooled and evaporated to dryness under nitrogen.

[3'-^{125}I]T_3, [3',5'-^{125}I]T_4, and L-[3'-^{125}I]rT_3 (specific activity >1200 μCi/μg) were from Amersham International, Aylesbury, UK. L-T_3 and L-T_4 were obtained from Sigma, St Louis MO.

The monoclonal antibody used in the human hepatocytes study (81-1A1-10), was raised against rat hepatocytes and selected on inhibition of iodide production from thyroid hormones in rat hepatocytes, as described earlier (3). A 1:200 dilution of mouse ascites fluid was used. All other reagents were of the highest purity commercially available.

2.2 METHODS

2.2.1 Human studies (Fructose)

Serum ^{131}I-T_4 kinetics in humans were studied two times. First during a control period and after one month this study was repeated; the only difference of the second study with the control study was an intravenous fructose loading of 0.5 g/kg, administered in 20 minutes as 20 % (w/v) solution. Tracer T_4 injection was done immediately following this 20 min period. In the control period an equal volume of saline was similarly given. The subjects showed no untoward symptoms or signs during the time of the fructose infusion. During the studies 10 drops of saturated KI-solution were administered 3 times a day to prevent thyroid uptake of radioactive iodide liberated during the study. The studies and protocols were approved

by the Ethical Committee of the medical centre and informed consent was obtained from all participants.

Fifty μCi ^{131}I-L-thyroxine (specific activity > 10 mCi/μg) in 1,5 mL of 2 % human serum albumin in phosphate-buffered saline, were administered as a bolus to the subjects via an intravenously inserted canula in the arm. Through a catheter in the contralateral arm, small blood samples were drawn at 10 min intervals during the first 6 hours, thereafter at 7, 8, 9, 10, 12, 15 and 24 hours and subsequently 1 sample/day during the following 10 days for determination of radioactivity. Percent tracer dose at the different time intervals and serum levels of T_4, free T_4 (equilibrium dialysis), T_3 and rT_3 were determined as outlined below.

Uptake of tracer T_4 into the REP, consisting mainly of the liver, was calculated using a three-compartmental model of thyroid hormone distribution and metabolism (4), applied by us in humans as reported previously (5). During the first six hours after tracer injection, integrated influx of tracer T_4 into REP minus efflux from REP was computed representing net uptake in this compartment (see Appendix).

2.2.2 Rat liver perfusion studies

2.2.2.1 Rat liver perfusion

Rat livers were isolated and perfused in a recirculating system at 37 C, essentially as described by Meijer et al. (6) (Figure 1). The perfusion procedure was in short as follows: the liver of an anesthetized rat was isolated from ligaments and surrounding tissue after cannulation of the common bile duct, the portal vein and the thoracic inferior vena cava. The liver with its connected cannulae was then transferred to the perfusion cabinet and connected to the perfusion system. All perfusions started between 9 and 10 hr. After isolation, the liver was preperfused for 0.5 h.

The perfusion medium used in all experiments was 150 mL Krebs-Ringer medium (118 mmol/L NaCl, 5 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, and 25 mmol/L $NaHCO_3$) with addition of 10 mmol/L glucose and 1 % bovine serum albumin (BSA; Boseral, Organon Teknika, The Netherlands).

In the experiments mimicking refeeding after fasting, glucose (10

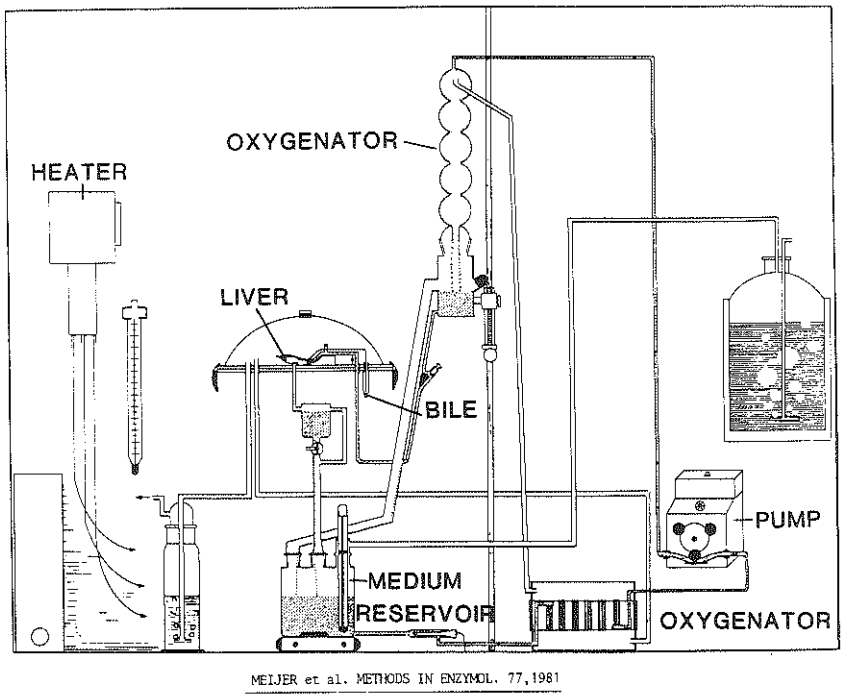

Figure 1

Isolated rat liver perfusion apparatus. Flow of medium occurs from medium reservoir via oxygenator to pump, via 6-bulb glass oxygenator to liver, and back to medium reservoir. Flow of gas occurs from bottle with saline (right) to medium reservoir, to 6-bulb glass oxygenator, down to the other oxygenator, and finally to the liver.

mmol/L), insulin (12 U/L), cortisol (500 nmol/L) or combinations of these were added to the medium.

In the experiments on the influence of fructose, medium was used which contained 10 mmol/L fructose.

In the studies with PTU, 100 μmol/L of PTU was added to the medium at the start of the preperfusion period.

The medium was gassed with carbogen (95% O_2, 5% CO_2; 400 mL/min). The functional state of the organ was monitored by its outer appearance, measurement of bile flow (about 0.7 mL/h), pH of medium (7.43) and enzyme release (LDH, GOT, GPT, < 10 units (μmol/min)/60 min) into the perfusion medium. No significant increase in enzyme release was seen

during the time of the perfusions. The perfusion pressure was determined by the height of a hydrostatic overflow reservoir above the liver chamber; changing the difference in height resulted in a change of the medium flow through the liver. The experiments were started by the addition of T_3 (300 pmol to 150 mL medium with 1 % BSA, and 1200 pmol to 150 mL medium with 4 % BSA) and [^{125}I]-T_3 or T_4 (1500 pmol to 150 mL medium with 1 % BSA and 1500 or 6000 pmol to 150 mL medium with 4 % BSA) and [^{125}I]-T_4 to the medium in the magnetically stirred main reservoir (time 0). Medium aliquots of 0.5 mL were taken at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60 min, (sometimes also 70, 80 and 90 min). Bile was collected during 10 min intervals. Medium and bile samples were stored at -20 C untill further analysis.

For analysis of medium aliquots, mixtures were prepared consisting of 0.5 mL medium and 0.5 mL 1 mol/L HCl. These were applied to small Sephadex LH-20 columns (bed volume 1 mL), equilibrated with 3 mL of 0.1 mol/L HCl. Iodide was eluted with 3 x 1 mL 0.1 mol/L HCl; T_3 glucuronide, T_3 sulfate and T_3 with 8 x 1 mL sodium acetate (0.1 mol/L, pH 4), 6 x 1 mL H_2O and 3 x 1 mL NaOH (0.1 M)/ethanol 50:50 (vol/vol), respectively. T_4 conjugates and T_4 were eluted with 8 x 1 mL H_2O and 3 x 1 mL NaOH/ethanol, respectively. Fractions of 1 mL were collected and counted for radioactivity. Bile was also analysed on Sephadex LH-20 after addition of 950 μl 0.1 mol/L HCl to a 50 μL aliquot. The same chromatographic procedure as for medium analysis was followed.

Free T_3 and T_4 fractions in medium aliquots were determined by equilibrium dialysis according to the method of Sterling and Brenner (7) or by ultrafiltration (8). For ultrafiltration, to 500 μL perfusion medium, Sephadex LH-20 purified ^{125}I-T_4 or ^{125}I-T_3 was added. The mixture was equilibrated for 0.5 h at 37 C and centrifuged at 4500 g for 15 min at 37 C in disposable microconcentrators (Centricon 10, Amicon, USA) yielding about 250 μL of ultrafiltrate. To 200 μL of ultrafiltrate, 1 mg unlabeled T_4 and 0.5 mL $MgCl_2$ (10% (w/vol)) was added, to separate intact tracer and free ^{125}I. The free fraction of the thyroid hormones was computed as the counts per minute in the $MgCl_2$ fraction divided by the counts per minute per 200 μL of the starting solution before ultrafiltration.

Measurement of the free T_3 and T_4 fraction in liver cytosol was also done by ultrafiltration (8): after adding 2 volumes of 50 mmol/L Tris-HCl buffer (pH 7.4), containing 0.25 mol/L sucrose, livers were disrupted by homogenization. After ultracentrifugation at 105000 g for 80 min, the resulting cytosol was 2 times diluted in Tris-HCl buffer. The free hormone fraction in the resulting cytosol was assayed by ultrafiltration (see above).

Total DNA was measured fluorimetrically in fed and fasted livers.

2.2.2.2 Data analysis liver perfusion

For analysis of thyroid hormone kinetics, the tracer disappearance curve from the medium of each experiment was fitted to a two-exponential model of thyroid hormone distribution and metabolism (Figure 2), as

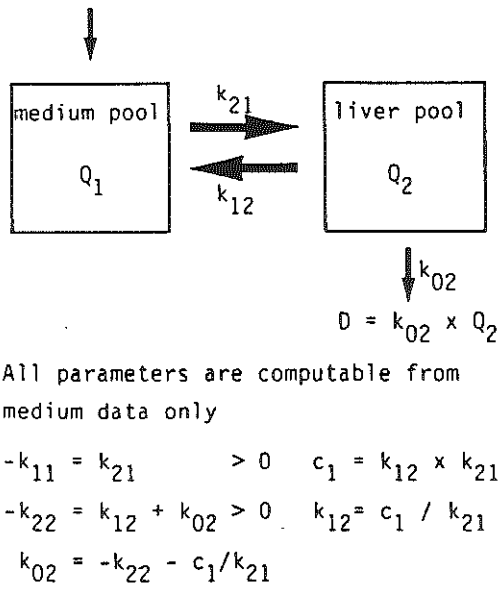

Figure 2
Two pool model of T_3 distribution and metabolism. K_{ij} values ($i \neq j, i,j = 1,2$, all $k_{ij} > 0$) are fractional transfer rates (min^{-1}) from pool j to pool i. K_{11}, k_{22} are fractional turnover rates (min^{-1}) of pool Q_1 and pool Q_2, respectively.

described by Docter et al. (9). Using this model, consisting of a medium and a liver pool, fractional transfer rate constants for influx (k_{21}), efflux (k_{12}),

and disposal (k_{02}), total mass transfer and the total disposal were calculated. Disposal could be corrected for differences in total mass transfer into the intracellular liver compartment, resulting in the
metabolic capacity = disposal / intracellular mass transfer

With the aid of experiments with 1 and 4 % BSA in the medium and using the two-pool model, it is possible to discriminate between transport of thyroid hormone to a non-metabolizing, presumably extracellular, liver compartment and transport into the intracellular liver compartment. During the perfusion with 1 % BSA total mass transfer (TMT) is:

$$TMT_{(1\%\ BSA)} = X + Y \qquad (1)$$

where X is transport of thyroid hormone into the intracellular compartment, Y is transport of thyroid hormone to the extracellular compartment, and TMT is the total amount of thyroid hormone (TH) transported to the liver during the perfusion. As X is linearly related to medium free (F)TH and Y is linearly related both to medium FTH and the medium albumin concentration (9), it follows that during the perfusion with 4 % BSA in the medium

$$TMT_{(4\%\ BSA)} = (FTH_{(4\%\ BSA)}/FTH_{(1\%\ BSA)})X + 4(FTH_{(4\%\ BSA)}/FTH_{(1\%\ BSA)})Y \qquad (2)$$

In this way two equations with two unknowns (X and Y) are obtained, of which both unknowns can be solved.

2.2.2.3 Calculation of the free hormone gradient over the plasma membrane

The free hormone clearances for the influx and efflux steps were calculated according to Weisiger et al. (8). These values are the volumes of medium or cytosol cleared of free thyroid hormone per second per gram.
For the *influx* step, the free clearance was calculated as:

$$(k_{21} \times MV) / (MFF \times LW) \text{ in } mL.s^{-1}.g^{-1}$$

k_{21} = influx rate constant (s^{-1}); MV = medium volume (mL); MFF = medium free hormone fraction; LW = liver weight (g).
For the *efflux* step, the free clearance was calculated as:

$$(k_{12} \times CV/g) / (FFDC \times (1/DF) \times ((CV/g)/(TV/g))) \text{ in } mL.s^{-1}.g^{-1} = k_{12} / (FFDC \times (1/DF) \times (1/(TV/g))) \text{ in } mL.s^{-1}.g^{-1}$$

k_{12} = efflux rate constant (s^{-1}); CV/g = cytosol volume per gram liver (mL/g); DF = dilution factor of liver piece; FFDC = free hormone fraction in diluted cytosol; TV/g = total volume per gram liver (mL/g); assumed to be 1 mL/g; (CV/g)/(TV/g) = fraction of total liver composed of cytosol.

If the overall uptake process is active, then the ratio between these two values for the free clearances should be greater than one.

2.2.2.4 ATP determination

ATP was extracted from liver pieces excised and snap-frozen in liquid nitrogen after 20 and 90 min perfusion with either glucose (10 mmol/l) or glucose and fructose (both 10 mmol/l). ATP was extracted using a solution of 0.1 M perchloric acid at 0 C and was determined using the luciferine/luciferase method (Adenylate Energy Charge Kit from Lumac, Landgraaf, the Netherlands).

2.2.3 Human hepatocytes

Liver cells were isolated from human livers, obtained through the Auxiliary Partial Liver Transplantation Program at the University Hospital Dijkzigt (Rotterdam, The Netherlands). Permission was given by the medical ethics committee to use the remaining part of the donor livers for scientific research. The livers were taken from physically healthy organ donors, who died from brain injury. During resection of the left lobe, the livers were perfused by portal venous cannulation with the preservation fluid Euro-Collins at 4 C. After resection, the left liver lobes were transported to the perfusion site within 45 min in a cold buffer (4 C), containing 10 mmol/L HEPES, 142 mmol/L NaCl, 16.7 mmol/L KCl, and 0.5 mmol/L ethylene glycol-O-O'-bis(2-amino-ethyl)-N,N,N',N',-tetraacetic acid (EGTA). Perfusion with 3 L of this buffer at a rate of 40 mL/min per catheter was started after insertion of four polyethylene catheters into the vascular orifices that were identified at the dissection surface. After preperfusion, each liver was perfused successively with 1) 500 mL of a HEPES buffer containing 5 mmol/L of $CaCl_2$ without recirculation, 2) with 200 mL of this buffer containing 0.05 % collagenase with recirculation during 20 min, and 3) this buffer containing 0.1 % collagenase with recirculation during 20 min. Liver tissue was dissociated in Hanks' buffer containing 2 % BSA. Cells were filtered through a 250 μm filter, centrifuged (50 times g for 30 sec) and washed three times in a cold culture medium (4 C) to remove non-parenchymal and damaged cells. At this stage, the cells came to our labaratory. After centrifugation, medium on the cells was replaced with

Ham's F-10 culture medium, containing 10.6 mmol/L PIPES, 11.2 mmol/L BES, 8.9 mmol/L HEPES, 12 U/L insulin, 2 mmol/L $CaCl_2$, 10 U/mL penicillin-streptomycin, and 10 % fetal calf serum. Cells were seeded in 6-well plates at a density of 2.10^6 cells/well and were maintained overnight (14 h) in 2 mL culture medium/well at 37 C in air. The next day, to remove unattached, nonviable cells, medium was replaced by 1 or 2 mL Ham's F-10 medium supplemented with 0.5 % BSA. In this medium the experiments were performed, the viability of the cells attached to the dishes and used in the different experiments was 60 to more than 90 % (trypan blue exclusion).

Uptake and subsequent deiodination of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 was investigated according to the well-defined assay conditions for these processes in isolated rat hepatocytes. In studies on cell-associated radioactivity, 1 mL culture medium with 0.5 % BSA was placed on the cells. At time zero, radioiodinated tracer (% FT_4 = 0.4, FT_4 = 0.3 pmol/L; % FT_3 = 2.4, FT_3 = 0.9 pmol/L; and % FrT_3 = 0.8, FrT_3 = 0.3 pmol/L) was added in the presence or absence of unlabeled hormone (1 μmol/L T_4, 5 μmol/L T_3, and 2 μmol/L rT_3). To study the time course of uptake, radioactivity in the cells was determined after 0, 1, 2, 5, 10, 30, and 60 min of incubation at 37 C, during which the wells were rotating on a tableau at 15°, followed by washing with 2 mL ice-cold phosphate buffered saline and lysis of the cells in 1 mL 0.1 N NaOH. In the initial uptake studies with ouabain (0.5 mmol/L) and fructose (10 mmol/L), cells were preincubated for 30 min with medium to which one of these compounds was added; uptake studies were performed in the presence of the compound after renewal of the medium. In the studies on intracellular metabolism during 20 h incubation, 2 mL culture medium with 0.5 % BSA were placed on the cells. Radioiodinated thyroid hormones were added in the presence or absence of ouabain (0.5 mmol/L), PTU (100 μmol/L), or a monoclonal antibody (ascites fluid dilution 1:200) directed against thyroid hormone uptake systems in rat hepatocytes. Parallel incubations without cells were performed to correct for spontaneous deiodination. After 20 h incubation at 37 C, medium was taken from the incubates and analyzed for remaining thyroid hormones, iodide, and conjugates.

All tracers used were always LH-20 purified on the day of the

experiment. Studies in cells of one liver were performed in triplicate. All studies were repeated in two to four different livers. Free hormone concentrations were estimated by equilibrium dialysis (7).

2.2.4 Rat hepatocytes

Methods used for isolation and culture of hepatocytes (2.10^6 cells/dish) were according a modification of the protocol by Berry and Friend (10). Four or 20 hours after isolation, culture medium (Ham's F10 with 10 % fetal calf serum) was removed and replaced with incubation medium (Ham's F10 with 0.5 % BSA) in which the experiments were performed at 37 C.

Uptake of ^{3}H-α-amino-iso-butyrate (α-AIB) (50 mmol/L and 5.5 μmol/L) was determined by measurement of cell associated (radio)activity (CAA = total uptake) at several time points up to 120 min, in the presence or absence of a) unlabeled T_3 (5.10^{-9} mol/L and 5.10^{-6} mol/L), b) unlabeled T_4 (5.10^{-9} mol/L and 5.10^{-6} mol/L), c) 2 monoclonal antibodies that inhibit thyroid hormone uptake by about 60 % in the used concentration (11), d) methyl(Me)-α-AIB (50 mmol/L) after 1 h preincubation (Me-α-AIB is a known A-system substrate and acts as a competitive inhibitor of α-AIB uptake), e) dibutyryl(db)-cAMP (0.1 mmol/L) (an A-system stimulator), and f) both T_3 (5.10^{-6} mol/L) and db-cAMP.

Furthermore, ^{125}I-T_3 uptake was determined by measuring cell associated radioactivity at several time points up to 60 min, in the presence or absence of Me-α-AIB (50 mmol/L, 1 h preincubation).

For each experiment: $n \geq 3$. Results are given as % of the 120 min (α-AIB) or 60 min (T_3) control value.

2.2.5 Statistical evaluation

This was performed using student's t-test and analysis of variance followed by comparison of class means (12).

REFERENCES

1 Ad hoc committee on standards for nutritional studies 1977 Report of the American Institute of Nutrition. J Nutr 7:1340-1348

2 Greenwood FC, Hunter WM, Glover JS 1963 The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem J 89:114-123

3 Mol JA, Krenning EP, Docter R, Rozing G, Hennemann G 1986 Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. J Biol Chem 261:7640-7643

4 DiStefano III JJ, Malone TK, Jang M 1982 Comprehensive kinetics of thyroxine distribution and metabolism in blood and tissue pools of the rat from only six blood samples: dominance of large, slowly exchanging tissue pools. Endocrinology 111:108-117

5 Van der Heijden JTM, Docter R, van Toor H, Wilson JHP, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251:E156-E163

6 Meyer DKF, Keulemans GTP, Mulder GJ 1981 Isolated perfused rat liver technique. In: Jakobi (ed), Methods in Endocrinology. New York Academic, 77, p 81-94

7 Sterling K, Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J Clin Invest 45:153-163

8 Weisiger RA, Luxon BA, Cavalieri RR 1992 Hepatic uptake of 3,5,3'-triiodothyronine: electrochemical driving forces. Am J Physiol 262:G1104-G1112

9 Docter R, De Jong M, Van der Hoek HJ, Krenning EP, Hennemann G 1990 Development and use of a mathemetical two pool model of distribution and metabolism of 3,3',5-triiodothyronine in a recirculating rat liver perfusion system: Albumin does not play a role in cellular transport. Endocrinology 126:451-459

10 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS lett 91:113-116

11 De Jong M, Docter R, Bernard HF, Van Toor H, Vos RA, Schoenmakers CHH, Hennemann G 1989 Monoclonal antibodies inhibit thyroid hormone transport into rat liver cells. Annales Endocrinol 50:118

12 Snedecor GW, Cochran WG 1967 One-way classifications. Analysis of variance. In: Statistical Methods, 6th ed. 258-298. The Iowa Univ. Press, Ames, Iowa USA

APPENDIX

Rat liver perfusion: calculation of liver uptake was done on the basis of the equations used for the human studies except that all parameters related to SEP were taken as zero.

Human studies: Calculation of the uptake in the rapid equilibrating pool (REP).
The following set of equations was used:

$$\frac{dQ_1(t)}{dt} = -(k_{21} + k_{31})Q_1(t) + k_{12}Q_2(t) + k_{13}Q_3(t) \qquad \%D/t \quad \text{plasma} \quad (I)$$

$$\frac{dQ_2(t)}{dt} = k_{21}Q_1(t) - (k_{12} + k_{02})Q_2(t) \qquad \%D/t \quad \text{REP} \quad (II)$$

$$\frac{dQ_3(t)}{dt} = k_{31}Q_1(t) - (k_{13} + k_{03})Q_3(t) \qquad \%D/t \quad \text{SEP} \quad (III)$$

$$Q_1(t) = y(t)V_p \qquad \%D \quad \text{plasmapool} \quad (IV)$$

$$y(t) = A_1e^{-\lambda 1t} + A_2e^{-\lambda 2t} + A_3e^{-\lambda 3t} \qquad \%D/L \quad \text{plasmaconc.} \quad (V)$$

According to the methods as outlined in refs. (4) and (5) the constants in the equations I - V were evaluated. Cumulative uptake in the REP (Q_2) was calculated by numerical solution of equation II for the time interval from 0 to 360 min, in 1 min steps, as follows:

$$Q_2(t_n) = Q_2(t_{n-1}) + k_{21}Q_1(t_{n-1}) - (k_{12} + k_{02})Q_2(t_{n-1})$$

$$= k_{21}Q_1(t_{n-1}) + (1 - k_{12} - k_{02})Q_2(t_{n-1}) \qquad \%D \qquad (VI)$$

At $t_n = 0$: $Q_2(t_0) = 0$ and $Q_1(t_0) = 100$ %D

therefore at 1 min (n=1)

$$Q_2(t_1) = k_{21}Q_1(t_{n-1}) = k_{21}.100 \qquad \%D$$

Subsequently $Q_2(t_n)$ is calculated for each minute from 2 to 360 min by evaluating VI, using the previously found $Q_2(t_{n-1})$; and using $Q_1(t_{n-1})$ after evaluation of IV and V.

CHAPTER 3

TRANSPORT OF T_3 INTO THE PERFUSED RAT LIVER AND SUBSEQUENT METABOLISM ARE INHIBITED BY FASTING.

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ABSTRACT

The effects of 48 h fasting on transport of T_3 and subsequent metabolism in isolated perfused rat livers were investigated. Tracer T_3 disappearance curves from the recirculating medium consisted of a fast component (FC) and a slow component (SC). Using a two-compartment model, both transport [expressed as the fractional transport rate constant from medium to liver (k_{21})] and disposal of T_3 were calculated. After fasting, k_{21}, total metabolism and metabolism corrected for differences in mass transfer were diminished, pointing to both decreased transport and metabolism, presumably caused by depletion of liver ATP. Concerning transport, it was shown that only transport into the intracellular liver compartment and not transport to the extracellular liver compartment was decreased after fasting. As for metabolism, T_3-glucuronidation was diminished, T_3-sulfation and subsequent deiodination were not affected. All mentioned decreased parameters normalized after addition of a combination of insulin, cortisol and/or glucose to the medium, possibly by (partially) restoration of cellular energy stores.

INTRODUCTION

Starvation induces alterations in iodothyronine concentrations in serum in both man and rats. Under these circumstances, the serum T_3 concentration decreases, and rT_3 levels increase, while serum T_4 concentrations mostly remain within normal range (1-5). It has been shown that these changes are effected by decreased peripheral conversion of T_4 into T_3. Metabolic clearance rate of rT_3 is also diminished with unaltered production rate (6,7). The causes of these changes have not been fully clarified. Both T_4 to T_3 conversion and rT_3 deiodination are catalyzed by 5'-deiodinase, and in these deiodinative processes the liver plays an important role (8,9). Decreased 5'-deiodinase activity has been claimed to be present during early and late starvation and has been accounted for by either a diminished activity of the enzyme itself or a deficiency of cytosolic cofactors such as NADPH or glutathione (10-12). In starvation beyond 48

hours in rats, tissue hypothyroidism, leading to diminished 5'-deiodination, may play a role in this aspect (13). These results were obtained primarily by the use of rat liver homogenates, not necessarily reflecting the situation in the intact liver.

As the deiodinases are located in the endoplasmatic reticulum, it is clear that T_4 has to cross the plasma membrane as a first and important step in its activation. Furthermore, in the liver the T_3 occupying the nuclear receptors is mainly derived from plasma, so for T_3 also, plasma membrane transport is necessary before it can regulate gene expression or be degraded. We have demonstrated the presence of specific energy-dependent cellular uptake mechanisms for T_4, T_3 and rT_3 in rat hepatocytes in primary culture (14-16). According to kinetic experiments T_4 and rT_3 appear to enter the cell by a common pathway, which is different from that for T_3. These observations led us to consider the possibility that fasting induces inhibition of transmembraneous transport of thyroid hormone into the liver, leading to diminished T_3 production, because of decreased availability of T_4, and to decreased metabolic clearance of rT_3. Recently, we demonstrated that this mechanism was operative in humans during caloric deprivation (17). Jennings et al. (18) reported a diminished uptake of T_4 and a normal 5'-deiodinase activity after fasting using a rat liver perfusion system.

To further investigate the aspect of changes in transmembraneous transport and subsequent metabolism of thyroid hormones after fasting, we studied these processes related to T_3 using a recirculating rat liver perfusion system, as in this model the cellular and structural integrity of the organ is maintained. Using this system, we were able to divide transport into the liver pool into transport to an extracellular, non-metabolizing liver compartment and transport into the intracellular liver compartment, where metabolism takes place. Transport to the former liver compartment of T_3 is dependent on the albumin concentration in the medium, whereas the amount of T_3 transported into the cells for further metabolism is determined by the free T_3 (FT_3) concentration and is independent of the albumin concentration in the medium (19).

Finally, the effects of refeeding on the isolated, fasted liver were

investigated by addition of glucose, insulin, cortisol and combinations of these to the perfusion medium.

MATERIALS AND METHODS

Animals: Male Wistar rats, weighing 200-250 g were used in all experiments. Weights of all groups of rats were similar before the experiments. In studies on the effect of fasting, food was withdrawn for 48 h, but free access to drinking water was maintained.

Experimental procedures: In the experiments mimicking refeeding after fasting, glucose (10 mmol/L), insulin (12 U/L), cortisol (500 nmol/L) or combinations of these were added to the medium.

After isolation, the liver was preperfused for 0.5 h or 2.5 h with medium in the presence or absence of glucose, insulin and/or cortisol. The experiments were started by the addition of T_3 (300 pmol/% BSA) and ^{125}I-T_3 to the medium (time 0).

See further Chapter 2.

RESULTS

I: Influence of 48-h fasting on transport and metabolism of T_3.

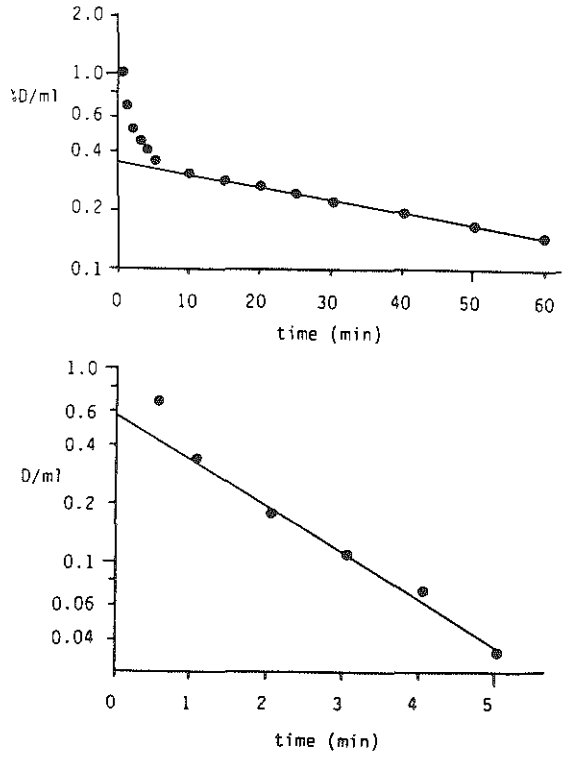
Fasting resulted in a decrease in liver weight between control (10.62 ± 1.59 g) and fasted rats (6.45 ± 0.312 g). Total liver DNA was not significantly different in control and 48-h fasted rats (100 ± 10.2 % and 104.5 ± 9.2 %, respectively, n = 8).

A typical example of a T_3 medium disappearance curve is shown in Figure 1 as a semi-log plot of T_3 concentration expressed as percentage dose per mL against time. In all experiments this medium disappearance can be described as the sum of two exponentials, which indicates that the use of a two-exponential disappearance curve is adequate to describe the changes in tracer T_3 in the medium. To investigate whether this kinetic behaviour could be an aspecific effect caused by binding to components of the perfusion system, medium with T_3 tracer was circulated for 2 h in the system without a liver. This resulted in T_3 disappearance smaller than 1 % after an initial distribution in the system. This demonstrates that this biphasic

Figure 1

Upper panel: typical example of biphasic T_3 disappearance from the medium, expressed as % dose of the added tracer/mL. The straight line is fitted on the second part of the curve (slow component).

Lower panel: first part of the total disappearance curve (fast component), after correction for the slow component (peel-off system).



disappearance is not caused by binding of T_3 to the system. With the parameters of the two-exponential disappearance curve, the two-pool model of distribution and metabolism was calculated for each experiment.

Transport: The results of the transport parameters of T_3 for the experiments with 1 % BSA are given in Table 1. After 48-h fasting, k_{21}, the fractional transfer rate constant from the medium to the liver, was decreased compared to controls, pointing to a decreased transport into the liver. Furthermore, the total and free hormone concentrations in the medium increased after fasting, because of the inhibited entry of T_3 into the fasted liver. To evaluate the transport capacity of the liver, mass transfer has to be corrected for the free hormone concentration as this moiety is the sole

Table 1

Fractional transport rate constants from medium to liver (k_{21}), and from liver to medium (k_{12}), total mass transfer (TMT), mean TT_3 in the medium, mean FT_3 in the medium, and TMT/FT_3, both for control and 48-h fasted livers perfused during 60 min (mean ± SD, all expressed in % of mean of control (n = 5), @ = p < 0.005 versus control).

parameter	control liver	48-h fasted liver
k_{21}	100 ± 8.8	63.2 ± 6.6@
k_{12}	100 ± 6.7	106.7 ± 6.8
TMT	100 ± 7.5	107.4 ± 4.2
TT_3	100 ± 6.3	136.7 ± 4.7@
FT_3	100 ± 6.6	138.3 ± 5.0@
TMT/FT_3	100 ± 7.1	75.5 ± 4.9@

extracellular determinant of transport in our system (19). The total mass transfer, as such unaltered after fasting, was decreased when corrected for the increasing free hormone concentration. Finally, k_{12}, the fractional transfer rate constant from the liver to the medium, was calculated. This parameter was not influenced after 48 h fasting.

The found decrease in transport after fasting (decreased k_{21} and decreased corrected total mass transfer) could theoretically be caused by diminished transport to the extracellular liver compartment, diminished transport into the intracellular liver compartment, or both. Recently, we described a method to discriminate between transport to the extracellular and intracellular liver pools (19). In this study we showed that albumin influenced only transport to the extracellular compartment of the liver and not that to the intracellular compartment. To calculate transport of T_3 to each of both pools, we tested our previously made assumption that transport to the extracellular pool is linearly related to the BSA concentration in the medium. Therefore, liver perfusions of control rats were performed with 1 %, 2 % and 4 % BSA in the medium. Total mass transfer/FT_3 (two-pool model) was calculated in these three conditions. The results are shown in Figure 2. In the left panel of Figure 2, the point of intersection with the abscissa corresponds with the total amount of T_3 transported into the intracellular liver compartment per pmol FT_3 (at this point BSA-concentration

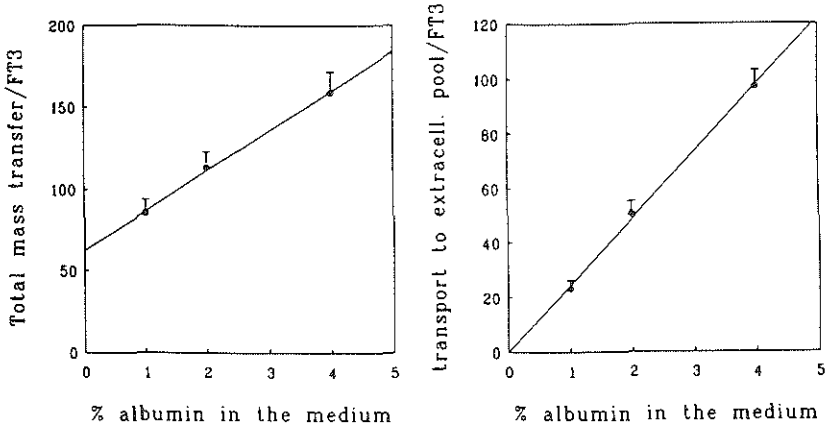


Figure 2
Left panel: relationship between albumin concentration in the medium and total mass transfer per pmol FT_3, expressed as pmol per pmol/L FT_3.
Right panel: relationship between albumin concentration in the medium and transport to the extracellular pool per pmol FT_3, expressed as pmol per pmol/L FT_3.

is 0, and the extracellular pool is consequently absent). The right panel shows the same data corrected for transport into the liver cells, leaving total transport to the extracellular liver compartment per pmol FT_3. It clearly appears that this transport is indeed linearly related to the amount of BSA in the medium, which shows that the assumption was correct.

To investigate the effect of fasting on transport to the extra- and intracellular compartments of the liver, we performed control and fasted rat liver perfusions with 1 % and 4 % BSA added to the medium. The results in Table 2 show that transport to the extracellular compartment of the liver is the same in livers of control and fasted rats, whereas transport into the intracellular compartment is decreased in livers of 48-h fasted rats.

Metabolism: After uptake into liver cells, T_3 can be conjugated to T_3G or T_3S (23). Of these conjugated products, T_3G normally appears mostly in the bile. T_3S is deiodinated, and the end product, iodide, is excreted mostly into the medium.

Table 2

Mean TT_3 in the medium (nmol/L), mean FT_3 in the medium (pmol/L), TMT (pmol/60 min), TMT/FT_3 (pmol per pmol/L FT_3), and transport to intracellular liver pool/FT_3 (=TrI) and extracellular liver pool/FT_3 (=TrE) (pmol per pmol/L FT_3) in control and 48-h fasted livers perfused with medium with 1 and 4 % BSA during 60 min (mean ± SD, n = 5, @ = p < 0.01 versus control with the same BSA concentration).

	control 1 % BSA	control 4 % BSA	48-h fasted 1 % BSA	48-h fasted 4 % BSA
TT_3	0.6 ± 0.04	3.5 ± 0.2	0.8 ± 0.04@	4.8 ± 0.4@
FT_3	13.4 ± 0.9	24.7 ± 1.1	18.6 ± 0.9@	32.9 ± 1.7@
TMT	1293 ± 96	6112 ± 212	1389 ± 58	6976 ± 378
TMT/FT_3	96.4 ± 6.8	247.1 ± 11.2	74.8 ± 3.7@	212.3 ± 14.8@
TrI/FT_3	47.7 ± 4.3	47.8 ± 4.2	29.0 ± 1.4@	29.0 ± 1.6@
TrE/FT_3	48.7 ± 3.3	195.0 ± 11.7	45.8 ± 3.4	183.3 ± 10.9

Table 3

Total production in medium and bile of iodide (I^-, % dose), T_3 glucuronide (T_3G, % dose) and T_3 sulfate (T_3S, % dose), total disposal (% control) and corrected disposal (= disposal/total mass transfer, % control) after 60 minutes of perfusion both for control and 48 h fasted livers (mean ± SD, n = 5, @ = p < 0.01 versus control).

	control	48-h fasted
I^-	12.8 ± 1.3	12.3 ± 1.1
T_3G	13.6 ± 1.7	8.8 ± 1.1@
T_3S	3.5 ± 0.2	3.8 ± 0.4
disposal	100 ± 4.5	61.6 ± 4.2@
corrected disposal	100 ± 7.0	57.4 ± 5.3@

The results of analysis of medium and bile by LH-20 chromatography and the integrated T_3 disposal during 60 minutes, i.e. the total amount of metabolized T_3 as calculated according to the two-pool model, are depicted in Table 3. Iodide production is not influenced by fasting, and sulfate production also remains constant. Glucuronide production decreases after fasting ($p<0.01$). Thus, the total disposal of T_3 is diminished compared to that in control livers, caused by the decrease in T_3 glucuronidation but not in deiodination. Disposal is not corrected for differences in liver weight

between controls and fasted rats (see Discussion).

The amount of metabolized T_3 is influenced by the total mass transfer of T_3 (= k_{21} x volume medium x integrated T_3-concentration) into the cells during the experiment (see Discussion). Therefore, the disposal of T_3 in the different groups has to be corrected for possible differences in total mass transfer. As total mass transfer is not significantly different in control and fasted livers, T_3 metabolism corrected for differences in total mass transfer parallels the uncorrected disposal of T_3 (Table 3).

II: Influence of "refeeding" of 48 h fasted livers on transport and metabolism of T_3.

The effect of subsequent refeeding on transport and metabolism of T_3 was investigated by experiments in which fasted and control rat livers were preperfused with media with or without addition of glucose, insulin, and cortisol for 0.5 h or 2.5 h, after which the experiment was started by addition of T_3 and [^{125}I]-T_3.

Transport: In Table 4 the k_{21} values are shown for all experimental

Table 4

Influence of different treatment regimes (preperfusion) on k_{21}-values (% control) of T_3 transport using livers of fasted rats (mean ± SD, @ = $p < 0.005$ versus control).

addition to medium		0.5-h preperfusion	2.5-h preperfusion
-	(n=5)	62.7 ± 4.8@	68.3 ± 4.5@
G	(n=5)	63.2 ± 6.6@	62.3 ± 5.9@
I	(n=4)	66.9 ± 5.8@	61.8 ± 5.7@
C	(n=3)	67.5 ± 4.9@	69.7 ± 4.7@
G + C	(n=3)	68.2 ± 5.9@	68.9 ± 7.2@
G + I	(n=4)	102.3 ± 7.6	92.8 ± 7.4
I + C	(n=4)	94.5 ± 5.3	101.8 ± 6.8
G + I + C	(n=4)	99.3 ± 6.9	95.5 ± 8.6

G = [glucose] = 10 mmol/L, I = [insulin] = 12 U/L, C = [cortisol] = 500 nmol/L.

conditions. The experiments with *fasted* livers without additions to the medium or with addition of either glucose, insulin, or cortisol alone or cortisol plus glucose all have significantly decreased k_{21} values (not significantly different from each other) compared to those of fed livers (shown in Table 1). The results of these fasting experiments were combined to simplify presentation, forming group 1A for 0.5-h preperfusion and group 1B for 2.5-h preperfusion. When combinations of insulin and glucose and/or cortisol were added to the medium in the fasting experiments, the k_{21}-values after 0.5-h (group 2A) and 2.5-h preperfusion (group 2B) appear to be increased compared to those in groups 1A and 1B and were, in fact, not different from those of fed control livers. Therefore, we will present the obtained data in six (sub)groups: group 1A and 1B represent the data of fasted livers perfused with "poor" medium after 0.5- and 2.5-h preperfusion. Group 2A and 2B consist of fasted livers perfused with "rich", i.e. insulin-combination-containing media, after 0.5- and 2.5-h preperfusion. Group 3 consists of livers of control rats perfused with "poor" medium, whereas the control livers in group 4 are perfused with "rich" medium.

The results of all parameters influencing transport: k_{21}, TMT and k_{12},

Table 5

Fractional transport rate constants from medium to liver (k_{21}), from liver to medium (k_{12}), and total mass transfer (TMT) (mean ± SD, all results expressed in % of mean of control, $n \geq 5$, @ = $p < 0.005$ versus control).

	Liver	med.	PRE	k_{21}	k_{12}	TMT
1A	fas	poor	0.5	65.7 ± 5.6@	105.8 ± 7.6	110.1 ± 4.8
1B	fas	poor	2.5	66.2 ± 5.6@	98.7 ± 6.8	112.0 ± 4.9
2A	fas	rich	0.5	98.7 ± 6.6	97.8 ± 3.4	114.1 ± 6.2
2B	fas	rich	2.5	96.7 ± 7.6	92.6 ± 5.4	111.7 ± 5.8
3	fed	poor	0.5	100 ± 8.8	100 ± 6.7	100 ± 7.5
4	fed	rich	0.5	98.5 ± 8.9	108.6 ± 4.3	104.3 ± 8.4

Fas = fasted, PRE = preperfusion in h., med. = medium, rich medium = medium with insulin, glucose and/or cortisol.

are depicted in Table 5 and show that after 48-h fasting, the diminished transport of T_3 is normalized by the addition of insulin and glucose and/or cortisol to the medium. Perfusion of control livers with "rich", i.e. insulin combination-containing media has no influence on the transport parameters.

Metabolism: As shown in Table 6, iodide production was the same in all groups, except for group 2B, where iodide production is strongly increased after 2.5-h preperfusion with insulin in combination with glucose and/or cortisol. Glucuronide production, which was decreased after fasting,

Table 6

Total production in medium and bile of iodide (I^-, % dose), T_3 glucuronide (T_3G, % dose) and T_3 sulfate (T_3S, % dose), total disposal (% control) and corrected disposal (= disposal/total mass transfer, % control) after 60 minutes perfusion (mean ± SD, n ≥ 5, all expressed in % of mean of control, @ = p < 0.01 versus control). Groups are constructed the same way as in Table 5.

	I^-	T_3G	T_3S	disposal	corr. disp.
1A	12.2 ± 1.2	8.9 ± 1.1@	4.0 ± 0.4	65.2 ± 4.4@	59.6 ± 5.1@
1B	13.8 ± 1.0	10.1 ± 1.0@	4.1 ± 0.2	72.2 ± 4.0@	64.6 ± 5.1@
2A	14.4 ± 1.3	9.5 ± 1.2@	4.5 ± 0.5	87.3 ± 3.9@	75.8 ± 5.1@
2B	25.6 ± 2.3@	12.0 ± 1.9	3.9 ± 0.3	115.4 ± 9.9	103.3 ± 5.2
3	12.8 ± 1.3	13.6 ± 1.7	3.5 ± 0.2	100 ± 4.5	100 ± 7.0
4	13.4 ± 1.5	13.8 ± 1.2	3.8 ± 0.2	110.2 ± 6.8	105.7 ± 8.9

increased again when insulin combinations with glucose and/or cortisol were added to the medium and the liver was preperfused over 2.5 h. Sulfate production remained constant in all groups. For disposal, as calculated by the two-pool model, normalization was reached by the addition of insulin and glucose and/or cortisol to the medium after 2.5-h preperfusion.

DISCUSSION

I: Influence of 48-h fasting on transport and metabolism of T_3.

An explanation for altered thyroid hormone metabolism after fasting may be inhibition of transport across the cell membrane, diminished

deiodinase activity (9,10,24), or both.

Our results investigating transport parameters show that transport of T_3 to the liver cells is decreased, and these findings are in concert with those of Jennings et al. for T_4 (18). Furthermore, it is shown that transport inhibition is due to a decreased entrance of T_3 into the intracellular, i.e. the metabolizing, liver compartment and not to a decreased transport to the extracellular compartment.

To investigate if decreased disposal is due to inhibited transport or metabolism, the disposal was corrected for differences in total mass transfer. It appeared that disposal of T_3 is also decreased after 48-h fasting, compatible with a decreased glucuronidation, but not sulfation, and subsequent deiodination of T_3. It has been shown that transport of thyroid hormones into rat hepatocytes is rate limiting in total cellular uptake and metabolism, and a decrease in metabolism could theoretically directly be related to an inhibition of cellular uptake (25) (see below). Correction of T_3 disposal for total mass transfer allows estimation of metabolism independent of differences in transport. In our perfusion system, total mass transfer is not significantly different in the various groups. This is caused by the fact that in recirculating liver perfusion, inhibited transport leads to a higher (free) T_3 concentration in the medium during the experiment. This will counteract the effects of transport inhibition on total mass transfer by increasing the driving force, thereby keeping the total mass transfer about constant. Thus, mass flux into the liver, not corrected for the free hormone concentration, is the same in control and fasted livers. Furthermore, k_{12}, the fractional transfer rate constant from liver to medium, is not influenced after fasting, thereby not influencing the intracellular free hormone concentration, which is the limiting determinant of the rate of metabolism. Therefore, disposal corrected for transport parallels the differences in uncorrected disposal between the groups before and after fasting. Thus, we can conclude that T_3 metabolism, i.e. T_3-glucuronidation, is impaired after 48-h fasting, while sulfation and deiodination remain unaltered. It has been shown that starvation in rats leads to diminished glucuronidation, but not to attenuation of sulfation (26-31), which is in concert with our findings.

As for the cause of the inhibition of transport into the liver and

subsequent metabolism, we have no definite evidence at this stage. Diminished intracellular ATP content, as seen in livers from fasted rats (32,33), can possibly account for the decreased transport of T_3. Furthermore, starvation may lead to attenuation of membrane fluidity, as such influencing transport or thereby causing a decrease in Na^+,K^+-ATP-ase activity, as shown in hepatocytes in primary culture (34). This mechanism may also be operative in fasted rat livers, since the uptake of thyroid hormone into isolated rat hepatocytes is inhibited by ouabain, an inhibitor of this ATP-ase.

It has been reported that the transport and metabolism of T_4 in the liver are reduced after fasting (15,35). This will lead to a reduced total plasma T_3 production, since 80 % of serum T_3 is derived from extrathyroidal T_4 deiodination, in which the liver plays a major role (8). As T_3 is the most biologically active thyroid hormone, inhibition of both the production and transport of T_3 into the liver will lead to a proportional lowering of intracellular FT_3 and, therefore, a reduced occupancy of the nuclear receptors. This is in concert with our findings in isolated rat hepatocytes, where 30 % inhibition of transport by ouabain resulted in 50 % inhibition of nuclear binding (36).

The fact that unidirectional transfer of thyroid hormone into the liver exceeds the metabolized amount does not argue against a rate-limiting role of the plasma membrane transport process in the converting process, assuming that the intracellular hormone binding capacity and metabolic capacity are constant. Then, the influx rate of thyroid hormone determines the intracellular free hormone concentration, as efflux (k_{12} in our model study) is unchanged and is a passive process (36). The rate of intracellular deiodination in a cellular system, with assumed constant deiodinating capacity, is determined by the occupancy of the deiodinating enzymes by the substrate and, therefore, by the intracellular free hormone concentration, the latter being determined by the rate of thyroid hormone transport over the plasma membrane. Changes in the influx rate of thyroid hormone can be effected by changes in the extracellular free hormone concentration or changes in the transport process, as is the case in the study reported here.

Unlike others (15,35), Kinlaw et al. (37) did not find decreased T_3

production from T_4 in starved rats. They performed non-compartmental kinetics in intact rats that were starved for 4 days. It has been shown that if starvation proceeds from 1 - 4 days, progressive tissue hypothyroidism develops, being a major factor in alterations in T_4 to T_3 conversion at 4 days (38), as in hypothyroid rats liver 5'-deiodinating activity (type I deiodinase) is decreased. Kinlaw et al. themselves state that any decrease in hepatic T_4 to T_3 conversion in their studies could have been obscured by an increase in extrahepatic (type II deiodinase) conversion by citing Kaplan and Yaskoski, who found a 60 % increase in deiodination in hypothalamic tissue during starvation in rats (39). Furthermore, Kinlaw et al. did not find a decrease in MCR of T_4 in fasting rats per unit (100 g) BW. As fasting obviously leads to a decrease in weight, the MCR not corrected for weight loss should be diminished in their studies, which is in accordance with our findings for the liver (see k_{21}).

Our results reported here underline the potential importance of regulation of transport of iodothyronines into cells in determination of the bioavailibility of thyroid hormone. Transport of iodothyronines into other tissue cells is also of importance in the regulation of biologic expression of thyroid hormone. It has been shown that different organs depend differently on T_3 derived from plasma or derived from local T_4 conversion (40). For instance, muscle depends for the major part, if not completely, on uptake of plasma T_3 for nuclear occupancy, whereas for pituitary and brain nuclear an important part of T_3 is derived from intracellular T_4 (41,42).

The transport processes of iodothyronines have been shown not only to exist in hepatocytes from rats (14-16), but also in many different cell types from different species, including humans (for review, see Ref. 43). Further studies in animals and man are needed to evaluate more precisely the role of transport mechanisms in the overall regulation of thyroid hormone activity.

II: Influence of refeeding of 48-h fasted livers on transport and metabolism of T_3

The stimulatory effects of insulin in combination with cortisol and/or

glucose on T_3 transport may be related to rapid restoration of ATP stores, depleted during starvation (see above). This is in accordance with the fact that in normally fed livers, with presumably optimal ATP levels, no effect of insulin with glucose and/or cortisol was seen on the transport parameters.

We did not correct for decrease in liver weight after 48-h fasting; this weight loss is mainly due to glycogen depletion and does not play an active role in thyroid hormone transport and metabolism. Total liver DNA is not significantly different in livers of control and 48-h fasted rats, indicating that the total number of cells does not change. In principle, it could be possible that a decrease in intracellular binding sites (because of the loss of binding protein after fasting) caused the decreased uptake and metabolism in the liver after fasting. We showed, however, that after 0.5-h preperfusion with insulin, glucose, and/or cortisol the impaired uptake was normalized. It is very unlikely that changes in the concentration of intracellular binding proteins by means of protein synthesis during this 0.5-h period could account for this normalization. In this respect, it is also important to note that no changes occurred on the k_{12} values after treatment of fasted livers with insulin, glucose and/or cortisol. If intracellular binding would have changed alteration in k_{12} would have occurred as well. It can, thus, be concluded that normalization of transport in fasted livers caused by insulin, glucose, and/or cortisol is independent of hepatic mass.

As for metabolism, a stimulating effect of insulin on deiodination, as we found, is also reported by Grau et al. (44) and Sato and Robbins (45) for T_4 deiodination in rat liver microsomes. The time dependence of this stimulation is demonstrated by the fact that no stimulation of deiodination is apparent after 0.5 h of preperfusion with insulin combinations, but only after 2.5 h in fed and fasted livers (not shown for fed livers). This suggests that protein synthesis of deiodinase might be involved. Insulin is apparently necessary to facilitate the effects of glucose and cortisol (a stimulator of liver gluconeogenesis) on uptake and metabolism of T_3. This is in concert with the findings of Gavin et al. (46), who demonstrated that glucose and insulin together reverse the effects of fasting on T_3 generation from T_4 in isolated hepatocytes.

Normalisation of metabolism occurred only after 2.5-h preperfusion

with insulin, cortisol, and/or glucose in the medium. Probably some protein synthesis related to T_3 glucuronidation may take place, normalizing this process. We found no inhibition of sulfation, or, more importantly, of deiodination after fasting. In other words, in our conditions, deiodination per se is not affected by fasting. This implies that after fasting of rats for a short period of time, i.e. 48 h, it is unlikely that decrease of deiodination plays an important role in lowered T_3 production from T_4. This makes our findings of inhibition of transport of thyroid hormone into the liver being a primary event during fasting the more significant. More knowledge, however, is necessary to more precisely evaluate the role of plasma membrane transport of thyroid hormone in the regulation of thyroid hormone activation and metabolism. Recently, we have identified the putative T_3 plasma membrane transporter in HepG2 hepatocytes. This protein has a mol wt of 66 kilodaltons and specifically binds T_3, but not T_4 (47). Further work is in progress that should ultimately result in studies concerning the regulation of gene expression of thyroid hormone transporters in physiological and pathological conditions.

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REFERENCES

1 Suda AK, Pittman CS, Shimizu T, Chambers JB 1978 The production and metabolism of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine in normal and fasting subjects. J Clin Endocrinol Metab 47:1311-1319

2 Cavalieri RR, Rapoport B 1977 Impaired peripheral conversion of thyroxine to triiodothyronine. Annu Rev Med 28:57-65

3 Balsam A, Ingbar SH, Sexton F 1978 The influence of fasting, diabetes and several pharmacological agents on the pathways of thyroxine metabolism in rat liver. J Clin Invest 62:415-424

4 Harris A, Fang SW, Vagenakis AG, Braverman LE 1978 Effect of starvation, nutrient

replacement and hypothyroidism on in vitro hepatic T_4 to T_3 conversion in the rat. Metabolism 27:1680-1690

5 Gavin LA, Bui F, McMahon F, Cavalieri RR 1980 Sequential deiodination of thyroxine to 3,3'-diiodothyronine via 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine in rat liver homogenate: the effects of fasting versus glucose feeding. J Biol Chem 255:49-53

6 Azizi F 1978 Effect of dietary composition on fasting induced changes in serum thyroid hormones and thyrotropin. Metabolism 27:935-942

7 Spaulding SW, Chopra IJ, Sherwin RS, Lyall SS 1976 Effect of caloric restriction and dietary composition on serum T_3 and rT_3 in man. J Clin Endocrinol Metab 42:197-200

8 Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed), Thyroid hormone metabolism. Marcel Dekker, New York and Basel, p 189-229

9 Kaplan MM, Utiger RD 1978 Iodothyronine metabolism in rat liver homogenates. J Clin Invest 61:459-471

10 Kaplan MM 1979 Subcellular alterations causing reduced hepatic thyroxine-5'-monodeiodinase activity in fasted rats. Endocrinology 104:58-64

11 Harris ARC, Fang SL, Kinerfeld K, Braverman LE, Vagenakis AE 1979 The role of sulfhydryl groups on the impaired hepatic 3,3',5- triiodothyronine generation from thyroxine in the hypothyroid, starved, fetal, and neonatal rodent. J Clin Invest 63:516-524

12 Balsam A, Ingbar SH 1979 Observations on the factors that control the generation of triiodothyronine from thyroxine in rat liver and the nature of the defect induced by fasting. J Clin Invest 63:1145-1151

13 Balsam A, Sexton F, Ingbar SH 1981 The influence of fasting and the thyroid state on the activity of thyroxine 5'-deiodinase in rat liver: A kinetic analysis of microsomal formation of triiodothyronine from thyroxine. Endocrinology 108:472-477

14 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314-320

15 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1982. Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229-233

16 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett 91:113-116

17 Van der Heyden JTM, Docter R, Van Toor H, Wilson JHP, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251:E156-E163

18 Jennings AS, Duncan CF, Utiger RD 1979 Regulation of the conversion of thyroxine to triiodothyronine in the perfused rat liver. J Clin Invest 64:1614-1624

19 Docter R, De Jong M, Van der Hoek HJ, Krenning EP, Hennemann G 1990 Development and use of a mathematical two pool model of distribution and metabolism of 3,3',5-triiodothyronine in a recirculating rat liver perfusion system: albumin does not play a role in cellular transport. Endocrinology 126:451-459

20 Meijer KF, Keulemans K, Mulder GJ 1981 Isolated perfused rat liver technique. Methods in Enzymology 77:81-94

21 Sterling K, Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J Clin Invest 45:153-163

22 Snedecor GW, Cochran WG 1967 One-way classifications. Analysis of variance. In: Statistical Methods, ed 6. p 258-298. The Iowa State Univ Press, Ames

23 Otten MH, Mol JA, Visser TJ 1983 Sulfation preceeding deiodination of iodothyronines in rat hepatocytes. Science 221:81-83

24 Balsam A, Ingbar SH 1978 The influence of fasting, diabetes, and several pharmacological agents on the pathways of thyroxine metabolism in rat liver. J Clin Invest 62:415-424

25 Hennemann G, Krenning EP, Polhuis M, Mol JA, Bernard HF, Visser TJ, Docter R 1986 Carrier-mediated transport of thyroid hormones into rat hepatocytes is rate limiting in total cellular uptake. Endocrinology 119:1870-1872

26 Dill RL, Klaassen CD 1986 The effect of inhibitors of mitochondrial energy production on hepatic glutathione, UDP-glucuronic acid, and adenosine 3'-phosphate-5'-phosphosulfate concentrations. Drug Metab and Disposition 14:190-196

27 Dills RL, Klaassen CD 1986 Effect of reduced hepatic energy state on acetaminophen conjugation in rats. J Pharmacol Exp Ther 238:463-472

28 Reinke LA, Belinsky SA, Evans RK, Kauffman FC, Thurman RG 1981 Conjugation of p-nitrophenol in the perfused rat liver: the effect of substrate concentration and carbohydrate reserves. J Pharmacol Exp Ther 217:863-870

29 Glazenburg EJ, Jekel-Halsema IMC, Scholtens E, Baars AJ, Mulder GJ 1983 Effects of variation in the dietary supply of cysteine and methionine on liver concentration of glutathione and "active sulfate" (PAPS) and serum levels of sulfate, cystine, methionine and taurine: relation to the metabolism of acetaminophen. J Nutr 113:1363-1373

30 Schwarz LR 1980 Modulation of sulfation and glucuronidation of 1-naphtol in isolated rat liver cells. Arch Toxicol 44:137-145

31 Sundheimer DW, Brendel K 1984 Factors influencing sulfation in isolated rat hepatocytes. Life Sci 34:23-29

32 Schwenke WD, Soboll S, Seitz HJ, Sies H 1981 Mitochondrial and cytosolic ATP/ADP ratios in rat liver in vitro. Biochem J 200:405-408

33 Cunningham CC, Malloy CR, Radda GK 1986 Effect of fasting and acute ethanol administration on the energy state of in vivo liver as measured by 31P-NMR spectroscopy. Biochim Biophys Acta 885:12-22

34 Schachter D 1984 Fluidity and function of hepatocyte plasma membranes. Hepatology 4:140-151

35 Docter R, De Jong M 1988 Inhibition of computed liver T_4 uptake during caloric deprivation and after fructose administration in humans and in perfused (recirculating) rat liver. Ann Endocrinol (Paris) 3:183

36 Hennemann G, Krenning EP, Bernard B, Huvers F, Mol J, Docter R, Visser TJ 1984 Regulation of influx and efflux of thyroid hormones in rat hepatocytes: Possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity. Horm Metab Res 14:1-6

37 Kinlaw WB, Schwartz HL, and Oppenheimer JH 1985 Decreased serum triiodothyronine in starving rats is due primarily to diminished thyroidal secretion of thyroxine. J Clin Invest 75:1238-1241

38 Chopra IJ 1980 Alterations in monodeiodination of iodothyronine in the fasting rat: Effects of reduced nonprotein sulfhydryl groups and hypothyroidism. Metabolism 29:161-167

39 Kaplan MM, Yaskoski KA 1982 Effects of congenital hypothyroidism and partial and complete food deprivation on phenolic and tyrosyl ring iodothyronine deiodination in rat brain. Endocrinology 110:761-767

40 Hennemann G 1986 Thyroid hormone deiodination in healthy man. In: Thyroid hormone metabolism. G. Hennemann, editor. Marcel Dekker, New York and Basel. p 277-295

41 Van Doorn J, Van der Heide D, Roelfsema F 1984 Sources and quantity of 3,5,3'-triiodothyronine in several tissues of the rat. J Clin Invest 72:1778-1792

42 Silva JE, Larsen PR 1978 Contributions of plasma triiodothyronine and local thyroxine monodeiodination to triiodothyronine and nuclear triiodothyronine receptor saturation in pituitary, liver and kidney of hypothyroid rats. Further evidence relating saturation of pituitary nuclear triiodothyronine receptors and the acute inhibition of thyroid-stimulating hormone release. J Clin Invest 61:1247-1259

43 Krenning EP, Docter R 1986 Plasma membrane transport of thyroid hormone. In: Hennemann G (ed) Thyroid hormone metabolism. Marcel Dekker, New York and Basel, p 107-131

44 Grau R, Keck FS, Loos U, Pfeiffer EF 1984 The influence of glucose and insulin infusion on in vitro T_4 monodeiodination in rat liver microsomes. In: Loos U and Wartofsky RL (eds), Peripheral metabolism of thyroxine. Georg Thieme Verlag, New York, p 62-77

45 Sato K, Robbins J 1981 Thyroid hormone metabolism in primary cultured rat hepatocytes. J Clin Invest 68:475-483

46 Gavin LA, Cavalieri RR, Moeller M 1987 Glucose and insulin reverse the effects of fasting on 3,5,3'-triiodothyronine neogenesis in primary cultures of rat hepatocytes.

Endocrinology 121:858-864

47 Van Stralen PGJ, Van der Hoek HJ, Visser TJ, Hennemann G 1991 Partial purification of a $BrAcT_3$-specifically labelled protein in HepG2 plasma membranes using affigel 731. Thyroid 1:p46

CHAPTER 4

ADAPTIVE CHANGES IN TRANSMEMBRANE TRANSPORT AND METABOLISM OF T_3 IN PERFUSED LIVERS OF FED AND FASTED HYPO- AND HYPERTHYROID RATS.

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ABSTRACT

Transport and subsequent metabolism of T_3 was studied in isolated perfused livers of eu-, hypo-, and hyperthyroid rats, both fed and 48-h fasted. T_3 kinetics (transport and metabolism) during perfusion were evaluated by means of a two-pool model, whereas metabolism of T_3 was also investigated by determination of T_3 breakdown products by chromatography of medium and bile. For comparison of groups, metabolism was corrected for differences in transport.

In fed hypothyroid livers transport parameters were not significantly changed compared to euthyroid livers, whereas metabolism was decreased. In fed hyperthyroid livers fractional rate constants for influx and efflux were decreased, while metabolism, corrected for differences in intracellular mass transfer, was increased. Furthermore, concerning transport in hyperthyroid livers, it was shown that only total mass transfer into the metabolizing liver compartment and not into the non-metabolizing liver compartment was decreased.

In fasted hypothyroid livers transport and metabolic parameters were decreased compared to euthyroid fed livers. In fasted hyperthyroid livers transport and metabolism were not significantly different from euthyroid fed livers, so transport was increased versus hyperthyroid fed livers. It appeared therefore that fasting normalized the effects of hyperthyroidism on both transport and metabolic processes of T_3 in the liver.

The present study demonstrates normal transport and decreased metabolism in livers of hypothyroid fed rats and decreased transport and increased metabolism in livers of hyperthyroid fed livers. In livers of hypothyroid fasted rats, transport and metabolism were decreased, whereas in livers of hyperthyroid fasted rats transport and metabolism were not significantly different from euthyroid fed livers. These changes might favour tissue euthyroidism, in spite of the altered thyroid and nutritional state, and can therefore be seen as adaptation mechanisms to these altered states at the tissue level.

INTRODUCTION

Many investigations are made about the effects of hyper- and hypothyroidism on thyroid hormone metabolism in vitro and in vivo (1-5). However, these effects are as yet incompletely understood. In hyperthyroid humans both decreased (6) and increased (7) metabolic rates of thyroid hormones are reported, whereas hypothyroid humans are reported to have an increased fractional conversion rate of T_4 to T_3 (7,8). Conversion of thyroid hormone takes mostly place by deiodination and conjugation reactions (9). Deiodination of T_4, T_3 and rT_3 is catalyzed by at least 3 different deiodinating enzymes (type I, II and III), located in different tissues (10,11). The liver plays quantitatively an important role in deiodination, because of the presence of type I deiodinase (10). Hyperthyroidism increased deiodination of iodothyronines by rat liver homogenates (4,5). In hypothyroidism, rat tissue deiodinase activities for all iodothyronines were decreased as found in homogenates and tissue slice studies (4,5). The latter data, however, are obtained in broken cell preparations, which have several drawbacks. Sulfation, for example, occurs only in intact cells, and greatly potentiates deiodination, especially of T_3 (9). In intact, isolated liver cell cultures, however, there is no normal relationship between transport of iodothyronines from the plasma into the cells and subsequent metabolism. Intact liver perfusion is therefore presumably the most physiological in vitro technique, as in this system the importance of other processes in thyroid hormone metabolism, such as transport over the cell membrane, can be investigated.

As for transport, we have demonstrated the presence of specific energy dependent cellular uptake mechanisms for T_4, T_3 and rT_3 in rat hepatocytes (12,13,14). According to kinetic experiments T_4 and rT_3 appear to enter the cell by a common pathway, which is different from that for T_3. Evidence has been presented for two distinct pathways for T_4 and T_3 transport in the perfused rat liver (15) and in vivo in the human liver (16) as well. Recently, we reported discrimination between transport to a non-metabolizing, presumably extracellular, liver compartment and transport into an intracellular, metabolizing, liver compartment in the perfused rat liver (17,18).

To investigate if changes in plasma membrane transport or in

subsequent metabolism of T_3 occur in hyper- and hypothyroidism, we studied these processes using a recirculating rat liver perfusion system. The effects of fasting in combination with hypo- and hyperthyroidism were investigated as well, as little attention has been paid until now to the influence of the nutritional state on thyroid hormone handling during hypo- and hyperthyroidism.

MATERIALS AND METHODS

Rats: Male Wistar rats, weighing 200-250 g, were used in all experiments. Rats were made hyperthyroid by daily ip. injection of T_4 (10 µg/100 g body weight) for 15 days. Hypothyroidism was induced by addition of 0.05 % mercapto-methyl- imidazole (MMI) to the drinking water for at least 3 weeks. Hyper- and hypothyroidism was confirmed by estimation of T_4 and TSH levels in plasma by RIA. In studies on the effect of fasting, food was withdrawn for 48 h, but free access to drinking water was maintained.

See further Chapter 2.

RESULTS

In hyperthyroid rats serum T_4 levels were significantly higher than in the euthyroid group, whereas serum TSH was significantly lower. In hypothyroid rats serum T_4 was significantly lower and serum TSH significantly higher than during euthyroidism (Table 1). Free T_3 concentrations in medium aliquots were

Table 1
TSH and T_4 serum levels in euthyroid (EU), hypothyroid (HO) and hyperthyroid (HE) rats.

	EU (n=7)	HO (n=8)	HE (n=4)
TSH ng/mL	1.6 ± 0.3	13.8 ± 1.3[@]	0.4 ± 0.04[@]
T_4 nmol/L	54 ± 4	8 ± 3[@]	154 ± 14[@]

[@] : $p<0.001$ versus EU

determined by equilibrium dialysis according to the method of Sterling and Brenner (20), % FT_3 in medium was not significantly different in the 3 groups

(2.23 ± 0.16 % ; 2.28 ± 0.13 % ; 2.25 ± 0.08 %).

Thyroid hormone disappearance from medium was as published earlier (17,18). Two components of disappearance were present. The fast component (FC) is determined for a minor part by distribution through the perfusion system and the extracellular liver compartment and represents mainly uptake into the cellular compartment. The slow component (SC) represents metabolism in the liver. Two $t_{1/2}$-values were calculated from the two components of the curve of each experiment.

Table 2

Half times of the fast (FC) and slow components (SC) of T3 disappearance from the medium using livers of fed and 48 h fasted euthyroid (EU), hypothyroid (HO) and hyperthyroid (HE) rats, medium with 1 % BSA.

	$t_{1/2}$ FC (min)	$t_{1/2}$ SC (min)
Fed:		
EU (n=7)	1.32 ± 0.08	67.8 ± 4.5
HO (n=8)	1.40 ± 0.09	99.9 ± 7.8[@]
HE (n=4)	2.10 ± 0.16[@]	65.5 ± 5.9
Fasted:		
EU (n=3)	2.55 ± 0.2[@]	105.6 ± 8.7[@]
HO (n=3)	2.15 ± 0.18[@]	126.5 ± 10.2[@]
HE (n=5)	1.41 ± 0.10	71.3 ± 4.9

Mean ± SD, [@]: $p<0.001$ versus EU fed.

Fed livers-Transport: The $t_{1/2}$ FC of medium disappearance using hypothyroid fed livers was not significantly different from that of euthyroid livers, whereas with hyperthyroid fed livers it was prolonged (Table 2), representing a decreased transport of T_3 into the liver. For hypothyroid fed livers, other transport parameters as fractional transfer rate constants for influx (k_{21}) and efflux (k_{12}) were also the same as in euthyroid livers. In hyperthyroid livers, however, k_{21} and k_{12} were diminished in the same direction ($p<0.001$),

resulting in an unchanged ratio (Table 3).

Table 3
Fractional transfer rate constants of influx (k_{21}) and efflux (k_{12}) of T_3 transport in livers of fed and 48 h fasted euthyroid (EU), hypothyroid (HO) and hyperthyroid (HE) rats and their ratio, medium with 1 % BSA.

	k_{21} (min^{-1})	k_{12} (min^{-1})	k_{21}/k_{12}
Fed:			
EU (n=7)	0.38 ± 0.03	0.14 ± 0.02	2.7 ± 0.3
HO (n=8)	0.37 ± 0.03	0.14 ± 0.03	2.6 ± 0.4
HE (n=4)	0.25 ± 0.04[@]	0.09 ± 0.01[@]	2.8 ± 0.4
Fasted:			
EU (n=3)	0.20 ± 0.02[@]	0.17 ± 0.02	1.2 ± 0.1[@]
HO (n=3)	0.24 ± 0.02[@]	0.17 ± 0.01	1.4 ± 0.1[@]
HE (n=5)	0.34 ± 0.03[@@]	0.15 ± 0.02	2.4 ± 0.3

Mean ± SD, [@]: $p<0.001$ versus EU fed, [@@]: $p<0.005$ versus HE fed.

A decreased k_{21} in hyperthyroid livers represents a diminished fractional transport into the liver. This could theoretically be caused by diminished transport to the extracellular, non-metabolizing, liver compartment or by diminished transport into the intracellular liver compartment or both. Recently, we published a method to discriminate between transport to the extracellular and intracellular liver pool (17,18). In these studies we showed that albumin influenced only transport to the extracellular compartment of the liver and not to the intracellular compartment. To investigate transport to the extracellular and intracellular compartment of the liver, we performed liver perfusions with 1 and 4 % BSA added to the medium. The results in Table 4 show that transport to the extracellular compartment of the liver was the same in livers of euthyroid and hyperthyroid rats, whereas transport into the intracellular compartment was decreased in livers of hyperthyroid rats.

Fed livers-Metabolism: The $t_{1/2}$ SC of T_3 medium disappearance was prolonged using livers of hypothyroid rats compared to euthyroid livers, whereas that of hyperthyroid rats was not significantly different compared to euthyroid livers (Table 2).

Table 4

Mean TT_3 in the medium (nmol/L), mean FT_3 in the medium (pmol/L), TMT (pmol/60 min), TMT/FT_3 (pmol per pmol/L free T_3), and transport to intracellular liver pool/FT_3 (=TrI) and extracellular liver pool/FT_3 (=TrE) (both pmol per pmol/L free T_3) in euthyroid (EU) and hyperthyroid (HE) livers perfused with medium with 1 and 4 % BSA.

	EU (n=7) 1 % BSA	EU (n=7) 4 % BSA	HE (n=4) 1 % BSA	HE (n=4) 4 % BSA
Mean TT_3	0.6 ± 0.04	3.5 ± 0.2	0.6 ± 0.04	3.7 ± 0.4
Mean FT_3	13.4 ± 0.9	24.7 ± 1.1	13.5 ± 0.9	25.8 ± 1.7
TMT	1293 ± 96	5995 ± 212	865 ± 87[@]	4974 ± 323[@]
TMT/FT_3	96.5 ± 6.8	242.7 ± 11.2	64.1 ± 5.7[@]	192.8 ± 13.9[@]
TrI/FT_3	47.7 ± 4.3	47.7 ± 4.3	21.2 ± 2.6[@]	21.2 ± 2.6[@]
TrE/FT_3	48.7 ± 3.3	195.0 ± 11.7	42.9 ± 4.7	171.6 ± 16.8

Mean ± SD, [@] : $p<0.001$ versus EU with corresponding BSA concentration

After uptake into the liver cells, T_3 is conjugated to T_3 glucuronide or, prior to deiodination, to T_3 sulfate (9). T_3 glucuronide normally appears mostly in the bile, T_3 sulfate is subsequently deiodinated and the end product, iodide, is excreted mostly to the medium (17). Results of analysis of medium and bile are depicted in Table 5. In hypothyroid livers iodide excretion was diminished compared to euthyroid livers ($p<0.001$), in hyperthyroid livers iodide production was not significantly different from euthyroid livers. Glucuronide excretion in medium and bile in euthyroid livers was the same as in hypothyroid and hyperthyroid livers. Sulfate excretion in medium and bile was diminished in hypothyroid livers ($p<0.05$), and unaffected in hyperthyroid livers. In accordance with these findings, the fractional disposal rate constant (k_{02}) and the integrated T_3 disposal during 60 minutes, i.e. the total amount of metabolized T_3, were decreased in hypothyroid ($p<0.001$) and unchanged in hyperthyroid livers (Table 6). The availability of T_3 for the intracellular

Table 5
Iodide (I^-), T_3 glucuronide (T_3G) and T_3 sulfate (T_3S) excretion in medium and bile in fed and 48 h fasted euthyroid (EU), hypothyroid (HO) and hyperthyroid (HE) livers, medium with 1 % BSA.

	I^- % of dose	T_3G % of dose	T_3S % of dose
Fed:			
EU (n=7)	12.8 ± 1.0	13.6 ± 1.7	3.5 ± 0.2
HO (n=8)	5.1 ± 1.1$^{\#\#\#}$	13.3 ± 1.5	2.6 ± 0.9$^{\#}$
HE (n=4)	12.3 ± 0.5	13.7 ± 2.8	3.1 ± 0.5
Fasted:			
EU (n=3)	12.1 ± 1.2	9.0 ± 1.1$^{\#\#}$	4.0 ± 0.4
HO (n=3)	3.4 ± 0.8$^{\#\#\#,@}$	10.8 ± 1.2$^{\#}$	2.4 ± 0.8$^{\#\#}$
HE (n=5)	11.2 ± 3.4	14.8 ± 1.5	4.1 ± 0.7

Mean ± SD, $^{\#}$: $p<0.05$ versus EU fed, $^{\#\#}$: $p<0.01$ versus EU fed, $^{\#\#\#}$: $p<0.001$ versus EU fed, $^{@}$: $p<0.05$ versus HO fed.

metabolizing enzymes is among other things influenced by the mass transfer of T_3 into the intracellular liver compartment during the experiment (see discussion). Therefore, the disposal of T_3 in the 3 groups has to be corrected for possible differences in intracellular mass transport (= metabolic capacity). Metabolic capacity was decreased in hypothyroid ($p<0.001$) and increased in hyperthyroid livers ($p<0.001$) (Table 6).

Fasted livers-Transport: The $t_{1/2}$ FC of medium disappearance using euthyroid fasted or hypothyroid fasted livers was prolonged compared to that of euthyroid fed livers, whereas with hyperthyroid fasted livers there was no significant difference (Table 2). In accordance with these findings, k_{21} was, compared to euthyroid fed livers, diminished after fasting in euthyroid livers ($p<0.001$), and in hypothyroid livers ($p<0.001$), whereas k_{12} was unchanged. Compared to euthyroid fed livers, both k_{21} and k_{12} were not significantly different in fasted hyperthyroid livers, and thus increased after fasting versus fed hyperthyroid livers ($p<0.005$ versus hyperthyroid fed) (Table 3).

Table 6
Fractional disposal rate (k_{o2}), disposal and metabolic capacity (metab. cap. = disposal/intracell. mass transfer) of livers of fed and 48 h fasted euthyroid (EU), hypothyroid (HO) and hyperthyroid (HE) rats, medium with 1 % BSA.

		k_{o2} min^{-1}	disposal pmol	metab. cap. % of EU fed
Fed:				
EU	(n=7)	0.016 ± 0.001	155.2 ± 20.4	100 ± 10.8
HO	(n=8)	0.013 ± 0.001$^{\#\#}$	109.7 ± 10.7$^{\#\#}$	70.6 ± 7.9$^{\#\#}$
HE	(n=4)	0.016 ± 0.001	158.0 ± 9.4	228.1 ± 18.6$^{\#\#}$
Fasted:				
EU	(n=3)	0.012 ± 0.001$^{\#\#}$	102.9 ± 8.7$^{\#}$	76.8 ± 3.3$^{\#}$
HO	(n=3)	0.011 ± 0.001$^{\#\#,@}$	85.0 ± 5.6$^{\#\#,@}$	48.6 ± 5.1$^{\#\#,@}$
HE	(n=5)	0.016 ± 0.001	144.7 ± 4.6	89.8 ± 9.6

Mean ± SD, $^{\#}$: $p<0.005$ versus EU fed, $^{\#\#}$: $p<0.001$ versus EU fed, $^{@}$: $p<0.025$ versus HO.

Fasted livers-Metabolism: The $t_{1/2}$ SC of medium disappearance using euthyroid fasted and hypothyroid fasted livers was prolonged compared to that of euthyroid fed livers, whereas with hyperthyroid fasted livers it was not significantly different (Table 2). As is shown in Table 5, after fasting iodide production in euthyroid livers did not change. In hypothyroid livers, this production was diminished after fasting compared to euthyroid fed livers ($p<0.001$) and hypothyroid fed values ($p<0.05$). In hyperthyroid fasted livers the amount of iodide excreted was not significantly different from euthyroid fed and hyperthyroid fed livers. Glucuronide production after fasting was decreased in euthyroid livers ($p<0.01$ versus euthyroid fed), and in hypothyroid livers ($p<0.05$ versus euthyroid fed and hypothyroid fed), but not in hyperthyroid livers. In euthyroid livers sulfate production after fasting was unchanged, in fasted hypothyroid livers it was decreased ($p<0.01$ versus euthyroid fed, NS versus hypothyroid fed), whereas it was unaffected in hyperthyroid fasted livers (NS versus hyperthyroid fed and euthyroid fed). In Table 6 it is shown that

after 48-h fasting the T_3 disposal was decreased in euthyroid ($p<0.005$ versus euthyroid fed), and in hypothyroid ($p<0.001$ versus euthyroid fed, NS versus hypothyroid fed), but not in hyperthyroid livers (NS versus euthyroid and hyperthyroid fed). After fasting disposal corrected for differences in intracellular mass transport was decreased in euthyroid livers ($p<0.005$ versus euthyroid fed), and in hypothyroid livers ($p<0.001$ versus euthyroid fed, $p<0.05$ versus hypothyroid fed), but not in hyperthyroid livers (NS versus euthyroid fed, decreased with $p<0.001$ versus hyperthyroid fed).

At last, in Table 7 a summary of all changes in liver transport and metabolism of T_3 during hypo- and hyperthyroidism, before and after 48-h fasting, is shown.

Table 7
Changes in transport and metabolism of T_3 in livers of eu-, hypo-, and hyperthyroid rats.

	transport	metabolism
Fed:		
EU	-	-
HO	-	▼
HE	▼	▲
Fasted:		
EU	▼	▼
HO	▼	▼▼
HE	-	-

- = no changes versus EU fed, ▼ = decreased versus EU fed, ▲ = increased versus EU fed.

DISCUSSION

Analysis of thyroid hormone kinetics in hypo- and hyperthyroidism has been the subject of many investigations. However, the adaptation of thyroid

hormone handling to a changed thyroid hormone state is not fully understood. The metabolic rate of thyroid hormone during hyperthyroidism in humans has both been reported to be decreased (6) and increased (7), while in vitro investigations in rat liver homogenates pointed to an increased T_4-5'-deiodinase activity (4,5). This increased deiodination has been attributed to changes in 5'-deiodinase activity (22,23), or to changes in both 5'-deiodinase activity and in cytosolic cofactor (24). In hypothyroid humans, the fractional conversion rate of T_4 to T_3 is increased (7,8,25). However, studies using rat liver homogenates and liver slices showed decreased hepatic 5'-deiodinase activities (23). Increased total fractional conversion rate of T_4 to T_3 may be explained by increased deiodinase activity in the brain during hypothyroidism (26).

In most in vitro measurements, it was not possible to consider the role of active transport over the cell membrane. Studies in rat hepatocytes have shown transport of thyroid hormones to be rate limiting in total cellular uptake and metabolism (27). Also, studies in vivo in a human subject with a liver T_4 transport syndrome (16) and studies in the perfused fasted rat liver (28) showed that T_3 production out of T_4 was decreased because of diminished liver uptake of T_4, rather than as a result of impaired conversion of T_4 to T_3. Thus, changes in transport may be of importance in the regulation of the ultimate conversion of thyroid hormones.

Fed livers: In livers of hypothyroid rats transport parameters are not significantly altered, i.e. k_{21} and k_{12} are not significantly different compared to those in euthyroid livers. As for metabolism, iodide production is diminished in hypothyroid livers, in accordance with the already mentioned in vitro findings (23). When in liver T_3 metabolism only deiodination is inhibited, T_3 conjugate concentration (especially T_3S) will rise, because T_3 has to be sulfated before it is deiodinated by the type I deiodinase (9). This phenomenon is found in livers perfused with medium to which propylthiouracil is added (15), iodide production from T_3 was strongly inhibited by PTU, while T_3 conjugates accumulated. Also in perfused livers of amiodarone-treated rats, we recently found a decreased deiodination of T_3, whereas total metabolism was the same as that of control livers, as T_3 to T_3S conversion continued normally and therefore T_3S concentration was much higher than in control livers (15).

In the hypothyroid livers T_3S production was 2.59 ± 0.92 % dose, and in euthyroid livers 3.48 ± 0.15 % dose ($p<0.05$). This decrease of sulfation, instead of the expected increase due to the impaired deiodination, points to an inhibited sulfation as well. This is in accordance with a decreased total disposal in hypothyroid livers, whereas it was not in fed livers after PTU and amiodarone.

It has been reported that nuclear T_3 in eu-, hypo- and hyperthyroid livers is derived mainly from plasma, in spite of present local T_4 to T_3 conversion (3,29). In hypothyroidism total and free plasma T_3 concentrations are decreased, thereby decreasing the supply of hormone to the liver. In case of unchanged transport but decreased metabolism, T_3 degradation in the liver will slow down (metabolic capacity is 70 % of that of euthyroid fed livers), and more intracellular T_3 will be available for binding to the nuclear receptors, as the intracellular hormone concentration is determined by 1) the plasma (medium) flow rate, 2) the plasma (medium) concentration of hormone and hormone binding proteins, 3) the rate constant for hormone dissociation from its binding proteins, 4) rebinding to its binding proteins, 5) influx rate constant into the cell, 6) efflux rate constant and 7) metabolic rate constant (30). As the first 4 factors are constant in our liver perfusion system, only the ratio of influx (k_{21}) to efflux (k_{12}) rate constant and the metabolic rate constant (k_{02}) determine the intracellular hormone concentration. The observed effects of hypothyroidism on liver uptake and metabolism of T_3 might favour tissue euthyroidism despite the hypothyroid state.

In hyperthyroid livers, the ratio of fractional influx rate (k_{21}) versus efflux rate (k_{12}) was unchanged. However, both k_{21} and k_{12} are decreased as compared to euthyroid livers, whereas the medium pool is the same for both livers, leading to a decreased total mass transfer into the intracellular liver compartment, which may subsequently diminish intracellular availability for binding to the nuclear receptors, as discussed above. This is also shown by the fact that metabolism corrected for differences in intracellular mass transport (metabolic capacity) was increased to 230 % of that of euthyroid fed livers. The cause of the decreased mass transfer and influx rate constant is as yet not known. Synthesis of hepatic mitochondrial ATP is increased during hyperthyroidism, increasing the availability of ATP for energy-consuming

processes. However, in vivo the energy state is reduced in the hyperthyroid liver, reflecting a new balance between increased ATP synthesis and even more increased consumption (31). This reduced ATP availability may be the cause of the found decreased transport of thyroid hormone into the hyperthyroid liver. Jennings et al. (32), concerning T_4 uptake and metabolism in the perfused rat liver, also found a decrease in the hepatic uptake of T_4 in thyrotoxicosis, opposite to an increase in deiodination, in agreement with our findings. As for the increased for transport corrected conjugation activity in hyperthyroid livers, at present there are to our knowledge no other studies published in which hepatic conjugation of thyroid hormones during hyperthyroidism has been investigated. However, many other hepatic enzymatic processes, including e.g. glucuronidation of several substances, show an increased activity during hyperthyroidism (33-36). The attenuated transport during hyperthyroidism protects the liver at least partially against tissue hyperthyroidism, and together with the increased metabolism this will result in a decrease of hepatic nuclear availability of T_3. A decreased transport and an increased degradation of T_3 in the liver are favourable adaptations to high serum T_3 concentrations during hyperthyroidism, because due to decreased transport less T_3 will enter the cell and because of increased degradation of T_3 less intact T_3 will be available for binding to the nuclear receptors. This is in concert with our findings in isolated rat hepatocytes, where inhibition of transmembrane transport by ouabain resulted in inhibition of nuclear binding (37).

Fasted livers: Fasting results in a decrease of both the fractional influx rate constant (k_{21}) and total mass transfer into the intracellular liver compartment and subsequent metabolism of T_3 in euthyroid perfused rat livers, as previously described (17), probably due to decrease of intracellular ATP after fasting (38). This results in a decrease of the metabolic capacity to 77 % of euthyroid fed livers. In hypothyroid fasted livers both k_{21} and metabolism are decreased compared to euthyroid and hypothyroid fed livers, whereas k_{12} was not significantly changed. Compared to euthyroid fasted livers, because of an even further decrease of metabolism after fasting in the hypothyroid liver (metabolic capacity is 50 % of that of euthyroid fed livers), availability of liver

T_3 to nuclear binding may be increased, which is favourable during hypothyroidism. Mariash et al. reported a marked overlap between the changes in rat hepatic mRNA activity profile induced by either hypothyroidism or starvation (39). This finding suggests that hypothyroidism and fasting work in the same direction with regard to this aspect of the metabolic state of the liver, which is in accordance with the fact that after fasting in the hypothyroid liver T_3 metabolism is further decreased compared to that in the hypothyroid fed liver. Transport, which is unaffected in the hypothyroid fed liver, is also decreased after fasting. It must be noted that the results of this study apply to the rat, a species which becomes hypothyroid after fasting beyond 48 h (24). Although our rats are fasted for 48 h and not longer, one has to be careful to extrapolate these results directly to other species, including humans.

In fasted hyperthyroid livers, transport is increased as compared to fed hyperthyroid livers and in fact not different from transport in fed euthyroid livers. In other words, the decrease in transport effected by hyperthyroidism in fed livers is abolished by fasting. This effect is difficult to explain and will await further investigation, especially on factors regulating cellular transport activity. It may be related to the fact that normally by fasting tissue hypothyroidism is induced (24,40), which may counteract the effects of the already existing hyperthyroidism. Metabolic capacity, which is increased in fed hyperthyroid livers is decreased in additional fasting and comparable to T_3 metabolism in the fed euthyroid liver. It thus appears that alterations in transport and metabolism induced by hyperthyroidism are normalized by fasting and these findings are in accordance with the fact that hyperthyroidism and fasting have an opposite effect on patterns of rat hepatic mRNA activity (39). So, also during fasting the availability of liver T_3 to nuclear binding show adaptations during hypo- and hyperthyroidism, which are favourable during these conditions.

In summary, the present study demonstrates normal transport and decreased metabolism in livers of hypothyroid fed rats and decreased transport and increased metabolism in livers of hyperthyroid fed rats. In livers of hypothyroid fasted rats, transport and metabolism were decreased, whereas in livers of hyperthyroid fasted rats transport and metabolism were not

significantly different from euthyroid fed livers. These changes might favour tissue euthyroidism, in spite of the altered thyroid and nutritional state, and can therefore be seen as adaptation mechanisms to these altered states at the tissue level.

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REFERENCES

1 LoPresti JS, Warren DW, Kaptein EM, Croxson MS, Nicoloff JT 1982 Urinary immunoprecipitation method for estimation of thyroxine to triiodothyronine conversion in altered thyroid states. J Clin Endocrinol Metab 55:666-670

2 Dratman MB, Crutchfield FL, Gordon JT, Jennings AS 1983 Iodothyronine homeostasis in rat brain during hypo- and hyperthyroidism. Am J Physiol 245:E185-E193

3 Van Doorn J, Roelfsema F, van der Heide D 1982 Contribution from local thyroxine to 3,5,3'-triiodothyronine to intracellular 3,5,3'-triiodothyronine in several organs of hypothyroid rats at isotope equilibrium. Acta Endocrinol (Copenh) 101:386-392

4 Kaplan MM, Utiger RD 1978 Iodothyronine metabolism in liver and kidney homogenates from hyperthyroid and hypothyroid rats. Endocrinology 103:156-161

5 Grussendorf M, Hufner M 1977 Induction of the thyroxine to triiodothyronine converting enzyme in rat liver by thyroid hormones and analogs. Clin Chim Acta 80:61-66

6 Shimizu TC, Pittman CS, Chambers JJB, Buck MW, Thurston CC 1976 The effect of thyroxine on the periferal conversion rate of thyroxine to triiodothyronine in man. In: Robbins J, Braverman LE (eds) Thyroid Research, Proceedings of the seventh international thyroid conference, American Elsevier, New York, p 263

7 Maguire SB, Dennehy A, Cullen MJ 1976 The effect of thyrotoxicosis and hypothyroidism on the extrathyroidal on the extrathyroidal conversion of thyroxine to triiodothyronine in man. In: Robbins J, Braverman LE (eds) Thyroid Research, Proceedings of the seventh international thyroid conference, American Elsevier, New York, p 259

8 Inada M, Kanji K, Kurata S, Kazama Y, Takayama H, Torizuka K, Fukase M, Soma T 1975 Estimation of thyroxine and triiodothyronine distribution and of the conversion rate of thyroxine to triiodothyronine in man. J Clin Invest 55:1337-1344

9 Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid hormone metabolism. Marcel Dekker, New York. pp 189-229

10 Visser TJ, Van der Does-Tobe I, Docter R, Hennemann G 1975 Conversion of thyroxine into triiodothyronine by rat liver homogenate. Biochem J 150:489-493

11 Visser TJ, Kaplan MM, Leonard JL, Larsen PR 1983 Evidence for two pathways of iodothyronine 5'-deiodination in rat pituitary that differ in kinetics, propylthiouracil sensitivity, and response to hypothyroidism. J Clin Invest 71:992-1002

12 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314-320

13 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1982 Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229-233

14 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett 91:113-116

15 De Jong M, Docter R, Van der Hoek HJ, Krenning EP, Van der Heide D, Quero C, Plaisier P, Vos RA, Hennemann G 1993 Different effects of amiodarone on transport of T_4 and T_3 into the perfused rat liver. Accepted for publication in Am J Physiol

16 Hennemann G, Vos RA, De Jong M, Krenning EP, Docter R 1992 Decreased peripheral T_3 production from T_4; A syndrome of impaired thyroid hormone activation due to transport inhibition of T_4 into T_3 producing tissues. In press (J Clin Endocrinol Metab)

17 Docter R, de Jong M, van der Hoek HJ, Krenning EP, Hennemann G 1990 Development and use of a mathematical two pool model of distribution and metabolism of 3,3',5-triiodothyronine in a recirculating rat liver perfusion system: albumin does not play a role in cellular transport. Endocrinology 126:451-459

18 De Jong M, Docter R, van der Hoek HJ, Vos RA, Krenning EP, Hennemann G 1992 Transport of T_3 and subsequent metabolism in the perfused rat liver are inhibited by fasting. Endocrinology 131:463-470

19 Meijer KF, Keulemans K, Mulder GJ 1981 Isolated perfused rat liver technique. Methods in Enzymology 77:81-93

20 Sterling K, Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J Clin Invest 45:153-163

21 Snedecor GW, Cochran WG 1967 One-way classifications. Analysis of variance. In: Statistical Methods, 6th ed. 258-298. The Iowa Univ. Press, Ames, Iowa USA

22 Harris ARC, Fang SL, Hinderfeld L, Braverman LE, Vagenakis AG 1979 The role of sulphydryl groups on the impaired hepatic triiodothyronine generation from thyroxine in the hypothyroid, starved, fetal, and neonatal rodent. J Clin Invest 63:516-522

23 Kaplan MM 1979 Changes in the particulate subcellular component of hepatic thyroxine-5'-monodeiodinase in hyperthyroid and hypothyroid rats. Endocrinology 105:548-554

24 Balsam A, Sexton F, Ingbar SH 1981 The influence of fasting and the thyroid state on the activity of thyroxine 5'-monodeiodinase in rat liver: a kinetic analysis of microsomal formation of triiodothyronine from thyroxine. Endocrinology 108:472-477

25 Lum SMC, Nicoloff JT, Spencer CA, Kaptein EM 1984 Peripheral tissue mechanism for maintenace of serum triiodothyronine values in a thyroxine-deficient state in man. J Clin Invest 73:570-575

26 Kaplan MM, Yaskoski KA 1982 Effects of congenital hypothyroidism and partial and complete food deprivation on phenolic and tyrosyl ring iodothyronine deiodination in rat brain. Endocrinology 110:761-767

27 Hennemann G, Krenning EP, Polhuis M, Mol JA, Bernard HF, Visser TJ, Docter R 1986 Carrier-mediated transport of thyroid hormones into rat hepatocytes is rate limiting in total cellular uptake. Endocrinology 119:1870-1872

28 Jennings AS, Duncan CF, Utiger RD 1979 Regulation of the conversion of thyroxine to triiodothyronine in the perfused rat liver. J Clin Invest 64:1614-1623

29 Van Doorn J, Van der Heide D, Roelfsema F 1984 The contribution of local thyroxine monodeiodination to intracelluiar 3,5,3'-triiodothyronine in several tissues of hyperthyroid rats at isotopic equilibrium. Endocrinology 115:174-182

30 Mendel CM, Cavalieri RR, Weisiger RA 1988 Uptake of thyroxine by the perfused rat liver: implications for the free hormone hypothesis. Am J Physiol 255:E110-E119

31 Muller MJ, Seitz HJ 1984 Thyroid hormone action on intermediary metabolism. Part I: respiration, thermogenesis and carbohydrate metabolism. Klin-Wochenschrift 62:11-18

32 Jennings AS, Crutchfield FL, Dratman MB 1984 Effect of hypothyroidism and hyperthyroidism on triiodothyronine production in perfused rat liver. Endocrinology 114:992-997

33 Kosuge T, Beppu T, Kodama T, Hidai K, Idezuki Y 1987 Serum bile acid profile in thyroid dysfunction and effect of medical treatment. Clinical Science 73:425

34 Muller MJ, Seitz HJ 1984 Thyroid hormone action on intermediary metabolism. Part II: Lipid metabolism in hypo- and hyperthyroidism. Klin-Wochenschrift 62:49-55

35 Fernandez V, Simizu K, Barros SB, Azzalis LA, Pimentel R, Junqueira VB, Videla LA 1991 Effects of hyperthyroidism on rat liver glutathione metabolism: related enzymes' activities, efflux and turnover. Endocrinology 129:85-91

36 O'Connor P, Feely J 1987 Clinical pharmacokinetics and endocrine disorders. Therapeutic implications. Clinical Pharmacokinetics 13:345-364

37 Hennemann G, Krenning EP, Bernard B, Huvers F, Mol J, Docter R, Visser TJ 1984 Regulation of influx and efflux of thyroid hormones in rat hepatocytes: Possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity. Horm Metab Res 14:1-6

38 Cunningham CC, Malloy CR, Radda GK 1986 Effect of fasting and acute ethanol administration on the energy state of in vivo liver as measured by 31P-NMR

spectroscopy. Biochim Biophys Acta 885:12-22

39 Mariash CN, Oppenheimer JH 1985 Interaction of thyroid hormone and nutrional signals on thyroid hormone action. Molec Cell Endocrinol 43:3-13

40 Chopra IJ 1980 Alterations in monodeiodination of iodothyronine in the fasting rat: Effects of reduced nonprotein sulfhydryl groups and hypothyroidism. Metabolism 29:161-167

CHAPTER 5

DIFFERENT EFFECTS OF AMIODARONE ON TRANSPORT OF T_4 AND T_3 INTO THE PERFUSED RAT LIVER.

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ABSTRACT

Uptake and metabolism of T_4 and T_3 were studied in isolated perfused livers of control and amiodarone-treated (40 mg/kg BW/day, 22 days) rats. Using this perfusion system and a two-pool model describing thyroid hormone kinetics, total uptake was evaluated by the $t_{1/2}$ of the fast component of the biphasic thyroid hormone disappearance from the medium and by k_{21}, the fractional influx rate constant. Metabolism was assessed by the $t_{1/2}$ of the slow component, by determination of breakdown products in medium and bile and by thyroid hormone disposal according to the two-pool model. Disposal was corrected for differences in mass transfer into the metabolizing pool. In amiodarone-treated rats both uptake and metabolism of T_4 were decreased. Furthermore, it was shown that only transport into the metabolizing liver compartment and not uptake in the non-metabolizing liver compartment was decreased. Both uptake and total metabolism of T_3 were unaffected by amiodarone.

The results showed that the different transport systems for T_4 and T_3, described in isolated rat hepatocytes, may also be operative in the intact rat liver. Furthermore, it can be concluded that the low T_3 syndrome caused by treatment with amiodarone may be due to both impaired transport and impaired 5'-deiodination.

INTRODUCTION

Amiodarone, 2-n-butyl-3-[4-(2-diethylaminoethoxy)3,5-diiodobenzoyl]-benzo-furan, is used as an anti-arrhythmic drug. It contains 39.4 % iodine on a weight basis and bears structural resemblance to thyroid hormones (3). Changes in clinical thyroid function were recognized soon after the introduction of this drug (8) and alterations in serum levels of iodothyronines, not reflecting changes in thyroid activity, have been described as well (9,10,19,23). Volunteers, receiving short-term administration of amiodarone, developed a low-T_3 syndrome with high total and free serum T_4 and rT_3, and low total and free T_3. Furthermore, the rate of thyroxine disappearance from serum was reduced compared to normal (12,16). However, it is not clear if this diminution

in serum T_4 clearance and the decrease in serum T_3 concentration is due to a reduction in T_4 to T_3 conversion by amiodarone (16,17,21), in which process the liver plays an important role in vivo (11,18), or to disturbances in other regulatory mechanisms, e.g. uptake of T_4 into the liver.

We have demonstrated the presence of two specific energy dependent cellular uptake mechanisms (one for T_4 and rT_3, and one for T_3) in rat hepatocytes in primary culture (13,14,15). These observations led us to consider the possibility that amiodarone inhibits transmembrane transport of T_4 into the liver, leading to a reduced availability in the cell and thereby to a reduced conversion to T_3. This latter possibility will ultimately lead to a low plasma T_3, as extrathyroidal conversion of T_4 accounts for up to 80 % of plasma T_3 in control situation. After amiodarone treatment this value is even more than 95 %, as amiodarone has been reported to inhibit T_3 production by the thyroid (25). In vitro studies indicated an impaired uptake of T_4 into isolated rat hepatocytes caused by amiodarone (1,15), but this was an acute effect of amiodarone and does not necessarily reflect the effects of longer treatment in vivo. In acute studies only the effects of amiodarone itself are measurable, and not the effects of possibly bioactive metabolites, as desethylamiodarone.

We investigated therefore the effects of 22 days of amiodarone administration to rats on transmembrane transport systems of T_4 and T_3 and subsequent metabolism of both hormones in a recirculating rat liver perfusion system. Using this system and employing a two-compartment model describing thyroid hormone kinetics, it is possible to investigate both uptake and metabolic processes in the liver. Furthermore, it is also possible to discriminate between transport to a non-metabolizing, presumably extracellular liver compartment and transport into an intracellular, metabolizing liver compartment (4,6).

We also performed some studies in livers of control rats with 6-propyl-thiouracil (PTU) added to the medium, because of the known inhibitory effects of PTU on the type I 5'-deiodinase (18), in order to compare them with the effects of amiodarone.

MATERIALS AND METHODS

Animals: Male Wistar rats, weighing 300-350 g were used in all experiments. Amiodarone-treated rats received the drug (40 mg/kg BW/day, 22 days) added to their diet. This diet, recommended by the American Institute of Nutrition (2), consisted of a semisynthetic, powdered substance, which was mixed with water to a homogeneous paste. The control rats received potassium iodide in their food, in an amount similar to the amount of iodide released from amiodarone (0.3 mg/day), to ensure an iodide intake similar to that of the rats receiving amiodarone. Control and amiodarone-treated rats exhibited a normal increase in body weight (control 2.5 ± 0.3 g/day; amiodarone 2.4 ± 0.4 g/day).

See further Chapter 2.

RESULTS

After 22 days of amiodarone administration serum total T_4 in the rats increased from 54.0 ± 4.7 to 74.2 ± 11.5 nmol/l ($n=10$, $p<0.01$), whereas serum T_3 decreased from 1.32 ± 0.17 to 1.07 ± 0.16 nmol/l ($n=10$, $p<0.05$). The serum free fraction of T_4 remained virtually the same (0.025 ± 0.001 % and 0.026 ± 0.001 %). % FT_4 in the perfusion medium with 1 % BSA was not significantly different in the control and amiodarone group (0.29 ± 0.03 % and 0.29 ± 0.04 %, respectively), and also % FT_3 in medium with 1 % BSA was not significantly different in the 3 groups (control, amiodarone and PTU: 2.18 ± 0.21 %, 2.05 ± 0.22 % and 2.28 ± 0.33 %, respectively). Liver weights were 11.9 ± 0.8 g for the control group and 11.8 ± 0.7 g for the amiodarone-treated group.

Thyroid hormone disappearance from medium using livers of control and amiodarone-treated rats is shown in Figure 1A and 1B for T_4 and T_3, respectively. Two components of disappearance were present. The fast component (FC) is determined for a minor part by distribution through the perfusion system and the vascular space of the liver, but represents mainly uptake in the cellular liver compartment. The slow component (SC) represents metabolism in the liver. Two $t_{1/2}$-values can be calculated from the two components of the curve, according to the least squares regression analysis (6).

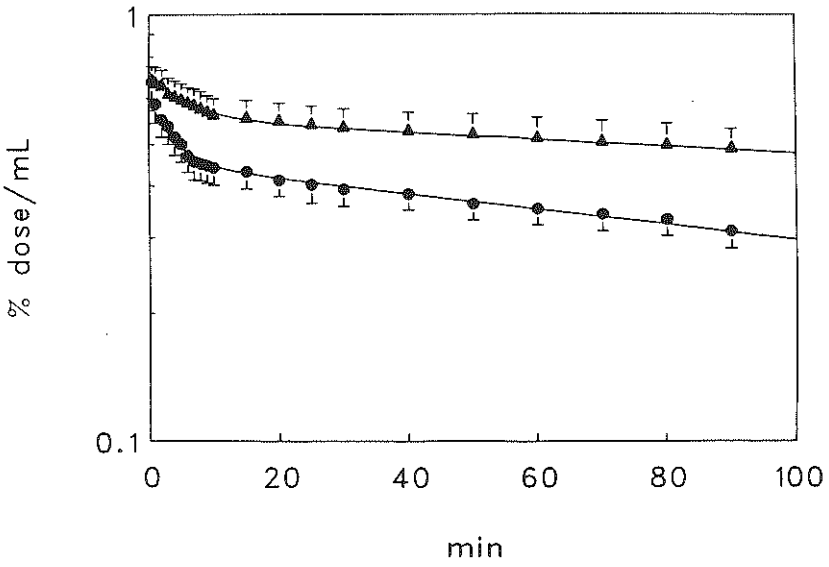

Figure 1A
Mean ^{125}I-T_4 medium disappearance curves, both for livers of control (•) and amiodarone-treated (▲) rats (mean ± SD).

Results T_4: It can be seen that in experiments with livers of amiodarone-treated rats T_4 disappearance from the medium is slower than with control livers (Figure 1A). The $t_{1/2}$ FC of T_4 medium disappearance was prolonged to 122 % ($p<0.01$) using livers of amiodarone treated rats compared to $t_{1/2}$ FC using livers of control rats (Table 1), representing a decreased uptake of T_4 into the liver. The $t_{1/2}$ SC was also prolonged after amiodarone ($p<0.001$).

Several other parameters of T_4 uptake, the fractional influx and efflux rate constants, k_{21} and k_{12}, were calculated according to the two-pool model. After amiodarone treatment, k_{21} is decreased to 71 % ($p<0.005$) of the control value, whereas k_{12} was unchanged (Table 1). This is in accordance with the prolongation of the $t_{1/2}$ FC, because a decreased k_{21} (with unchanged k_{12}) and a prolonged $t_{1/2}$ FC both represent a diminished uptake in the liver. This observed diminution of uptake could theoretically be caused by diminished uptake in the non-metabolizing liver compartment or by diminished transport

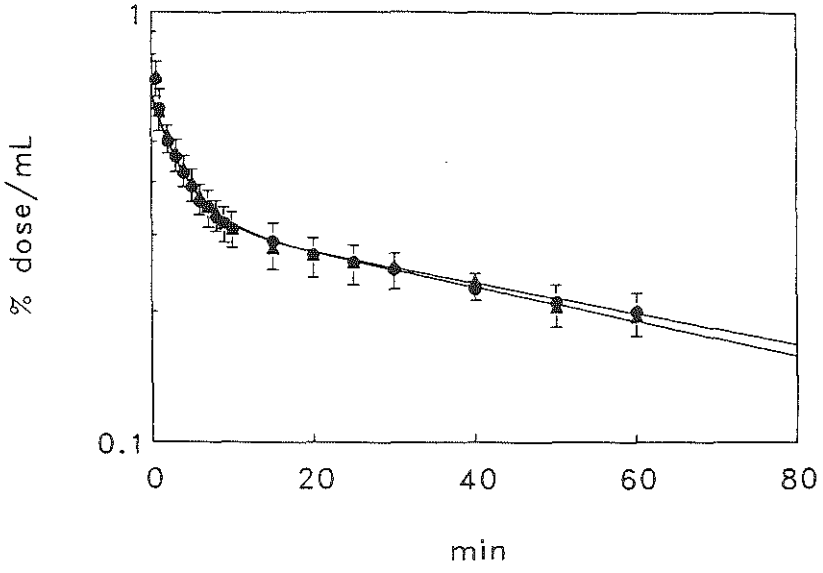

Figure 1B:
Mean ^{125}I-T_3 medium disappearance curves, both for livers of control (•) and amiodarone-treated (▲) rats (mean ± SD).

into the metabolizing liver compartment or both. Recently, we published a method to discriminate between transport to the non-metabolizing and metabolizing liver compartment (4-6). In these studies we showed that albumin influenced only uptake in the non-metabolizing compartment of the liver and not to the metabolizing compartment. To investigate the influence of amiodarone on uptake in the non-metabolizing and metabolizing compartment of the liver, we performed liver perfusions with 1 and 4 % BSA added to the medium (see methods). The results in Table 2 show that uptake in the non-metabolizing compartment of the liver was the same in control livers and livers of amiodarone-treated rats, whereas transport into the metabolizing compartment was decreased in livers of amiodarone-treated rats ($p<0.001$).

After uptake in liver cells, thyroid hormones can be conjugated to glucuronides or sulfates (22). The glucuronides are normally excreted into bile, whereas the sulfates are excreted into the medium or further deiodinated (6).

Table 1

Parameters of transport ($t_{1/2}$ fast component (FC), k_{21}) and metabolism ($t_{1/2}$ slow component (SC), disposal, metabolic capacity (M.C., i.e. disposal/mass transfer into the metabolizing compartment)) of 90 min T_4 and 60 min T_3 handling in livers of control (C) and amiodarone (A)-treated rats.

parameter	T_4C (n=5)	T_4A (n=6)	T_3C (n=6)	T_3A (n=4)
$t_{1/2}$ FC (min)	3.03 ± 0.3	3.78 ± 0.2$^{@}$	2.13 ± 0.3	2.14 ± 0.3
k_{21} (min^{-1})	0.083 ± 0.01	0.055 ± 0.005$^{@@}$	0.25 ± 0.05	0.25 ± 0.05
k_{12} (min^{-1})	0.13 ± 0.02	0.14 ± 0.02	0.09 ± 0.01	0.09 ± 0.01
$t_{1/2}$ SC (min)	179.5 ± 18.3	262.0 ± 24.2$^{@@@}$	88.9 ± 11.1	85.0 ± 10.3
disposal (pmol)	292.4 ± 22.9	208.7 ± 22.3$^{@@@}$	127.9 ± 12.5	135.5 ± 13.1
M.C. (% of C)	100 ± 12.0	87.6 ± 8.9	100 ± 10.9	104.2 ± 7.3

$^{@}$: p<0.01 compared to controls, $^{@@}$: p<0.005 compared to controls, $^{@@@}$: p<0.001 compared to controls, mean (SD), medium with 1 % BSA.

Table 2

Mean free (F) T_4 in the medium, total mass transfer (TMT), TMT/FT_4, and transport to metabolizing liver pool/FT_4 (=TM) and non-metabolizing liver pool/FT_4 (=TNM) in 90 min in control (C) and amiodarone-treated (A) livers perfused with medium with 1 and 4 % BSA.

	C (n=5) 1 % BSA	C (n=5) 4 % BSA	A (n=6) 1 % BSA	A (n=6) 4 % BSA
Mean FT_4 (pmol/L)	10.86 ± 0.82	16.66 ± 1.23	12.10 ± 0.98	18.19 ± 1.16
TMT (pmol)	4738.6 ± 320.2	21590.8 ± 754.2	4406.4 ± 341.4	20945.8 ± 1968
TMT/FT_4 (pmol/pM)	436.3 ± 21.6	1295.9 ± 98.7	364.4 ± 16.5$^{@}$	1151.3 ± 82.5$^{@}$
TM/FT_4 (pmol/pM)	149.7 ± 11.2	149.7 ± 11.2	104.0 ± 10.3$^{@}$	104.0 ± 10.3$^{@}$
TNM/FT_4 (pmol/pM)	286.7 ± 19.6	1146.3 ± 78.5	268.6 ± 22.5	1075.2 ± 81.6

$^{@}$: p<0.001 versus control with corresponding BSA concentration, mean (SD).

Iodide, an end product of thyroid hormone deiodination, is released mostly into the medium (6). After amiodarone treatment, iodide production from T_4, calculated from LH-20 chromatography of medium and bile, appeared to be decreased from 4.1 ± 0.4 to 1.0 ± 0.2 % of dose of added T_4 (p<0.01). Conjugate accumulation was increased from 3.2 ± 0.4 to 5.9 ± 0.4 % of dose

($p<0.001$) (not depicted). The total disposal of T_4 was decreased in livers of amiodarone-treated rats ($p<0.005$, Table 1). The amount of T_4 metabolized is influenced by the amount of T_4 transferred into the metabolizing compartment. Therefore, the disposal of T_4 in the two groups has to be corrected for possible differences in transfer into the metabolizing compartment, resulting in the metabolic capacity (see Methods). This metabolic capacity was not different in amiodarone-treated and control livers (Table 1).

A decrease in T_4 deiodination was also found when PTU was added to the perfusion medium (Table 3), while T_4 conjugates increased. Uptake was not affected by PTU, as $t_{1/2}$ FC was not different from that in control experiments (Table 3).

Table 3
Parameters of transport ($t_{1/2}$ fast component (FC)) and metabolism ($t_{1/2}$ slow component (SC)), iodide and conjugate production after 60 min T_3 and 90 min T_4 handling in control livers perfused in the presence or absence of PTU (100 µmol/L) in the medium.

	T_3		T_4	
	Control	PTU	Control	PTU
$t_{1/2}$ FC (min)	2.13 ± 0.3	2.15 ± 0.3	2.60 ± 0.18	2.66 ± 0.20
$t_{1/2}$ SC (min)	88.9 ± 11.1	92.3 ± 8.6	288.3 ± 35.4	331.8 ± 34.6
iodide (% of dose)	12.8 ± 0.9	0.02 ± 0.00[@]	3.6 ± 0.3	0.4 ± 0.1[@]
conjugates (% of dose)	17.1 ± 1.2	33.4 ± 2.8[@]	2.7 ± 0.4	5.4 ± 0.5[@]

[@] : $p<0.001$ versus control, mean ± SD, n = 3-6, medium with 1 % BSA.

Results T_3: In experiments with livers of amiodarone-treated rats T_3 disappearance from the medium is the same as that using control livers (Figure 1B), $t_{1/2}$ FC and $t_{1/2}$ SC of T_3 medium disappearance are both unchanged after amiodarone treatment (Table 1). In accordance with these findings, k_{21} and k_{12} were not changed either (Table 1). Iodide production from T_3 was decreased in livers of amiodarone rats from 8.6 ± 1.9 to 2.9 ± 0.8 % of dose ($p<0.01$), but conjugates increased from 8.1 ± 0.7 to 14.5 ± 0.9 % of dose ($p<0.001$) (not depicted). Total disposal, therefore, was not changed

(Table 1).

This phenomenon is also found in experiments with PTU added to the medium (Table 3). Deiodination of T_3 was strongly inhibited by PTU, while T_3 conjugates increased in the perfusion medium. Uptake and total metabolism were not affected by PTU, as $t_{1/2}$ FC and $t_{1/2}$ SC were not significantly different from control experiments.

DISCUSSION

Patients receiving short-term treatment with amiodarone develop high total and free serum T_4 and low total and free T_3 (9,10,19,23), the so-called low T_3 syndrome. Our results obtained in the rat are in accordance with these findings; an increase of total and free T_4 and a decrease of total T_3 after 22 days amiodarone treatment was seen.

In humans, also caloric deprivation and treatment with d-propranolol elicit a low T_3 syndrome, but it appeared that the mechanisms that are the cause of the low T_3 syndrome are different. During caloric deprivation uptake of T_4 and T_3 into tissues is diminished. It was concluded that regardless of any possible change in 5'-deiodinase activity, inhibition of T_4 uptake per se contributed to low T_3 production and therefore low T_3 serum levels, due to less substrate (i.e. T_4) availability for T_3 production in tissues (27). No inhibition of T_4 or T_3 uptake in the tissues could be found during d-propranolol treatment, although the production of T_3 from T_4 was substantially diminished. It was therefore concluded that d-propranolol-induced changes in thyroid hormone metabolism resulting in a low T_3 syndrome, were due to inhibition of thyroid hormone deiodination (28). As pointed out in the introduction, it is not clear whether the reduced T_4 disappearance from plasma and the low T_3 plasma levels during amiodarone treatment as described in man, are due to a decreased metabolism per se, or to a decreased uptake of T_4 into extra-thyroidal tissues (e.g. the liver).

We recently described a regulatory role of thyroid hormone transport into the perfused rat liver in overall thyroid hormone metabolism after 48 h fasting, after perfusion with medium containing fructose and during perfusion with medium containing serum of patients with non thyroidal illness (4,5,29).

In the experiments reported here uptake of T_4 into the liver was decreased after amiodarone, as $t_{1/2}$ FC was increased and k_{21} was decreased, whereas k_{12} was not. It appeared that the decrease in total uptake was caused by an inhibition of transport to the metabolizing pool, whereas uptake in the non-metabolizing pool was unaffected after amiodarone. These data also show that discrimination between transport to the metabolizing and non-metabolizing liver pool is important, because the actual inhibition of transport to the metabolically active pool (30 %) by amiodarone is greater than expected from the data of total mass transfer to the liver (10 % inhibition).

As to the cause of the inhibition of transport of T_4 after amiodarone treatment, we have no definite explanation at this stage. At the level of the plasma membrane, transport inhibition may be effected on the basis of competition by the structural relationship between amiodarone and T_4. Other possibilities like a decrease of intracellular energy stores, plasma membrane fluidity or Na^+-K^+-ATPase activity induced by amiodarone and leading to attenuated T_4 transport may also play a role.

Iodide production from T_4 is also decreased after amiodarone treatment. This is in concert with the findings of Larsen et al. (17) and Obregon et al. (21), who described that drugs with diiodo- and triiodo-substituted benzene rings are competitive inhibitors of 5'-deiodinase in all tissues. A decreased deiodination will lead to the fact that the intracellular T_4 concentration will stay higher and therefore conjugate production will be increased. This is also the case in our experiments with PTU and amiodarone; the increase in conjugate production compensates for the reduction in deiodination caused by both substances. After amiodarone treatment, metabolic capacity, i.e. disposal corrected for differences in mass transfer into the metabolizing compartment and calculated according to the two-pool model (see methods), is not significantly affected after amiodarone treatment. These findings are in accordance with the fact that the increase in $t_{1/2}$ SC (31 %) is exactly the same as the decrease of transport into the metabolizing pool (32 %), pointing to the fact that the increase in $t_{1/2}$ SC, and thus the decrease in total metabolism, can be accounted for by an inhibition of transport into the metabolizing liver pool. This means that a decrease in 5'-deiodinase activity does not affect total disposal of T_4, underscoring the fact that transmembrane transport may be the

process that regulates intracellular availability of T_4.

The results of amiodarone on T_3 uptake in the liver, not being affected by amiodarone, are in contrast to those obtained with T_4. Total metabolism is not changed either, although deiodination is inhibited. This apparent discrepancy can again be explained by an increased conjugate accumulation (conjugates are 180 % of control values as found by LH-20 chromatography of medium, bile and liver), which counterbalances the diminutive effect of attenuated deiodination. This is due to the fact that prior to 5'-deiodination in rat liver, T_3 has to be sulfated, and therefore T_3 conjugates will accumulate when deiodination is inhibited. T_3 conversion to T_3 conjugates is apparently unaffected by amiodarone.

This phenomenon is also found in livers perfused with medium to which PTU is added (Table 3). Deiodination of T_3 is strongly inhibited by PTU, but T_3 conjugates accumulate in a compensatory manner in the perfusion medium, therefore total metabolism ($t_{1/2}$ SC) is not significantly different from control experiments. These findings are in also concert with those obtained for T_4 in this study, that inhibition of deiodination does not affect disposal of thyroid hormones in the liver, because of a compensating increase in conjugate concentration.

The differences in amiodarone effects on T_4 and T_3 uptake could be explained by the existence of two different transport systems (one for T_4 and rT_3 and one for T_3), as has been described in rat hepatocytes in primary culture (13,14,15). Both systems have the characteristics of an active transport system: ATP dependence, temperature dependence and inhibition by metabolic blockers. However, there are considerable differences between the two systems e.g. concerning K_m and V_{max} for the substrates. Furthermore, mild alterations in ATP concentration have more profound effects on T_4 than on T_3 transport (13,14,15). The effects of amiodarone on transport of different thyroid hormones suggest that the different transport systems for T_4 and T_3 as detected in vitro, are apparently also operative in the intact rat liver. Recently, we also reported evidence for 2 different transport systems for T_4 and T_3 in vivo in a human subject, suffering from a T_4 and rT_3 transport inhibition syndrome in the liver, whereas T_3 transport was not affected at all (7).

The described effects of amiodarone on thyroid hormone serum levels

may (at least in part) be explained by the observed differential effects on thyroid hormone transport systems in the liver. Thus, the increase in serum total and free T_4 may be explained by inhibition of T_4 entry into tissues by the action of amiodarone on transmembrane T_4 transport. This leads to decreased availability of T_4 for conversion into T_3 in T_3-producing tissues. Extrathyroidal T_3 production is further diminished by direct effects of amiodarone on the 5'-deiodinative process. The finding that uptake of T_3 into the liver and subsequent degradation are unaffected by amiodarone further contributes to maintenance of low plasma T_3.

The present findings from studies in rat liver, show that the low T_3 syndrome caused by treatment with amiodarone may be due to a combination of both impaired uptake and impaired 5'-deiodination.

REFERENCES

1 Aanderund, S., J. Sundsfjord, and J. Aarbakke. Amiodarone inhibits the conversion of thyroxine to triiodothyronine in isolated rat hepatocytes. Endocrinology 115: 1605-1608, 1984.

2 Ad hoc committee on standards for nutritional studies. Report of the American Institute of Nutrition. J. Nutr. 7: 1340-1348, 1977.

3 Burger, A., D. Dinichert, P. Nichod, N. Jenny, T. Lemarchand-Beraud, and M.B. Valloton. Effects of amiodarone on serum T_3, rT_3, T_4, and thyrotropin. A drug influencing peripheral metabolism of thyroid hormone. J. Clin. Invest. 58: 255-259, 1976.

4 De Jong M., R. Docter, H.J. van der Hoek, R.A. Vos, E.P. Krenning, and G. Hennemann. Transport and metabolism of T_3 into the perfused rat liver are inhibited by fasting. Endocrinology 131: 463-470, 1992.

5 De Jong, M., R. Docter, R.A. Vos, H.F. Bernard, D. Hadian, and E.P. Krenning. Transport of T_4 to an extracellular, non-metabolizing, and an intracellular, metabolizing, liver pool: influence of fructose and of serum of patients with non-thyroidal illness. J. Endocrinol. Invest. 15 :133 (abstr), 1992.

6 Docter, R., M. de Jong, H.J. van der Hoek, E.P. Krenning, and G. Hennemann. Development and use of a mathematical two pool model of distribution and metabolism of T_3 in a recirculating rat liver perfusion system: albumin does not play a role in cellular transport. Endocrinology 126: 451-459, 1990.

7 Hennemann, G., R.A. Vos, M. de Jong, E.P. Krenning, and R. Docter. Decreased peripheral T_3 production from T_4; A syndrome of impaired thyroid hormone activation

due to transport inhibition of T_4 into T_3 producing tissues. In press (J Clin Endocrinol Metab), 1993.

8 Jonckheer, M.H., P. Blockx, R. Kaivers, and G. Wyffels. Hyperthyroidism as a possible complication of the treatment of ischaemic heart disease with amiodarone. Acta Cardiol. (Brux.) 36 :192-200, 1973.

9 Jonckheer, M.H., P. Block, I. Proekaert, C. Cornette, and C. Beckers. Low T_3 syndrome in patients treated with an iodine chronically containing drug, amiodarone. Clin. Endocrinol. (Oxf) 9: 27-35, 1978.

10 Jonckheer, M.H. Amiodarone and the thyroid gland. A review. Acta Cardiol. (Brux.) 36: 199-205, 1981.

11 Kaplan, M.M., and R.D. Utiger. Iodothyronine metabolism in rat liver homogenates. J. Clin. Invest. 61: 459-471, 1978.

12 Kaptein, E.M., P.M. Egodage, M.T. Hoopes, and A.G. Burger. Amiodarone alters thyroxine transfer and distribution in humans. Metabolism 37: 1107-1113, 1988.

13 Krenning, E.P., R. Docter, H.F. Bernard, T.J. Visser, G. Hennemann. Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett. 91: 113-116, 1978.

14 Krenning, E.P., R. Docter, H.F. Bernard, T.J. Visser and G. Hennemann. Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim. Biophys. Acta 676: 314-320, 1981.

15 Krenning, E.P., R. Docter, H.F. Bernard, T.J. Visser, and G. Hennemann. Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett. 140: 229:233, 1982.

16 Lambert, M.J., A.G. Burger, R.L. Galeazzi, and D. Engler. Are selective increases in serum T_4 due to iodinated inhibitors of T_4 monodeiodination indicative of hyperthyroidism? J. Clin. Endocrinol. Metab. 55: 1058-1065, 1982.

17 Larsen, P.R., T.E. Dick, B.P. Markovitz, M.M. Kaplan, and T.G. Gard. Inhibition of intrapituitary thyroxine to 3,5,3'-triiodothyronine conversion prevents the acute suppression of thyrotropin release by thyroxine in hypothyroid rats. J. Clin. Invest. 64: 117-128, 1979.

18 Leonard, J.L., and T.J. Visser. Biochemistry of deiodination. In Thyroid hormone metabolism. Hennemann, editor. Dekker, New York and Basel. 189-229, 1986.

19 Melmed S., K. Nademanee, A.W. Reed, J.A. Hendrickson, B.N. Singh, and J.M. Hershman. Hyperthyroxinemia with bradycardia and normal thyrotropin secretion after chronic amiodarone administration. J. Clin. Metab. 53: 997-1001, 1981.

20 Meijer, D.K.F., K. Keulemans, and G.J. Mulder. Isolated perfused rat liver technique. Methods in Enzymology 77: 81-93, 1981.

21 Obregon, M.J., A. Pascual, J. Mallol, G. Morreale de Escobar, and F. Escobar del Rey. Evidence against a major role of L-thyroxine at the pituitary level: Studies in rats

treated with iopanoic acid (telepaque). Endocrinology 106: 1827-1836, 1980.

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

23 Pritchard D.A., B.N. Singh, and P.J. Hurley. Effects of amiodarone on thyroid function on patients with ischaemic heart disease. Br. Heart. J. 37: 856-860, 1975.

24 Rutgers, M., F. Bonthuis, W.W. de Herder, and T.J. Visser. Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J. Clin. Invest. 80: 758-762, 1987.

25 Schröder-van der Elst, J.P., and D. van der Heide. Thyroxine, 3,5,3'-triiodothyronine, and 3,3',5-triiodothyronine concentrations in several tissues of the rat: effects of amiodarone and desethylamiodarone on thyroid hormone metabolism. Endocrinology 127: 1656-1664, 1990.

26 Sterling, K., and M.A. Brenner. Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J. Clin. Invest. 45: 153-163, 1966.

27 Van der Heijden, J.T.M., R. Docter, H. van Toor, J.P.H. Wilson, G. Hennemann, and E.P. Krenning. Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am. J. Physiol. 251: E156-E163, 1986.

28 Van der Heijden, J.T.M., E.P. Krenning, H. van Toor, G. Hennemann, and R. Docter. Three compartmental analysis of the effects of d-propranolol on thyroid hormone kinetics. Am. J. Physiol. 255: E80-E86, 1988.

29 Vos R.A., M. de Jong, R. Docter, H. van Toor, H.F. Bernard, E.P. Krenning, and G. Hennemann. Morbidity-dependent thyroid hormone transport inhibition by serum of patients with non-thyroidal illness (NTI) in rat hepatocytes and in the perfused rat liver. In: Progress in thyroid research, Gordon, Gross and Hennemann (eds) 1991. Balkema Rotterdam. 693-696, 1991.

CHAPTER 6

T_4 TRANSPORT INTO THE PERFUSED RAT LIVER AND COMPUTED LIVER T_4 UPTAKE IN HUMANS ARE INHIBITED BY FRUCTOSE.

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ABSTRACT

Recently, we described a two-pool model for T_3 transport and metabolism in isolated perfused rat livers. Now, we applied this model to investigate transmembrane T_4 transport and its possible ATP dependence in vivo. These studies are performed in perfused rat livers during control and fructose perfusion, as it has been shown that intracellular ATP is decreased after fructose loading. Furthermore, we studied serum T_4 tracer disappearance curves and computed T_4 hepatic uptake in 4 human subjects before and after intravenous fructose loading. In the perfused rat liver, we found a decrease in liver ATP concentration and a decrease in medium T_4 disappearance and T_4 uptake in the liver pool after fructose. Furthermore, it was shown that when corrected for differences in the free hormone concentration only transport to the metabolizing liver pool was decreased after fructose perfusion, whereas transport to the non-metabolizing pool was unaffected. Disposal, corrected for differences in transport into the metabolizing pool, was also not affected after fructose. In the human studies intravenous fructose administration induced a rise in serum lactic acid and uric acid, indicating a decrease in liver ATP. This was observed concomitant with a decrease in serum tracer T_4 disappearance and computed T_4 uptake in the liver during the first hours after fructose administration. These results suggest ATP-dependence of transport of iodothyronines into the liver in vivo, and show that in the rat liver and in humans, uptake of T_4 may be regulated by intracellular energy stores and in this way the tissue uptake process may affect intracellular metabolism and bioavailability of thyroid hormone.

INTRODUCTION

The low T_3 syndrome is characterized by a lowered serum T_3 level concomitant with increased serum rT_3, due to a decrease in T_3 production rate and rT_3 metabolic clearance rate, respectively. A variety of factors and compounds may lead to a low T_3 syndrome in man, e.g. caloric deprivation (33,38), non-thyroidal illness (4), dexamethasone (6,15), propylthiouracil (PTU) (1,17) and X-ray cholecystographic agents (5,37,44). Studies in rat liver

homogenates from starved rats (19) or from rats treated with PTU (41) or dexamethasone (23), revealed a decreased 5'-deiodinase activity. It was hence suggested that inhibition of this deiodinative step was responsible for the observed changes in serum iodothyronines as seen in the low T_3-syndrome. However, decreased deiodination was not always explanatory of the low T_3 syndrome (39). An alternative explanation, therefore, was given by Krenning et al. (25), who showed in rat hepatocytes in primary culture that iodothyronines were taken up by two active, *ATP dependent*, transport mechanisms: T_4 and rT_3 enter the cell via a common pathway, which is different from that of T_3. Inhibition of notably the T_4-rT_3 transport pathway into the liver due to e.g. ATP decrease would result in an increased serum rT_3 concentration and decreased serum T_3, because of reduced intracellular T_4 availability for conversion to T_3 (39), as the liver plays a dominant role in plasma T_3 production and rT_3 metabolic clearance (2,40). However, ATP dependence of the transport process is an vitro finding (cultured hepatocytes), and does not necessarily reflect the in vivo situation.

To investigate the changes in transmembrane transport and subsequent metabolism of thyroid hormones, because of ATP decrease, in a more physiological model, we studied these processes in a recirculating rat liver perfusion system. This was done before and after addition of fructose to the perfusion medium, as it has been shown that fructose induces a prompt and significant fall in liver ATP (3,34). Using this liver perfusion system, in which the cellular and structural integrity of the organ is maintained, and using a two-compartment model of T_3 transport and metabolism, we recently reported to be able to divide transport into the liver pool into transport to a non-metabolizing, presumably extracellular liver compartment and transport to the intracellular liver compartment, where metabolism takes place. Transport of T_3 to the former liver compartment appeared to be dependent on the albumin concentration in the medium, whereas the amount of T_3 transported into the cells for further metabolism was determined by the free T_3 concentration and independent of the albumin concentration in the medium (11,14). Now, we applied this two-compartment model to investigate T_4 uptake into the liver and the influence of fructose on this process.

Furthermore, early T_4 tracer disappearance from serum (representing

transport to the liver) was examined in humans in two conditions: before and after an intravenous bolus of fructose. From the same studies T_4-tracer liver uptake was computed using a three-compartment model of thyroid hormone distribution and metabolism (13,39).

MATERIALS AND METHODS

Subjects: Four (three women) healthy subjects, age 27-36 years (mean 30.8 years) participated in this study. All had normal thyroid function as measured by serum T_4, T_3-resin uptake and thyrotropin response to an intravenous bolus of thyrotropin releasing hormone. None of the persons had recently (within three months) used any medication.

Rats: Male Wistar rats, weighing about 250 g were used in the experiments. Liver weights were 10.2 ± 0.8 g in the group of glucose perfused livers and 10.9 ± 1.1 g in the glucose and fructose perfused group (NS).

See further Chapter 2.

RESULTS

Rat liver perfusion: After 20 and 90 min perfusion with glucose and fructose liver ATP content was decreased more than 60 % compared to livers perfused with only glucose (Table 1).

Table 1

ATP content in rat livers at 20 and 90 min after the start of the perfusion with medium with either 10 mmol/L glucose or both glucose and fructose (both 10 mmol/L). Data are expressed as % of 20 min value of glucose perfusion.

	Glucose	Glucose + Fructose
ATP (20 min)	100 ± 6.5 % (= 2.3 μmol/g liver)	34.9 ± 6.3 %[@]
ATP (90 min)	89.9 ± 9.2 %	22.4 ± 5.0 %[@]

Mean ± SD, n = 5-7 for each experiment. [@]: $p<0.001$ versus corresponding control (only glucose).

In all experiments the T_4 medium disappearance could be described as the sum of two exponentials, compatible with a two-compartment model of T_4 distribution and metabolism. In Figure 1 medium disappearance of T_4-tracer is depicted in perfusions with and without fructose in the medium. It can be seen

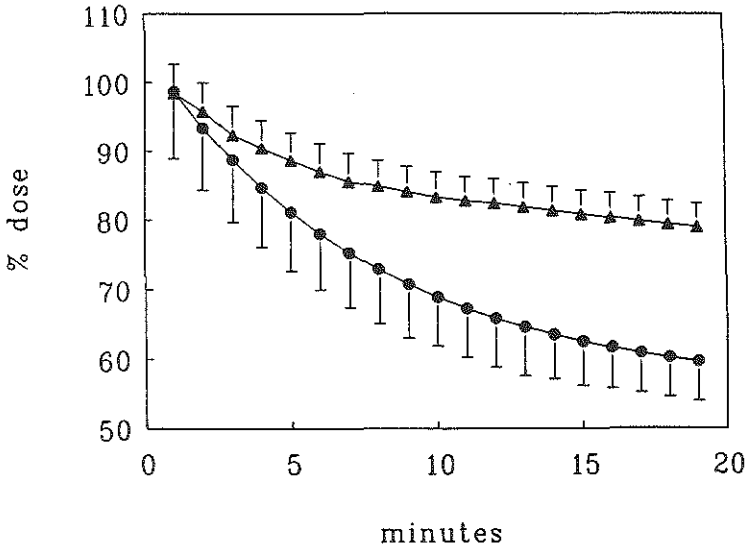

Figure 1
Medium ^{125}I-T_4 disappearance into rat liver during glucose (10 mmol/L, n=5, •) and glucose and fructose perfusion (both 10 mmol/L, n=5, ▲). Medium contains 1 % BSA. Values are mean ± SD, p<0.001.

that in the presence of fructose T_4-tracer disappearance from medium occurs more slowly than during control perfusion ($p<0.001$). From the parameters of the two-compartment model, the uptake into the liver compartment can be calculated. Figure 2 shows the computed liver uptake of T_4, which is decreased during the fructose study ($p<0.001$), in accordance with the diminished medium disappearance. In Table 2 some parameters of the two-compartment model are given. It can be seen that the transport parameters, i.e. total mass transfer and k_{21}, the fractional influx rate constant, are both decreased during fructose, whereas k_{12}, the fractional efflux rate constant, is not affected by

fructose.

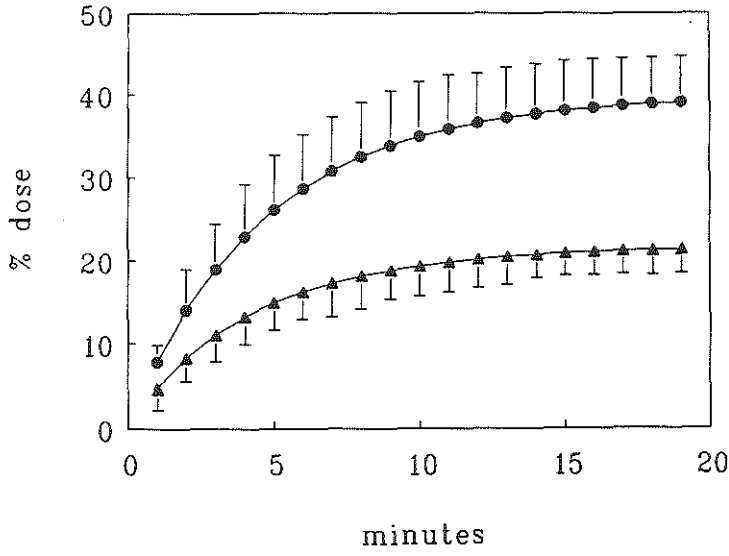

Figure 2
^{125}I-T_4 liver uptake in rat livers during glucose (10 mmol/L, n=5, •) and glucose and fructose perfusion (both 10 mmol/L, n=5, ▲). Medium contains 1 % BSA. Values are mean ± SD, p<0.001.

The found decrease in uptake after fructose could theoretically be caused by decreased transport to the extracellular liver compartment or by diminished transport into the intracellular compartment or both. Recently, we published a method, using the rat liver perfusion, to discriminate between transport of thyroid hormone to the extracellular and intracellular liver pool (11,14). To investigate the influence of fructose on transport to the extracellular and intracellular compartment of the liver for T_4, we applied this method by performing liver perfusions with 1 and 4 % BSA and T_4 tracer added to the medium, in the presence or absence of fructose. The transport and disposal parameters for the glucose perfusions are shown in Table 3. After raising the BSA concentration in the medium from 1 to 4 %, the integrated mean free hormone concentration in the medium decreases from 16.6 ± 1.1 pmol/L when 1 % BSA is used to 7.8 ± 0.5 pmol/L when the medium BSA

Table 2
T_4 kinetics in perfused rat livers after 90 min of perfusion with medium with 1 % BSA, with either glucose (10 mmol/L) or glucose and fructose (both 10 mmol/L). Calculated with two compartmental model.

		Glucose	Glucose + Fructose
Medium pool			
mean free hormone conc.	pmol/L	16.6 ± 1.1	18.6 ± 1.3$^{@@}$
mean total hormone conc.	nmol/L	6.1 ± 0.4	6.4 ± 0.5
Liver pool			
total mass transfer	pmol	5920 ± 378	4872 ± 248$^{@}$
total disposal	pmol	231.0 ± 17.7	118.7 ± 9.9$^{@}$
Fractional transfer rate constants			
k_{21} influx	min^{-1}	0.09 ± 0.008	0.04 ± 0.006$^{@}$
k_{12} efflux	min^{-1}	0.12 ± 0.02	0.13 ± 0.02
k_{02} disposal	min^{-1}	0.005 ± 0.001	0.006 ± 0.001

$^{@}$: $p<0.001$, $^{@@}$: $p<0.01$, mean ± SD, n = 5-7.

concentration is 4 %. Disposal shows a similar decrease as the decrease in the mean free T_4 concentration. However, transport to the extracellular pool increases significantly ($p<0.001$), despite the lower FT_4 concentration, while transport to the intracellular pool is diminished in parallel with the free hormone concentration. After correction for the differences in FT_4, it appears that the amount of T_4 metabolised and thus transported to the intracellular pool is not significantly different in the experiments with 1 % and 4 % BSA in the medium.

The transport and disposal parameters in the presence of fructose in the medium are also shown in Table 3. After raising the BSA concentration in the medium from 1 to 4 %, the mean free hormone concentration in the medium decreases from 18.6 ± 1.3 pmol/L when 1 % BSA is used to 7.2 ± 0.6 pmol/L when the medium BSA concentration is 4 %. When fructose is added to the medium, it appears that *qualitatively* transport and disposal show a similar pattern as the glucose perfused livers during perfusion with 1 % and 4

Table 3

Mean T_4 in the medium (nmol/L), mean FT_4 in the medium (pmol/L), TMT (pmol), TMT/FT_4 (pmol per pmol/L free T_4), disposal (pmol), transport to intracellular liver pool/FT_4 (=TrI) and extracellular liver pool/FT_4 (=TrE) (pmol per pmol/L free T_4) in rat livers perfused for 90 min with either glucose (10 mmol/L) or glucose + fructose (both 10 mmol/L), medium with 1 and 4 % BSA.

	Glucose 1 % BSA	Glucose 4 % BSA	Glucose + Fructose 1 % BSA	Glucose + Fructose 4 % BSA
FT_4	16.6 ± 1.1	7.8 ± 0.5	18.6 ± 1.3	8.5 ± 0.6
TMT	5920 ± 378	7676 ± 512	4872 ± 248$^{@}$	6930 ± 324$^{@@}$
Disposal	231.0 ± 17.7	95.2 ± 8.7	118.7 ± 9.9$^{@}$	54.3 ± 3.4$^{@}$
TrI/FT_4	148.5 ± 12.3	148.5 ± 12.3	75.6 ± 6.2$^{@}$	75.6 ± 6.2$^{@}$
TrE/FT_4	209.1 ± 16.5	836.2 ± 78.0	188.6 ± 14.5	752.5 ± 66.4

Mean ± SD, n = 5-7 for each group, $^{@}$ = $p<0.001$, $^{@@}$ = $p<0.05$ versus glucose with the same BSA concentration.

% BSA in the medium. Disposal shows a concomittant decrease, when the free T_4 concentration is decreased. Transport to the extracellular pool increases significantly ($p<0.001$), despite the lower FT_4 concentration, while transport to the intracellular pool diminishes in parallel with the free hormone concentration. After correction for the differences in FT_4, the amount of T_4 metabolised and transported into the intracellular pool is not significantly different in the experiments with 1 % and 4 % BSA in the medium, whereas transport to the extracellular pool is. However, looking *quantitatively* to the effects of fructose, it appears that compared to control livers transport to the extracellular pool is not affected, whereas transport to the intracellular pool is decreased ($p<0.001$). Disposal is decreased after fructose to the same extent as transport into the intracellular pool, so the ratio of disposal to transport into the intracellular pool (both corrected for the free hormone concentration) is not affected after fructose compared to the control situation (0.092 for control versus 0.085 for fructose, NS), pointing to the fact that the decrease in disposal may be caused by the decrease in transport, and not by a diminution of disposal itself.

Table 4
Serum thyroid hormone parameters before fructose infusion in four normal subjects. Samples were drawn just before the start of the kinetic studies.

		Control	Fructose	Refer. range
T_4	nmol/L	92.0 ± 10.8	95.8 ± 4.6	60 - 150
FT_4 (dial.)	pmol/L	18.6 ± 1.5	19.3 ± 1.0	15 - 26
T_3	nmol/L	1.7 ± 0.3	1.9 ± 0.4	1.1 - 3.0
rT_3	nmol/L	0.4 ± 0.06	0.3 ± 0.06	0.15 - 0.45
TSH (0 min)	mU/L	0.8 ± 0.3	0.9 ± 0.4	0.2 - 4.5
TSH (30 min)	mU/L	15.6 ± 3.1	after 200 µg TRH (iv)	

Mean ± SD.

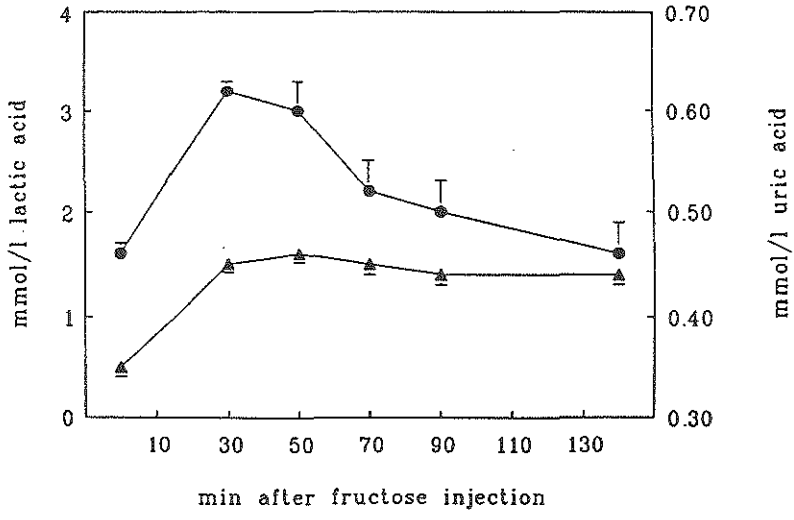

Figure 3
Serum lactic acid (•) and uric acid (▲) levels after i.v. fructose in 4 human subjects. Values are mean ± SEM.

Human studies: In the control and fructose periods no change in body weight occurred. Serum levels of T_4, FT_4, T_3, rT_3 and TSH were normal at the

time of kinetic studies in both periods (Table 4). Figure 3 shows mean serum levels of uric acid and lactic acid after intravenous fructose administration. A clear rise of both was achieved, suggesting a decrease in liver ATP (3,34). In Figure 4 the mean decline in serum tracer T_4 is given, expressed as percentage of the dose injected until six hours after injection. It can clearly be seen that T_4 tracer disappears more slowly from the plasma compartment after fructose administration ($p<0.025$) as compared to control.

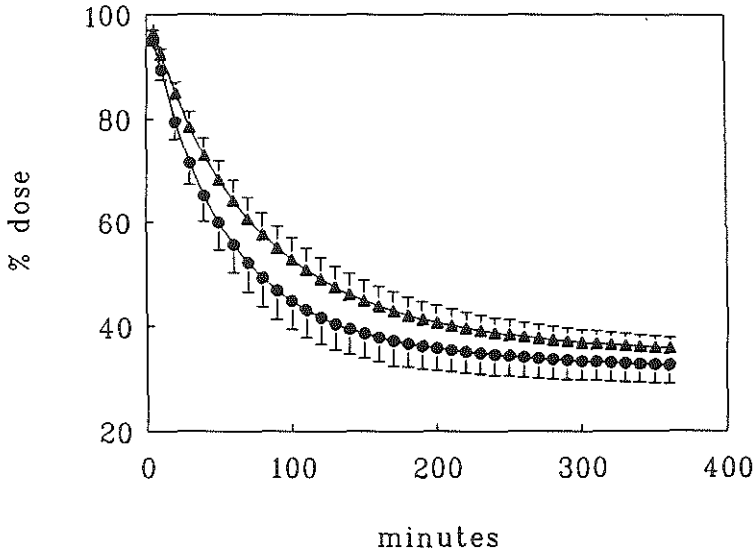

Figure 4
Early serum ^{131}I-T_4 disappearance before (•) and after i.v. fructose (▲) in 4 human subjects. Values are mean ± SEM, $p<0.025$.

T_4-tracer serum disappearance curves of each subject in each condition could be fitted to the sum of 3 exponentials, therefore, a three-compartment model of distribution and metabolism of thyroxine (with a plasma pool, a rapidly equilibrating pool (REP) and a slowly equilibrating pool (SEP) (13,39)) could be used. It can be concluded from Table 5 that overall T_4 kinetics are not significantly different from control during the study with fructose infusion, because fructose is only given during 20 min at the start of the T_4 kinetic

Table 5
T_4 kinetics before and after fructose infusion in four normal subjects. Mean ± SEM.

		Control	Fructose
Production rate	nmol/day	112.8 ± 16.4	116.9 ± 15.1
MCR	L/day	1.3 ± 0.1	1.2 ± 0.2
Plasma pool			
free hormone conc.	pmol/L	17.6 ± 1.1	18.6 ± 1.1
total hormone conc.	nmol/L	88.2 ± 3.5	97.4 ± 1.9
size	L	3.8 ± 0.6	4.0 ± 0.6
pool	nmol	337.0 ± 64.4	392.0 ± 53.5
Rapidly equilibrating pool			
size	L	6.0 ± 0.8	6.1 ± 0.6
pool	nmol	524.0 ± 65.6	593.0 ± 51.7
mass transfer rate from plasma	nmol/h	223.0 ± 17.3	192.0 ± 15.2
fractional transfer rate from plasma	h^{-1}	0.7 ± 0.2	0.5 ± 0.07
Slowly equilibrating pool			
size	L	4.3 ± 0.7	4.5 ± 0.6
pool	nmol	387.0 ± 71.1	436.0 ± 55.7
mass transfer rate from plasma	nmol/h	18.2 ± 6.5	11.5 ± 3.4
fractional transfer rate from plasma	h^{-1}	0.05 ± 0.02	0.03 ± 0.01

study, a very short period compared to the duration of the study, which is 9 days. The influence of fructose can only be measured during the first hours of the tracer T_4 kinetic studies. Figure 5 summarizes the computed uptake of T_4 during 360 min in the rapid equilibrating pool, mainly composed of the liver (39). After fructose, a significant decrease in initial hepatic uptake was found as compared to control ($p < 0.025$).

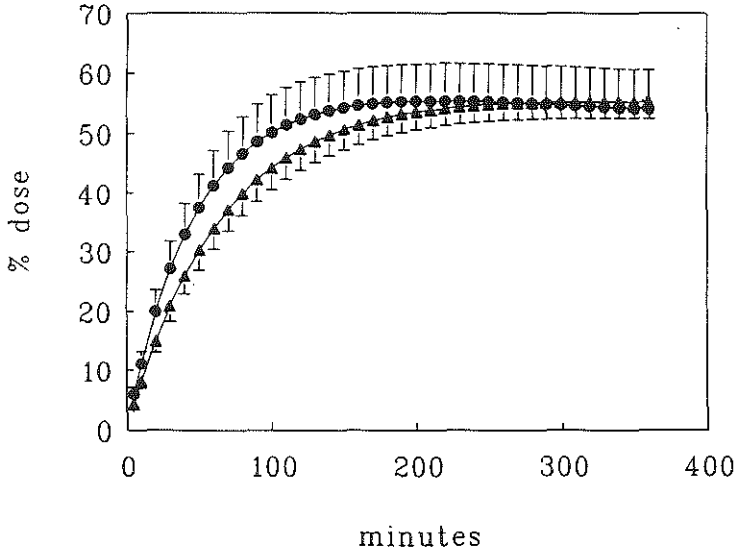

Figure 5
Computed ^{131}I-T_4 uptake in REP before (•) and after i.v. fructose (▲) in 4 human subjects. Values are mean ± SEM, $p<0.025$.

DISCUSSION

Rat liver perfusion: We have reported the use of a two-compartment model of T_3 distribution and metabolism in the isolated perfused rat liver (14). It appeared that the model adequately described the fate of thyroid hormone in the perfusion system, as it correctly calculated medium pool, liver pool and disposal, compared to direct measurements of these parameters without the use of the model. Using this model, the experiments with 1 % BSA in the medium show that of the total amount of T_4 transported to the liver about 60 % is transported to the extracellular pool, whereas 40 % is transported into the intracellular, metabolically active pool. Disposal in this latter pool amounts to 9 % of the T_4 transported into this pool. When the BSA concentration in the medium is raised to 4 %, only 15 % of the total amount of T_4 transported to the liver is transported into the intracellular, metabolically active pool (Table 3).

This indicates that the major part of the hormone transported to the liver does not enter the cells, but is sequestered in a metabolically inert pool, probably located outside the cell. Transport to this pool is dependent on both the free hormone concentration and the BSA concentration in the medium, in contrast to transport of hormone into the intracellular pool, which is only dependent on the free hormone concentration in the medium. These findings of transport to an extracellular and intracellular liver T_4 pool, with their respective characteristics are qualitatively, but not quantitatively the same as for T_3. Thus, it appeared that using 1 % BSA in the medium of the total amount of T_3 transported to the liver about 20 % was metabolized, whereas for T_4 this is only 9 %.

Fructose induced a decreased clearance of T_4 tracer from the medium and consequently attenuated liver uptake. Furthermore, it appeared that the decrease in total transport was caused by an inhibition of transport to the intracellular pool, whereas transport to the extracellular pool was unaffected after fructose (Table 3). Disposal, corrected for differences in transport to the intracellular pool, was also unaffected after fructose (Table 3). These data show that discrimination between transport to the intra- and extracellular liver pool is important, because the actual inhibition of transport to the metabolically active pool (40 %) is much greater than expected from the data of total mass transfer to the liver (20 % inhibition, Table 3).

Human studies: In man a marked difference in time for equilibration of T_4 tracer between liver and plasma was found (32). Several studies (7,16) showed equilibrium of radioactivity above the liver with serum T_4 tracer after 2-4 hours. Although including kidneys and lungs, the REP is predominantly constituted by the liver (12,39) which contains about 1/3 of the total body T_4 pool (20). Current thinking places the liver in a dominant role in both plasma T_3 production and rT_3 degradation (2,39). Because equilibrium of tracer T_4 between plasma and liver compartment will be reached between 2-4 hours (7,16), we examined the serum T_4 tracer disappearance curve during the first 6 hours after injection.

From Figure 3 it can be seen that intravenous fructose administration induces a prompt rise in lactic and uric acid, reflecting a marked decrease in

liver ATP concentration (3,34). No changes were observed in serum total and free T_4 (Table 4). From Figure 4 it appears that T_4 tracer disappears more slowly from the plasma compartment after fructose than in the control period. Figure 5 shows that computed T_4 uptake into the REP is significantly decreased in the fructose experiment compared to control. It can be concluded from Table 5 that overall T_4 kinetics are not significantly different from control during the study with fructose infusion, because fructose is only given during 20 min at the start of the T_4 kinetic study, a very short period compared to the duration of the study, which is 9 days. The influence of fructose can only be measured during the first hours of the tracer T_4 kinetic studies and this may explain the difference with overall kinetics in the shorter rat liver studies (90 min), where fructose is present during the whole experiment.

General: This study shows that uptake of T_4 in the liver, which in vitro has been shown to be ATP dependent, is decreased by fructose, both in perfused livers of rats and in humans. The effects of fructose on intracellular ATP levels have been described extensively (3,34). The mechanism of fructose-induced depletion of liver adenine nucleotides is dependent on the very rapid phosphorylation of fructose by the enzyme fructokinase. This is associated with the accumulation of fructose-1-phosphate and depression of hepatic ATP-levels, as also shown in our liver perfusion studies (Table 1). So, this study points to ATP dependence of T_4 transport in vitro as well as in vivo. That transport of T_4 is decreased as a result of depressed hepatic ATP levels, caused by fructose, is shown by liver perfusion experiments in which fructose was given for 20 min only during preperfusion. After this period livers were perfused with medium containing glucose. Whereas after 20 min fructose preperfusion liver ATP was about 30 % of control ($p<0.001$), after 90 min (i.e. 70 min of only glucose perfusion) ATP was restored to 95 % of control (NS) on that time-point. Disappearance of T_4 from the medium and computed liver uptake were decreased during the fructose period of the experiment compared to control experiments ($p<0.001$), whereas it was normalized during the perfusion with only glucose (not illustrated).

We could recently indicate transport inhibition of iodothyronines as an

underlying cause for inducing the changes in serum thyroid-hormones as seen in the low T_3 syndrome during caloric deprivation in humans (39). Furthermore, in perfused livers of 48 h starved rats, we showed an inhibition of transport of T_3 into the intracellular liver compartment (11). Possible causes for this decreased unidirectional transport into the perfused liver were a diminished intracellular ATP concentration and loss of intracellular T_3 binding proteins. However, preperfusion with medium with addition of insulin, glucose and/or cortisol for only 30 minutes fully normalized the impaired transport after fasting, pointing to the fact that loss of intracellular binding proteins did not play a role in this respect, as it is unlikely that protein synthesis could occur within 30 minutes. Therefore, it was hypothesized that decreased transport after fasting was caused by a decrease in intracellular ATP levels, which were presumably (partially) restored after perfusion with the combination of insulin and glucose and/or cortisol (11). Indeed it has been shown that during fasting intracellular ATP is decreased in vivo (10). The here reported decreased uptake of T_4-tracer in the liver after fructose due to decreased intracellular ATP levels is compatible with the same phenomena.

Decrease of intracellular ATP levels, although found in several states of non-thyroidal illness (NTI) (24,28,43), is not the only possible factor causing inhibition of transmembrane transport. From the work of Chopra et al. (8,9), it became evident that sera of patients with NTI and low serum T_4 may contain an inhibitor of thyroid hormone binding to serum proteins. Vos et al. (42) showed that sera of patients with NTI and a low T_4 state inhibit thyroid hormone uptake in hepatocytes in primary culture and in the perfused rat liver without a direct effect on deiodination. Lim et al. (26) showed that in NTI caused by uremia, CMPF and indoxyl sulfate, being present in high concentrations, cause T_4 transport inhibition in this condition. Furthermore, they reported that in non-uremic NTI non-esterified fatty acids and bilirubin may be the putative inhibitors (27).

In our studies we used compartmental analysis to calculate uptake of T_4 in the liver in humans and in the perfused rat liver. Current thinking allows use of this model only during steady state conditions, which is the case in the human studies but not in the rat liver perfusion experiments. However, this prerequisite of steady state conditions during model-based kinetic analysis is

most probably not necessary. It has been shown in the human that massive doses of unlabeled T_4 can be injected during a single compartmental analysis of T_4 kinetics, without any influence on the final results (35). Furthermore, Meinhold et al. (30) measured the metabolic clearance rate of diiodo-tyrosine (DIT) using non-compartmental kinetic analysis, by measuring the plasma disappearance of DIT by radio immunoassay after injection of a large amount of unlabeled DIT. The results were compared with similar studies performed with radiolabeled DIT. No differences in the final results were observed, if the serum DIT concentrations before injection of the unlabeled DIT were used to calculate the production rate and the total body pools. Considering these facts, we think that it is allowed to use compartmental analysis, even under non-steady state conditions, although it has been claimed that interpretation of data can cause some problems. In this light it is reassuring to note that the results of medium T_4 disappearance, which are measured directly, give similar results as compartmental analysis and subsequent calculation of liver uptake.

Although influx of T_4 into tissues exceeds T_4 metabolism in those tissues (this study in rat livers and ref. 14), this influx may still be rate-limiting in the determination of the intracellular free T_4 concentration, and therefore in T_4 metabolism, as shown in this study. The rate of disposal can generally be assumed to be a function of the intracellular hormone concentration, when metabolic disposal of hormones occurs within cells. The intracellular hormone concentration is determined by 1) the plasma (medium) flow rate, 2) the plasma (medium) concentration of hormone and hormone binding proteins, 3) the rate constant for hormone dissociation from its binding proteins, 4) rebinding to its binding proteins, 5) influx rate constant into the cell, 6) efflux rate constant and 7) metabolic rate constant (31). As the first 4 factors are constant in our liver perfusion system, only the ratio of influx (k_{21}) to efflux (k_{12}) rate constant and the metabolic rate constant (k_{02}) determine the intracellular hormone concentration. We found that in livers perfused with fructose and glucose the influx rate constant is decreased compared to livers perfused with glucose, whereas efflux (which is a passive phenomenon (21)) and metabolic rate constants are not affected (Table 2). So, there is a decrease of the ratio of influx to efflux rate constant, thereby decreasing intracellular total and free thyroid hormone concentration independently of the medium free hormone

concentration. This will lead to a decrease in substrate occupancy of metabolic enzymes and consequently attenuation of hormone metabolism. Furthermore, we found that disposal is decreased to the same extent as the inhibition of transport into the intracellular pool (Table 3). So, also on a theoretical basis it appears that the decrease in disposal may be accounted for by the inhibition in transport. Taking all these facts together, changes in uni-directional transport may be regulatory in hormone metabolism. Our study is in accordance with earlier in vitro studies that also showed that the carrier mediated uptake proces of thyroid hormones is rate limiting in hormone metabolism (22).

The present findings show that both in humans and in the rat liver uptake of thyroid hormones may be regulated by intracellular energy stores. It is conceivable that regulation of uptake of thyroid hormones in other organs and tissues is also operative in vivo and that these mechanisms may affect overall thyroid hormone metabolism and generation of bioactive thyroid hormone.

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REFERENCES

1 Abuid, J., and P.R. Larsen. Triiodothyronine and thyroxine in hyperthyroidism - Comparison of the acute changes during therapy with antithyroid drugs. J. Clin. Invest. 54: 201-208, 1974.

2 Bauer, A.G.C., J.P.H. Wilson, S.W.J. Lamberts, R. Docter, G. Hennemann, and T.J. Visser. Handling of iodothyronines by the liver and kidney in patients with chronic liver disease. Acta Endocrinol. 116: 339-346, 1987.

3 Bode, J.C.H., O. Zelder, H.J. Rump, and U. Wittkamp. Depletion of liver adenosine phosphates and metabolic effects of intravenous infusion of fructose or sorbitol in man and in the rat. Eur. J. Clin. Invest. 3: 436-441, 1973.

4 Braverman, L.E., and A.G. Vagenakis. Endocrine manifestations of systemic disease. The thyroid. Clinics Endocrinol. Metab. 8: 621-639, 1979.

5 Bürgi, H., C. Wimpfheimer, A. Burger, W. Zaunbauer, H. Rösler, and T. Lemarchand-Beraud. Changes of circulating thyroxine, triiodothyronine and reverse triiodothyronine after radiographic contrast agents. J. Clin. Endocrinol. Invest. 43: 1203-1210, 1976.

6 Burr, W.A., R.S. Griffiths, D. Ramsden, E.G. Black, R. Hoffenberg, H. Meinhold, and K.M. Wenzel. Effect of a single dose of dexamethasone on serum concentration of thyroid hormones. Lancet 2: 58-61, 1976.

7 Cavalieri, R.R., G.L. Searle, J.N. Castle, and R. Dickard. The kinetics of distribution between plasma and liver of ^{131}I-labeled L-thyroxine in man: observation of subjects with normal and decreased serum thyroxine-binding globulin. J. Clin. Invest. 45: 939-949, 1966.

8 Chopra, I.J., T.S. Huang, A. Beredo, D.H. Solomon, G.N. Chua Teco, and J.F. Mead. Evidence for an inhibitor of extrathyroidal conversion of thyroxine to 3,5,3'-triiodothyronine in sera of patients with nonthyroidal illness. J. Clin. Endocrinol. Metab. 60: 666-672, 1985.

9 Chopra, I.J., T.S. Huang, A. Beredo, D.H. Solomon, and G.N. Chua Teco. Serum thyroid hormone binding inhibitor in nonthyroidal illness. Metabolism 35: 152-159, 1986.

10 Cunningham, C.C., C.R. Malloy, and G.K. Radda. Effect of fasting and acute ethanol administration on the energy state of in vivo liver as measured by 31P-NMR spectroscopy. Biochim. Biophys. Acta 885: 12-22, 1986.

11 De Jong, M., R. Docter, H.J. van der Hoek, R.A. Vos, E.P. Krenning, and G. Hennemann. Transport of T_3 into the perfused rat liver and subsequent metabolism are inhibited by fasting. Endocrinology 131: 463-470, 1992.

12 DiStefano III, J.J., M. Jang, T.K. Malone, and M. Broutman. Comprehensive kinetics of triiodothyronine production, distribution, and metabolism in blood and tissue pools of the rat using optimized blood-sampling protocols. Endocrinology 110: 198-213, 1982.

13 DiStefano III, J.J., T.K. Malone, and M. Jang. Comprehensive kinetics of thyroxine distribution and metabolism in blood and tissue pools of the rat from only six blood samples: dominance of large, slowly exchanging tissue pools. Endocrinology 111: 108-117, 1982.

14 Docter, R., M. de Jong, H.J. van der Hoek, E.P. Krenning, and G. Hennemann. Development and use of a mathemetical two-pool model of distribution and metabolism of 3,3',5-triiodothyronine in a recirculating rat liver perfusion system: Albumin does not play a role in cellular transport. Endocrinology 126: 451-459, 1990.

15 Duick, D.S., D.W. Warren, J.T. Nicoloff, C.L. Otis, and M.S. Croxson. Effect of a single dose of dexamethasone on the concentration of serum triiodothyronine in man. J. Clin. Endocrinol. Metab. 39: 1151-1154, 1974.

16 Felicetta, J.V., W.L. Green, and W.B. Nelp. Inhibition of hepatic binding of thyroxine by cholecystographic agents. J. Clin. Invest. 65: 1032-1040, 1980.

17 Geffner, D.L., M. Azukizawa, and J.M. Hershman. Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin response in man. J. Clin. Invest. 55: 224-229, 1975.

18 Greenwood, F.C., W.M. Hunter, and J.S. Glover. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89: 114-123, 1963.

19 Harris, A.R.C., S.L. Fang, A.G. Vagenakis, and L.E. Braverman. Effect of starvation, nutriment replacement, and hypothyroidism on in vitro hepatic T_4 to T_3 conversion in the rat. Metabolism 27: 1680-1690, 1978.

20 Hennemann, G. Transport and distribution of thyroid hormones. In; Hesch RD (ed) The low T_3 syndrome, vol 40. Proceedings of the Serono Symposia, Academic Press, p 41-49, 1981.

21 Hennemann, G., E.P. Krenning, B. Bernard, F. Huvers, J. Mol, R. Docter, and T.J. Visser. Regulation of influx and efflux of thyroid hormones in rat hepatocytes: Possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity. Horm. Metab. Res. (suppl.) 14: 1-6, 1984.

22 Hennemann, G., E.P. Krenning, M. Polhuys, J.A. Mol, B.F. Bernard, T.J. Visser, and R. Docter. Carrier-mediated transport of thyroid hormone into rat hepatocytes is rate-limiting in total cellular uptake and metabolism. Endocrinology 119: 1870-1872, 1986.

23 Kaplan, M.M., and R.D. Utiger. Iodothyronine metabolism in rat liver homogenate. J. Clin. Invest. 61: 459-471, 1978.

24 Karlsson, J., J.T. Willerson, S.J. Leshin, C.B. Mullins, and J.H. Mitchell. Skeletal muscle metabolites in patients with cardiogenic shock or severe congestive heart failure. Scand. J. Clin. Lab. Invest. 35: 73-79, 1975.

25 Krenning, E.P., and R. Docter. Plasma membrane transport of thyroid hormone. In: Hennemann G. (ed.). Thyroid hormone metabolism. Marcel Dekker Inc, New York, p 107-132, 1986.

26 Lim, C.F., B.F. Bernard, M. de Jong, R. Docter, E.P. Krenning, and G. Hennemann. A furan fatty acid and idoxyl sulfate are the putative inhibitors of thyroxine metabolism in uremia. J. Clin. Endocrinol. Metab. 76: 318-324, 1993.

27 Lim, C.F., R. Docter, T.J. Visser, E.P. Krenning, B. Bernard, H. van Toor, M. de Jong, and G. Hennemann. Inhibitors of thyroxine hepatocytes transport in non-uremic critical illness. Effects of bilirubin and non-esterified fatty acids. In press. J. Clin. Endocrinol. Metab., 1993.

28 Manzke, H., K. Dorner, and J. Grunitz. Urinary hypoxanthine, xanthine and uric acid excretion in newborn infants with perinatal complications. Acta Paediatr. Scand. 66: 713-717, 1977.

29 Meijer, D.K.F., G.T.P. Keulemans, and G.J. Mulder. Isolated perfused rat liver technique. In: Jakobi (ed.), Methods in Endocrinology. New York: Academic, 77, p 81-94, 1981.

30 Meinhold, H., T. Olbright, and D. Schwartz-Porsche. Turnover and urinary excretion of circulating diiodothyrosine. J. Clin. Endocrinol. Metab. 64: 794-799, 1987.

31 Mendel, C.M., R.R. Cavalieri, and R.A. Weisiger. Uptake of thyroxine by the perfused rat liver: implications for the free hormone hypothesis. Am. J. Physiol. 255: E110-E119, 1988.

32 Nicoloff, J.T., and J.T. Dowling. Estimation of thyroxine distribution in man. J. Clin. Invest. 47: 26-37, 1968.

33 Portnay, G.I., J.T. O'Brian, J. Bush, A.G. Vagenakis, F. Azizi, R.A. Arky, S.H. Ingbar, and L.E. Braverman. The effect of starvation on the concentration and binding of thyroxine and triiodothyronine in serum and on the response to TRH. J. Clin. Endocrinol. Metab. 39: 191-194, 1974.

34 Raivio, K.O., M.P. Kekomäki, and P.H. Mäenpää. Depletion of liver adenine nucleotides induced by D-fructose. Biochem. Pharmacol. 18: 2615-2624, 1969.

35 Sterling, K., and B.B. Chodos. Radiothyroxine turnover studies in myxedema, thyrotoxicosis, and hypermetabolism without endocrine disease. J. Clin. Invest. 35: 806-810, 1956.

36 Sterling, K., and M.A. Brenner. Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J. Clin. Invest. 45: 153-163, 1966.

37 Suzuki, H., N. Kadema, K. Takeuchi, and S. Nakagawa. Effects of three-day oral cholecystography on serum iodothyronines and TSH concentrations. Acta Endocrinol. 92: 477-488, 1979.

38 Vagenakis, A.G., A. Burger, G.I. Portney, M. Rudolph, J.T. O'Brian, F. Azizi, R.A. Arky, P. Nicox, S.H. Ingbar, and L.E. Braverman. Diversion of peripheral thyroxine metabolism from activating to inactivating pathways during complete fasting. J. Clin. Endocrinol. Metab. 41: 191-194, 1987.

39 Van der Heijden, J.T.M., R. Docter, H. van Toor, J.P.H. Wilson, G. Hennemann, and E.P. Krenning. Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am. J. Physiol. 251: E156-E163, 1986.

40 Van der Heyden, J.T.M., E.P. Krenning, H. van Toor, G. Hennemann, and R. Docter. Three-compartmental analysis of effects of D-propranolol on thyroid hormone kinetics. Am. J. Physiol. 255: E80-E86, 1988.

41 Visser, T.J. A tentative review of recent in vivo observations of the deiodination of iodothyronines and its possible physiological implications. Mol. Cell. Endocrinol. 10: 241-247, 1978.

42 Vos, R.A., M. de Jong, R. Docter, H. van Toor, H.F. Bernard, E.P. Krenning, and G. Hennemann. Morbidity-dependent thyroid hormone transport inhibition by serum of patients with non-thyroidal illness (NTI) in rat hepatocytes and in the perfused rat liver. In: Progress in thyroid research, Gordon, Gross and Hennemann (eds.). Balkema Rotterdam. p 693-696, 1991.

43 Woolliscroft, J.O., H. Colfer, and I.H. Fox. Hyperuricemia in acute illness: a poor prognostic sign. Am. J. Med. 72: 58-62, 1982.

44 Wu, S.Y., I.J. Chopra, D.H. Solomon, and L.R. Bennet. Changes in circulating iodothyronines in euthyroid and hyperthyroid subjects given ipodate (Oragrafin) an agent for oral cholecystography. J. Clin. Endocrinol. Metab. 46: 691-697, 1978.

CHAPTER 7

TRANSPORT AND METABOLISM OF IODOTHYRONINES IN CULTURED HUMAN HEPATOCYTES.

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ABSTRACT

Thyroid hormone uptake into cultured human hepatocytes was studied using measurement of cell-associated radioactivity of radioiodinated thyroid hormones after 10-min incubation in culture medium with 0.5 % BSA. Furthermore, 20-h incubations were performed to study transport and further intracellular metabolism. The results indicate the presence of saturable active uptake systems for T_4, T_3, and rT_3, as addition of the unlabeled hormone (1, 5 and 2 μmol/L, respectively) to the medium resulted in a decrease in cell associated radioactivity of 20-30 %. Inhibition was also achieved after 30-min preincubation with fructose (10 mmol/L), which induces a decrease in intracellular ATP or ouabain (0.5 mmol/L), indicating energy dependence and the necessity for a sodium gradient for at least part of the transport process, respectively. After 20-h incubation, iodide production was inhibited in the presence of ouabain (0.5 mmol/L), PTU (100 μmol/L), or a monoclonal antibody (81-1A1-10; ascites dilution, 1:200) directed against thyroid hormone transport systems in rat hepatocytes.

These data indicate that there is a high degree of similarity between the properties of the uptake process and subsequent conversion of thyroid hormones in human and rat hepatocytes, although the rates of uptake and conversion are lower in human hepatocytes. Furthermore, regulation of thyroid hormone uptake at the level of the plasma membrane may also be operative in human hepatocytes.

INTRODUCTION

T_4, the main product of the thyroid, is activated by peripheral 5'-deiodination, resulting in the formation of bioactive T_3 (1,2). In this process, the liver plays quantitatively an important role by the presence of type I deiodinase (3). So, to become activated, T_4 has to reach the intracellular deiodinating enzymes, and therefore it has to be transferred over the liver cell membrane into the cell. This translocation process over the liver cell membrane is not only important for T_4, but also for plasma derived T_3, as

this hormone has to reach its receptors on the nucleus to exert its biological effects. The translocation process has long been thought to be a passive process. There is, however, evidence in isolated rat hepatocytes and perfused rat livers that T_3 and T_4 are transported into the cells by active, saturable, transport mechanisms, which may play a regulatory role in subsequent metabolism (4-7). Similar active transport mechanisms have also been found in human cells, such as lymphocytes (8) and fibroblasts (9), but there are no reports concerning the transport and metabolism of thyroid hormones in intact human hepatocytes. It is important to know whether active transmembrane transport of thyroid hormones is also present in the liver, because this organ plays a central role in extrathyroidal plasma T_3 production (3) and in the clearance of plasma rT_3 (10). If active transport is indeed present, then regulation of these processes may take place. We report here on the uptake of T_4, T_3, and rT_3 into cultured human hepatocytes and the influence of various compounds known to inhibit thyroid hormone uptake and/or metabolism in rat hepatocytes.

MATERIALS AND METHODS

Liver cells were isolated from human livers, obtained through the Auxiliary Partial Liver Transplantation Program at the University Hospital Dijkzigt in Rotterdam, The Netherlands.

See further Chapter 2.

RESULTS

Figure 1 shows the typical time course of cell associated radioactivity of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 during 60 min, expressed as a percentage of the dose. Cell viability in this experiment was more than 90 %. Uptake of all three thyroid hormones increased with time and did not show equilibrium within 60 min. Only after 60-min incubation with ^{125}I-rT_3 was radioactive iodide production was measurable (in this experiment, 12 fmol $^{125}I^-$ per pmol/L free rT_3/well; for ^{125}I-T_4 and ^{125}I-T_3 iodide production was not measurable within 60 min).

As uptake was about linear up to 10 min (Figure 1), we investigated

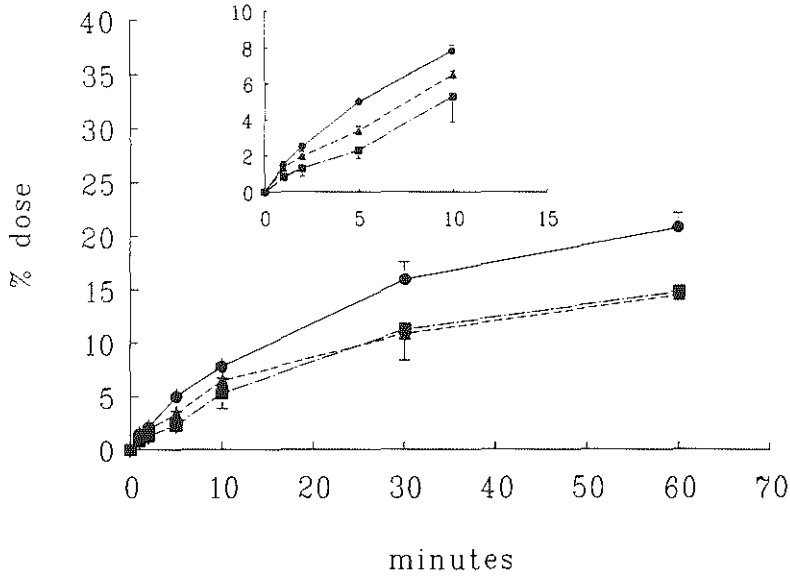

Figure 1

Typical example of cell-associated radioactivity of ^{125}I-T_4 (·), ^{125}I-T_3 (▲) and ^{125}I-rT_3 (■) as a function of time at 37 C and expressed as a percentage of the dose. Cultured human hepatocytes (2.10^6 cells/well) were incubated in 1 ml culture medium with 0.5 % BSA. Mean ± SD, each point was determined in triplicate. The inset shows a magnification of the first part of the curve untill 10 min.

the saturability of initial uptake after 10-min incubations with or without unlabeled hormone (1 μmol/L T_4, 5 μmol/L T_3, and 2 μmol/L rT_3) (Table 1). Data are expressed as a percentage of the control at 10 min. The inhibitory effect of unlabeled hormone ranged from 20 % for T_3 to 30 % for T_4 (Table 1). Also in Table 1, the effects of ouabain (0.5 mmol/L, inhibits Na^+,K^+-ATP-ase) or fructose (10 mmol/L, decreases intracellular ATP) during 30-min preincubation and 10-min incubation are shown. The uptake of all three thyroid hormones was decreased in the presence of both ouabain and fructose. The effect of ouabain was somewhat smaller than that of fructose, but uptake was significantly decreased in all cases.

In Table 2 a typical example of radioactive iodide and conjugate production from ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 is shown after 20-h incubation, expressed as femtomoles of $^{125}I^-$ or ^{125}I-labeled thyroid hormone conjugate per pmol/L free thyroid hormone/well. This experiment was performed with

Table 1

Influence of unlabeled hormone, ouabain (0.5 mmol/L), and fructose (10 mmol/L) on cell-associated activity of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 after 10-min incubation, expressed as a percentage of the control value. Concentrations of unlabeled hormone were 1 µmol/L T_4, 5 µmol/L T_3 and 2 µmol/L rT_3. Culture conditions are described in Figure 1. Mean ± SD, n = 2-4 different livers; each point in each liver in triplicate. $^{@}$: $p < 0.001$, $^{@@}$: $p < 0.025$, *: $p < 0.01$, **: $p < 0.05$.

	^{125}I-T_4	^{125}I-T_3	^{125}I-rT_3
control	100[$]	100[$]	100[$]
+ unlabeled hormone	69.9 ± 6.4[@@]	79.4 ± 6.2[*]	76.8 ± 10.7[**]
+ fructose	45.1 ± 9.4[@]	62.0 ± 11.4[*]	29.6 ± 5.8[*]
+ ouabain	56.5 ± 7.8[@]	75.5 ± 10.4[@@]	75.6 ± 10.3[**]

[$]: actual values are 8.15 ± 1.5, 6.5 ± 2.9 and 6.2 ± 2.8 % dose for T_4, T_3 and rT_3, respectively.

Table 2

Typical example of $^{125}I^-$ and ^{125}I-thyroid hormone conjugate excretion in medium from ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 in isolated human hepatocytes after 20-h incubation, expressed as femtomoles of $^{125}I^-$ or ^{125}I-thyroid hormone conjugate per pmol/L free thyroid hormone/2.10^6 cells, respectively. Mean ± SD, each point in triplicate. Experiments are performed in culture medium with 0.5 % BSA. ND = not detectable.

	$^{125}I^-$	^{125}I-conjugate
^{125}I-T_4	19.5 ± 2.5	1.0 ± 0.8
^{125}I-T_3	6.8 ± 0.7	ND
^{125}I-rT_3	65.9 ± 9.4	1.9 ± 0.9

cells from the same liver as that used in Figure 1. After uptake of the tracers, there was more iodide production from rT_3 than from T_4 and T_3. Furthermore, excretion of conjugates into the medium was very low (just above the detection limit of our assay for T_4 and rT_3), whereas T_3 conjugates were not detectable.

Table 3 summarizes the effects of 20-h incubation with ouabain or a monoclonal antibody known to inhibit the uptake of thyroid hormones into rat hepatocytes. These two compounds also inhibited iodide production in

Table 3

Iodide and conjugate release in medium after 20-h incubation from ^{125}I-T_4, ^{125}I-T_3 or ^{125}I-rT_3 in isolated human hepatocytes, expressed as a percentage of the control value, in the presence or absence of several substances (MAb = monoclonal antibody). Mean ± SD, n = 2-4 different livers, each point in each liver in triplicate. $^{@}$: $p < 0.001$, $^{@@}$: $p < 0.025$, $^{\#}$: $p < 0.01$, $^{\#\#}$: $p < 0.05$. Experiments are performed in culture medium with 0.5 % BSA. ND = not detectable.

	^{125}I-T_4	^{125}I-T_3	^{125}I-rT_3
Iodide:			
control	100$^{\$}$	100$^{\$}$	100$^{\$}$
+ ouabain (0.5 mmol/L)	50.4 ± 19.4$^{@@}$	52.7 ± 11.2$^{@@}$	70.9 ± 11.2$^{\#}$
+ PTU (100 µmol/L)	15.9 ± 0.5$^{@}$	12.7 ± 8.7$^{@}$	11.6 ± 1.5$^{@}$
+ MAb (1:200)	1.82 ± 0.9$^{@}$	21.0 ± 4.3$^{@}$	29.2 ± 3.5$^{@}$
Conjugates:			
control	100$^{\$}$	ND	100$^{\$}$
+ ouabain (0.5 mmol/L)	ND	ND	ND
+ PTU (100 µmol/L)	83.8 ± 14.2	ND	250.6 ± 90.5$^{\#\#}$
+ MAb (1:200)	ND	ND	ND

$^{\$}$: actual values are 16.3 ± 4.8, 5.5 ± 1.6 and 54.1 ± 15.6 fmol ^{125}I^{-}/pmol/L free thyroid hormone/2.10^{6} cells for T_4, T_3 and rT_3, respectively.

$^{\$}$: actual values are 0.9 ± 0.2 and 1.6 ± 0.4 fmol ^{125}I-thyroid hormone conjugate/pmol/L free thyroid hormone/2.10^{6} cells for T_4 and rT_3, respectively.

human hepatocytes. The monoclonal antibody inhibited iodide production from T_4 by 98 %, indicating that uptake via diffusion is virtually absent. Table 3 further shows the effect of PTU, an inhibitor of the type I deiodinase. In human hepatocytes, radioactive iodide production from all three tracers was inhibited by PTU. Table 3 also shows the conjugates excreted in the medium. For ouabain and the monoclonal antibody, the amount of conjugates released, was decreased to undetectable levels. This decrease was parallel to the decrease in deiodination, indicating that not the deiodinating process itself, but, rather, uptake is affected by these compounds (see also Discussion). After incubation with PTU, conjugate concentrations were not decreased, as was the case after incubation with

ouabain and the monoclonal antibody, but remained the same for T_4, whereas for rT_3 conjugate levels were much higher than those in the control situation. This indicates that PTU inhibits deiodination and not the uptake process (see also Discussion).

Table 4
Comparison of cultured human and rat hepatocytes with regard to isolation, uptake and metabolism of ^{125}I-T_4, ^{125}I-T_3 and ^{125}I-rT_3. Ouab = ouabain, fruc = fructose, MAb = monoclonal antibody, n = number of livers used.

	Human hepatocytes (n=2-4)			Rat hepatocytes (n>50)		
Isolation						
- attachment time	20 h			4 h		
Uptake and conversion	T_4	T_3	rT_3	T_4	T_3	rT_3
- saturability; inhibition by ouab, fruc, MAb, PTU	+	+	+	+	+	+
- % $^{125}I^-$ in medium after 20 h incubation	2	3	27	18	85	100
Conjugates excretion						
- increase after PTU	-	-	+	-	+	+

In Table 4, cultured human and rat hepatocytes are compared with regard to isolation, uptake process and metabolism of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3. The properties of uptake and intracellular deiodination were quite similar in both cell types; the data on iodide excretion into the medium after 20-h incubation show that the metabolism of thyroid hormones was much slower in human than in rat hepatocytes.

DISCUSSION

The major new finding of these studies is the existence of saturable energy-dependent uptake processes for ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 in cultured human hepatocytes. In rat hepatocytes, thyroid hormone uptake plateaus

after 5-min incubation (4), whereas in human hepatocytes this plateau is not reached after 60 min, indicating that the uptake process is slower in human hepatocytes.

In agreement with the results obtained in cultured rat hepatocytes, uptake of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 was (partially) saturable with unlabeled hormone (1,5 and 2 μmol/L, respectively), indicating that in human hepatocytes also, transport into the cells may involve a carrier-mediated mechanism. This is found for a variety of cells in several species, including human lymphocytes (8) and fibroblasts (9).

In the present study, cell associated radioactivity at 10 min was suppressed by the unlabeled hormone by about 20-25 % for ^{125}I-T_3 and ^{125}I-rT_3, and about 30 % for ^{125}I-T_4. It is noteworthy that this suppression of cell-associated radioactivity is presumably less than the actual inhibition of transport into the cells, as inhibition of cell associated radioactivity may be obscured by binding of thyroid hormone to the outer cell surface. This is in accordance with our findings in rat hepatocytes, in which the inhibitory effects of ouabain on total uptake were less than those on nuclear uptake (13).

To study the Na^+ dependence of the uptake process, incubations were performed in the presence of ouabain, which reduces the Na^+-gradient by inhibition of Na^+,K^+-ATP-ase. Ouabain reduced the cell-associated radioactivity of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 after 10 min up to 35 % of the control value. This indicates that a Na^+-gradient is necessary for (at least part of) the uptake process. Also in the 20-h incubation studies, when suppression of iodide production was about 50 % of the control value, it could be concluded that inhibition of iodide production in the presence of ouabain was caused by inhibition of uptake and not by an (additional) inhibition of the deiodination process itself. This is because conjugate concentrations in the medium are not increased after ouabain incubation, which would be expected when the deiodination process is inhibited. In rat hepatocytes, a marked increase (> 500 %) in T_3 conjugate excretion was found after inhibition of the deiodination process (14). Furthermore, we have shown that ouabain did not affect iodothyronine deiodination when tested in liver microsomes (15).

To study any energy dependence of the uptake process, incubations were performed in the presence of fructose. The effects of fructose on intracellular ATP levels have been described extensively (16,17) and shown by us in perfused rat liver (manuscript submitted for publication). The mechanism of fructose-induced depletion of liver adenine nucleotides is dependent on the very rapid phosphorylation of fructose by the enzyme fructokinase. This is associated with the accumulation of fructose-1-phosphate and depression of hepatic ATP levels. It is shown in the present study that cell-associated radioactivity of ^{125}I-T_4, ^{125}I-T_3, and ^{125}I-rT_3 was decreased in the presence of fructose, pointing to energy dependence of the uptake systems. The effect of fructose is most clear on the uptake of rT_3 and T_4 and less clear on uptake of T_3 (80 %, 70 %, and 50 % inhibition, respectively). It is noteworthy that fructose inhibits iodide production more than the unlabeled hormones. Therefore, it can be concluded that the concentrations of the unlabeled hormones that we used do not fully saturate the uptake systems.

We have also tested the effect of a monoclonal antibody, raised against rat hepatocytes and selected on the basis of its inhibitory activity on plasma membrane transport of thyroid hormones in rat hepatocytes. The monoclonal antibody also inhibited iodide production by all three radioiodinated thyroid hormones in human hepatocytes, pointing to the fact that structural similarities exist between thyroid hormone transport proteins in rat and human hepatocytes. Again, the inhibition of iodide production is caused by inhibition of uptake into the cells, whereas the deiodinating process itself is not affected. This is shown by the amount of conjugates excreted into the medium after incubation with the monoclonal antibody (Table 3), according to the explanation of transport inhibition by ouabain (see above). Furthermore, the 98 % inhibition of iodide production from T_4 by the monoclonal antibody shows that uptake by diffusion was virtually absent. That diffusion was responsible for only a very small part of the total amount of hormone taken up into the cells, whereas the remainder was taken up by active transport was also shown in human fibroblasts (9). We postulate that T_3 and rT_3 do also not diffuse through the plasma membrane and that residual uptake in the presence of the monoclonal antibody is due

to incomplete inhibition of the transport process.

As for intracellular conversion, it is obvious from Table 2 that rT_3 is the preferred substrate for the type I deiodinase, as previously shown by Visser et al. (18), who determined the type I deiodinase properties in human liver microsomes. They found a high degree of similarity with the enzyme in rat liver microsomes with regard to substrate preference, facilitatory effect of sulfation on deiodination of several iodothyronines, ping-pong-type kinetics, cofactors and inhibition by PTU (18). From their study they concluded that indeed the human liver is a major site for plasma rT_3 clearance, in accordance with our finding that already after 60 min incubation with ^{125}I-rT_3, radioactive iodide, because of intracellular conversion, was measurable in the medium, whereas for T_4 and T_3 this was not the case.

In human and rat liver microsomes, it was shown that sulfation of T_3 before deiodination increases the rate of deiodination by 30-fold (18,19). In human liver tumor cells (HepG2), only a small amount of T_3 is deiodinated, in spite of normal T_3 uptake into the tumor cells. In homogenates of these cells, T_3S is nearly a 100-fold more rapidly deiodinated than T_3 itself, pointing to a low rate of T_3 sulfation in these cells (20). Our results in Table 4 show a lower intracellular conversion of all three thyroid hormones in human hepatocytes compared to that in rat hepatocytes, in accordance with the findings of Visser et al. (18) in human liver microsomes that the conversion of all iodothyronines is 2-8 times slower in human than in rat liver. However, the lower rate of conversion as found in the present study may also be explained by the lower uptake into the cells, causing a lower intracellular availability. From Table 4 it is clear that especially ^{125}I-T_3 is very slowly deiodinated after uptake into the human cells, compared to rat hepatocytes. Therefore, it is suggested that although the overall deiodination of all thyroid hormones is slower, sulfation of T_3 occurs at a much lower rate in human than in rat hepatocytes, in accordance with the findings of Gong et al. (21), who investigated species differences in T_3 sulfation. They found a 8-20 times more rapid sulfation in male rats than in humans. This is also in agreement with the fact that after inhibition of the deiodination by PTU, T_3 conjugates do not increase in the medium to detectable levels, whereas in rat hepatocytes a marked increase (> 500 %) in T_3 conjugate

excretion is found after inhibition of the deiodination process by PTU (14). After incubation with PTU, an increase in conjugate release was found for rT_3, probably caused by increased glucuronidation as a result of increased intracellular availability due to the decreased deiodination process, in agreement with findings in rat hepatocytes (22).

Thus, qualitatively, there appears to be a high degree of similarity between thyroid hormone uptake systems and the process of further metabolism in human and rat hepatocytes, although uptake and deiodination (for all three thyroid hormones tested) and sulfation of T_3 occur at a lower rate in human hepatocytes. We found in rat hepatocytes and in the perfused rat liver that the transport process was rate-limiting in total cellular uptake and metabolism (15). The studies reported here suggest that in human liver cells also, thyroid hormone uptake is (partly) a carrier-mediated active process and may play a regulatory role in intracellular bioavailability.

ACKNOWLEDGMENTS

Human liver tissue was obtained through the Auxiliary Partial Liver Transplantation Program at the Department of Surgery of the University Hospital Dijkzigt in Rotterdam, The Netherlands. Consent was given by the medical ethical committee in this hospital. Cells used in the experiments described in this paper were obtained through the Human Liver Cell Foundation, which was established to make optimal use of human liver tissue, exchange ideas, stimulate collaboration, and provide members with isolated liver cells.

REFERENCES

1 Larsen PR, Silva JE, Kaplan MM. Relationships between circulating and intracellular thyroid hormones: Physiological and clinical implications. Endocr Rev 1981 2:87-102.

2 Engler D, Burger AG. The deiodination of the iodothyronines and of their derivatives in man. Endocr Rev 1984 5:151-184.

3 Leonard JL, Visser TJ. Biochemistry of deiodination. In: Thyroid hormone metabolism. G. Hennemann, editor. Marcel Dekker, New York and Basel. 1986 p189-229.

4 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G. Characteristics of

active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 1981 676:314-320.

5 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G. Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 1982 140:229-233.

6 De Jong M, Docter R, Van der Hoek HJ, Vos RA, Krenning EP, Hennemann G. Transport of T_3 into the perfused rat liver and subsequent metabolism are inhibited by fasting. Endocrinology 1992 131:463-470.

7 Blondeau JP, Osty J, Francon J. Characterization of the thyroid hormone transport system of isolated hepatocytes. J Biol Chem 1988 263:2685-2692.

8 Holm AC, Wong KY, Pliam NB, Jorgensen EC, Goldfine ID. Uptake of L-triiodothyronine into human cultured lympfocytes. Acta Endocrinol (Copenh) 1980 95:30-33.

9 Docter R, Krenning EP, Bernard HF, Hennemann G. Active transport of iodothyronines into human cultured fibroblasts. J Clin Endocrinol Metab 1987 65:624-628.

10 Bauer AGC, Wilson JHP, Lamberts SWJ, Docter R, Hennemann G, Visser TJ. Handling of iodothyronines by liver and kidney in patients with chronic liver disease. Acta Enocrinol 1987 116:339-346.

11 Mol JA, Krenning EP, Docter R, Rozing G, Hennemann G. Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. J Biol Chem 1986 261:7640-7643.

12 Sterling K, Brenner MA. Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J Clin Invest 1966 45:153-163.

13 Hennemann G, Krenning EP, Bernard B, Huvers F, Mol J, Docter R, Visser TJ. Regulation of influx and efflux of thyroid hormones in rat hepatocytes: Possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity. Horm Metab Res 1984 14:1-6.

14 Docter R, Krenning EP, Bernard HF, Visser TJ, Hennemann G. Inhibition of uptake of thyroid hormone into rat hepatocytes by preincubation with N-Bromoacetyl-3,3',5-triiodothyronine. Endocrinol 1988 123:1520-1525.

15 Hennemann G, Krenning EP, Polhuis M, Mol JA, Bernard HF, Visser TJ, Docter R. Carrier-mediated transport of thyroid hormones into rat hepatocytes is rate limiting in total cellular uptake. Endocrinology 1986 119:1870-1872.

16 Raivio KO, Kekomäki MP, Mäenpää PH. Depletion of liver adenine nucleotides induced by D-fructose. Biochem Pharmacol 1969 18:2615-2624.

17 Bode JCh, Zelder O, Rump HJ, Wittkamp U. Depletion of liver adenosine phosphates and metabolic effects of intravenous infusion of fructose or sorbitol in

man and in the rat. Eur J Clin Invest 1973 3:436-441.

18 Visser TJ, Kaptein E, Terpstra OT, Krenning EP. Deiodination of thyroid hormone by human liver. J Clin Endocrinol Metab 1988 67:17-24.

19 Visser TJ, Mol JA, Otten MH. Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinol 1983 112:547-557.

20 Van Stralen PGJ, Van der Hoek HJ, Docter R, De Jong M, Krenning EP, Lim CF, Hennemann G. Reduced T_3 deiodination by the human hepatoblastoma cell-line HepG2 caused by deficient T_3 sulfation. Biochim Biophys Acta 1993 1157:114-118.

21 Gong D-W, Murayama N, Yamazoe Y, Kato R. Hepatic triiodothyronine sulfation and its regulation by growth hormone and triiodothyronine in rats. J Biochem 1992 112:112-116.

22 Eelkman Rooda SJ, Van Loon MAC, Visser TJ. Metabolism of reverse triiodothyronine by isolated rat hepatocytes. J Clin Invest 1987 79:1740-1748.

CHAPTER 8

T_4 AND T_3 ARE NOT TRANSPORTED INTO RAT LIVER CELLS VIA AMINO ACID TRANSPORT SYSTEM A.

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INTRODUCTION

Amino acid transport processes in animal cells have been extensively studied. At least 8 distinct systems have been identified in normal hepatocytes on the basis of their kinetic properties and specificity toward the classes of amino acids (1). One of these, system A, a neutral amino acid transport carrier, is present in all nucleated cells and is characterised by its Na^+-dependence, the availability of specific substrates, such as α-AIB and its regulatory properties. The system is inhibited by pH values below 7.0 (2). There are various hormones, growth factors and second messengers, which are known to stimulate transport by system A in several tissues in the rat, e.g. insulin (3) and cAMP (4). The translocation of substrates via system A across the hepatocyte plasma membrane represents the rate limiting step in their overall metabolism (5).

The transport systems of thyroid hormones in rat hepatocytes have several characteristics in common with transport system A, e.g. temperature-, Na^+- and ATP-dependence and stimulation by insulin after fasting (6,7,8). Also transport of thyroid hormones into the liver is rate limiting on cellular uptake and metabolism, in other words: transport of thyroid hormones may play a regulatory role in these processes (9).

Because of these facts, the question was raised if amino acid transport system A and the thyroid hormone transport pathway in rat liver could be identical. Therefore, we tested the uptake of T_3 and α-AIB into isolated rat hepatocytes and their possible mutual influence.

MATERIALS AND METHODS

Methods used for isolation and culture of hepatocytes (2.10^6 cells/dish) were according a modification of the protocol by Berry and Friend (10). The following experiments are done: determination of ^{3}H-α-AIB (50 mmol/L and 5.5 μmol/L) uptake by measurement of cell associated (radio)activity (CAA = total uptake) at several time points up to 120 min, in the presence or absence of a) unlabeled T_3 (5.10^{-9} mol/L and 5.10^{-6} mol/L), b) unlabeled T_4 (5.10^{-9} mol/L and 5.10^{-6} mol/L), c) 2 monoclonal antibodies that inhibit thyroid hormone uptake by about 60 % in the used concentration (9), d) methyl(Me)-α-AIB (50 mmol/L) after 1 h preincubation, (Me-α-AIB is a known A-system

substrate and acts as a competitive inhibitor of α-AIB uptake), e) dibutyryl(db)-cAMP (0.1 mmol/L) (an A-system stimulator), and f) both T_3 and db-cAMP. Furthermore, ^{125}I-T_3 uptake was determined by measuring cell associated radioactivity at several time points up to 60 min, in the presence or absence of Me-α-AIB (50 mM, 1 h preincubation).

For each experiment: n ≥ 3. Results are given as % of the 120 min (α-AIB) or 60 min (T_3) control value (mean ± SD).

See further Chapter 2.

RESULTS

Figure 1 shows the time dependent uptake of ^{3}H-α-AIB, in the

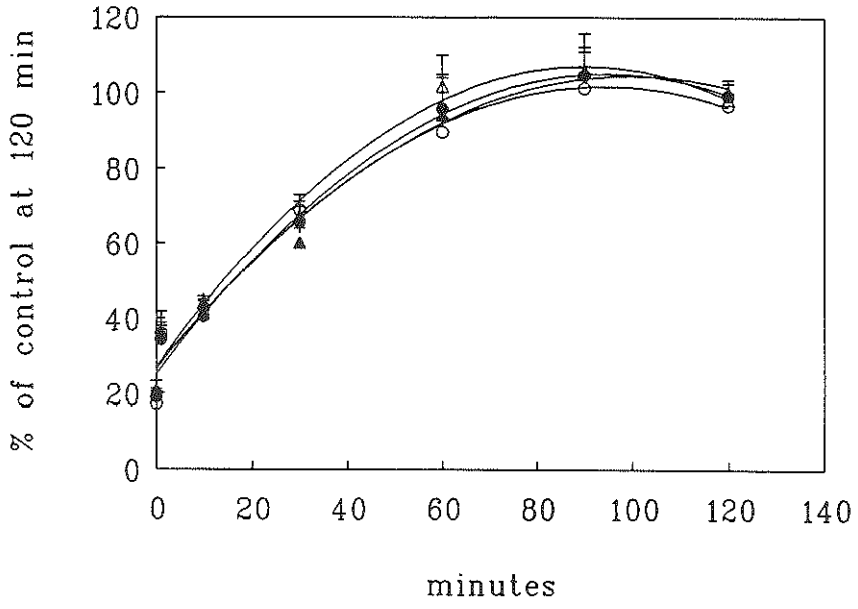

Figure 1
Time dependent cell associated radioactivity of ^{3}H-α-AIB, in the presence of 50 mM (▲) and 5.5 μM unlabeled α-AIB (•), and in the presence of unlabeled T_3 at concentrations of both 5.10^{-6} mol/L (○) and 5.10^{-9} mol/L (△), n ≥ 3, mean (SD).

presence of 50 mmol/L and 5.5 μmol/L unlabeled α-AIB. There is no difference in the uptake in the presence of the 2 concentrations, indicating

that the uptake system is not saturated at these concentrations. Addition of unlabeled T_3 at concentrations of both 5.10^{-6} mol/L and 5.10^{-9} mol/L has no effect on α-AIB uptake. The same holds for addition of unlabeled T_4 (not shown), indicating that T_4 and T_3 do not competitively inhibit the amino acid uptake system.

In Figure 2 the effects of Me-α-AIB and 2 monoclonal antibodies are

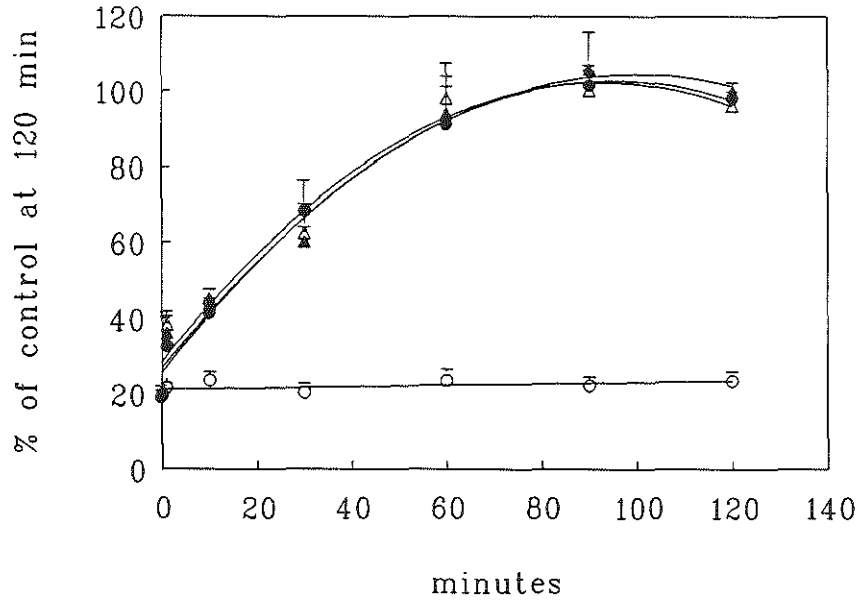

Figure 2
Time dependent cell associated radioactivity of ^{3}H-α-AIB, in the presence of 50 mmol/L unlabeled α-AIB (▲), 2 thyroid hormone transport inhibiting monoclonal antibodies (•) and (△), and 50 mmol/L Me-α-AIB (○), n ≥ 3, mean (SD).

shown. The 2 monoclonal antibodies, that inhibit transport of T_3 and T_4 into rat hepatocytes for about 60 %, have no effect on the uptake of α-AIB. Me-α-AIB, however, inhibits the uptake of α-AIB with 80 % ($p < 0.001$).

Figure 3 shows the effects of db-cAMP and T_3 + db-cAMP on α-AIB uptake. Db-cAMP stimulates the uptake of α-AIB to about 140 % ($p < 0.005$). Extra addition of unlabeled T_3 has no effect on the stimulation by db-

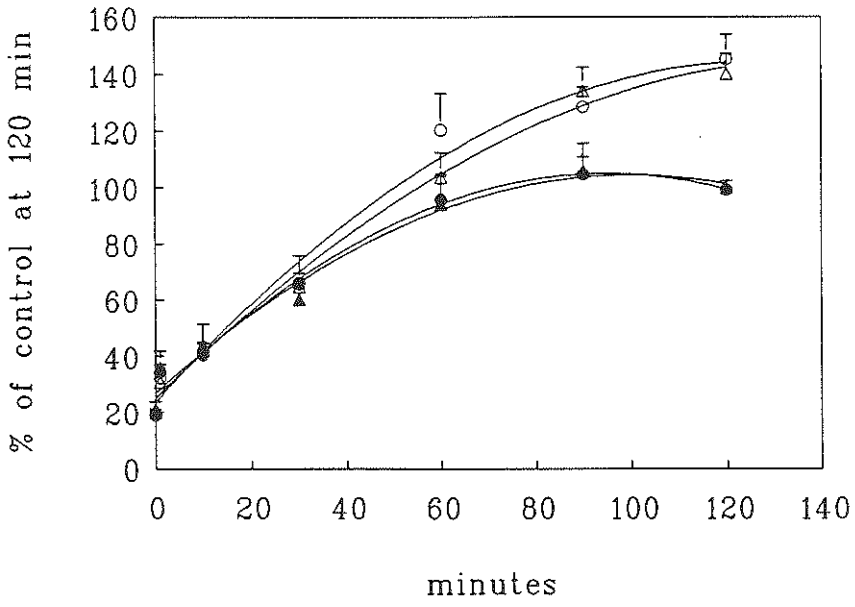

Figure 3
Time dependent cell associated radioactivity of ^{3}H-α-AIB, in the presence of 50 mmol/L (▲) and 5.5 μmol/L unlabeled α-AIB (•), db-cAMP (0.1 mM, △) and both db-cAMP and unlabeled T_3 (5.10^{-6} mol/L) (○), n ≥ 3, mean (SD).

cAMP.

The time dependent uptake of ^{125}I-T_3 is shown in Figure 4. It can be concluded from this Figure that Me-α-AIB has no effects on the uptake of T_3.

Finally, it was found that there was an effect of culture time on uptake of α-AIB: whereas uptake of T_3 is detectable after both 4 h and 20 h culture periods, uptake of α-AIB is only detectable after a 4 h culture period.

DISCUSSION

As described in the introduction, we compared the amino acid transport system A and thyroid hormone transport systems in isolated rat hepatocytes in primary culture, because of several common characteristics.

Uptake of α-AIB is time dependent and it is shown that in our test

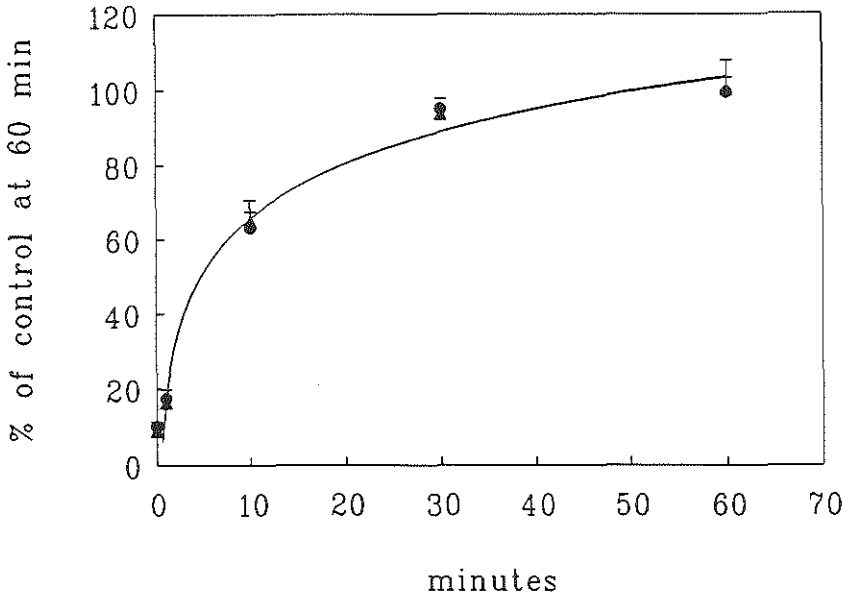

Figure 4
Time dependent cell associated radioactivity of $^{125}T_3$ (▲), in the presence of 50 mmol/L Me-α-AIB (•), n ≥ 3, mean (SD).

system this uptake can be stimulated by and inhibited with db-cAMP and Me-α-AIB, respectively, which validates the test system for investigation of the competitive effects of thyroid hormones on α-AIB uptake.

From the results, it can be concluded that there are no effects of T_3 and T_4 on α-AIB uptake and furthermore, it was found that a substrate of the A-system (Me-α-AIB) has no effects on the uptake of T_3.

These findings, which point to different transport systems for neutral amino acids and thyroid hormones in rat hepatocytes, are in accordance with reported differences in K_m of both systems. K_m of the A-system is reported to be in the millimolar range (12), whereas that of the thyroid hormones is in the nanomolar range (6). These facts led us to the conclusion that thyroid hormone transport systems appear to be distinct from the transport system A of amino acids.

REFERENCES

1 Christensen HN, De Cespedes C, Handlogten ME, Ronquist G 1973 Energization of amino acid transport, studies for the Ehrlich ascites tumor cell. Biochim Biophys Acta 300:487-522

2 Le Cam A, Freychet P 1977 Neutral amino acid transport. J Biol Chem 252:148-156

3 Le Cam A, Freychet P 1978 Effect of insulin on amino acid transport in isolated rat hepatocytes. Diabetologica 15:117-123

4 Goldfine ID, Simons CG, Ingbar SH 1975 Stimulation of the uptake of α-aminoisobutyric acid in rat thymocytes by L-triiodothyronine: a comparison with insulin and dibutyryl-cyclic-AMP. Endocrinology 96:802-805

5 Sips HJ, Groen AK, Tager JM 1980 PLasma-membrane transport of alanine is rate-limiting for its metabolism in rat-liver parenchymal cells. FEBS lett 119:271-274

6 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314-320

7 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1982 Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229-233

8 De Jong M, Docter R, van der Hoek HJ, Vos RA, Krenning EP, Hennemann G 1992 Transport of T_3 into the perfused rat liver and subsequent metabolism are inhibited by fasting. Endocrinology 131:463-470

9 De Jong M, Docter R, Bernard HF, Van Toor H, Vos RA, Schoenmakers CHH, Hennemann G 1989 Monoclonal antibodies inhibit thyroid hormone transport into rat liver cells. Annales Endocrinol 50 no2:118

10 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett 91:113-116

11 Snedecor GW, Cochran WG 1967 One-way classifications. Analysis of variance. In: Statistical Methods, 6th ed. The Iowa Univ. Press, Ames, Iowa USA. p 258-298

12 Fehlmann M, Le Cam A, Kitabgi P, Rey JF, Freychet P 1979 Regulation of amino acid transport in the liver. J Biol Chem 254:401-407

CHAPTER 9

THE PRESENCE OF A GRADIENT OF FREE T_4 AND T_3 OVER THE RAT LIVER CELL MEMBRANE. INFLUENCE OF MEDIUM FLOW RATE ON HANDLING OF T_4 AND T_3 IN PERFUSED RAT LIVERS.

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Submitted.

ABSTRACT

The possible existence of a gradient of free T_4 and T_3 over the liver cell membrane was studied in isolated perfused rat livers. Therefore, T_4 and T_3 disappearance from the medium was investigated using a two-pool model; both transport and metabolic parameters were determined. Using the fractional transfer rate constants for influx (k_{21}) and efflux (k_{12}), free hormone clearances were calculated for influx and efflux. For T_4 and T_3, it appeared that the ratio of these parameters was 18.2 and 18.8, respectively, pointing to a gradient of free T_4 and T_3 over the cell membrane.

Furthermore, we tested the influence of the perfusate flow rate on liver handling of T_4 and T_3. It appeared that for T_4 both transport and metabolic parameters were not significantly different at medium flow rates of 30, 40 or 50 mL/min. For T_3, however, at 30 mL/min both transport and metabolism were lower than at 40 or 50 mL/min. Between the last 2 flow rates no significant differences were found, indicating that at a medium flow rate of 40 mL/min is optimal for T_3 transport. These results show flow dependence of T_3 transport, which may also play a role in vivo. Interesting is also the finding that a decrease of transport into the cells due to a decrease in flow from 40 to 30 mL/min caused a decrease in total metabolism, while metabolic capacity (k_{02}) of the liver was unchanged. This indicates a regulatory role of transmembrane transport in hepatic T_3 metabolism, in agreement with earlier findings.

INTRODUCTION

3,3',5,5'-Tetraiodothyronine (T_4) is formed in the thyroid and becomes activated by deiodination to 3,3',5-triiothyronine (T_3) (1). In the deiodination process, which takes place mostly outside the thyroid, the liver plays quantitatively an important role because of its high type I deiodinase activity (1,2). So, in order to become activated, T_4 has to be transported from the plasma over the liver cell membrane into the intracellular compartment, as the deiodinating enzyme is located in the endoplasmic reticulum (1). Also for T_3 the plasma membrane transport process is very important, as in most organs T_3, that has to bind to its receptors on the nucleus to exert its effects, is derived

from plasma and not directly from intracellular T_4 conversion (3). It was long assumed that passive diffusion across the plasma membrane was sufficient to explain cellular thyroid hormone uptake. However, in the last decades the transport process of thyroid hormones over the liver cell membrane has been subject of many studies, and evidence has accumulated that cellular uptake of T_4 and T_3 into several organs is carrier mediated, and temperature and energy depedent (4-9). Therefore, this process may play a role in the regulation of the availability of T_4 to the deiodinating enzyme, thereby influencing the amount of bioactive T_3 formed. Indeed, we showed that inhibition of transport of T_4 by fructose resulted in a diminution of intracellular T_4 metabolism in the perfused rat liver (10), and also in isolated rat hepatocytes inhibition of thyroid hormone transport has been shown to be rate- limiting on intracellular conversion (11).

In contradiction with the reports regarding active transport of thyroid hormones into the liver and other organs (4-11) are the recent findings of Weisiger et al. (12). They investigated transport of T_3 into the perfused rat liver and as they were not able to find a significant difference between the free hormone clearances for influx and efflux into the liver, they concluded that in this organ T_3 uptake is equilibrative rather than actively driven by cellular energy metabolism.

In our recirculating rat liver perfusion model, in which thyroid hormone disappearance from the medium can be described by a two-pool model, it is possible to investigate both thyroid hormone transport and metabolism (13). Using this system, we were able to show energy dependent transport processes for both T_4 and T_3 (10,14). In the study presented here, we investigated and compared uptake of T_3 and T_4 and calculated the free hormone clearances for influx and efflux for both hormones.

Furthermore, in order to study to what extent intracellular thyroid hormone availability in the liver is dependent on perfusate flow rate in our perfusion system, we investigated T_4 and T_3 transport and subsequent metabolism at different flow rates (30, 40 and 50 mL/min = 3, 4 and 5 mL/min/g liver).

MATERIALS AND METHODS

Calculation of the free hormone gradient over the plasma membrane:

The free hormone clearances for the influx and efflux steps were calculated according to Weisiger et al. (8). These values are the volumes of medium or cytosol cleared of free thyroid hormone per second per gram.

For the *influx* step, the free clearance was calculated as:

$$(k_{21} \times MV) / (MFF \times LW) \text{ in } mL.s^{-1}.g^{-1}$$

k_{21} = influx rate constant (s^{-1}); MV = medium volume (mL); MFF = medium free hormone fraction; LW = liver weight (g).

For the *efflux* step, the free clearance was calculated as:

$$(k_{12} \times CV/g) / (FFDC \times (1/DF) \times ((CV/g)/(TV/g))) \text{ in } mL.s^{-1}.g^{-1} =$$
$$k_{12} / (FFDC \times (1/DF) \times (1/(TV/g))) \text{ in } mL.s^{-1}.g^{-1}$$

k_{12} = efflux rate constant (s^{-1}); CV/g = cytosol volume per gram liver (mL/g); DF = dilution factor of liver piece; FFDC = free hormone fraction in diluted cytosol; TV/g = total volume per gram liver (mL/g); assumed to be 1 mL/g; (CV/g)/(TV/g) = fraction of total liver composed of cytosol.

If the overall uptake process is active, then the ratio between these two values for the free clearances should be greater than one.

See further Chapter 2.

RESULTS

Gradient: Typical examples of T_4 and T_3 medium disappearance curves are shown in Figure 1A and 1B, respectively, as semi-log plots of the thyroid hormone concentration expressed as percentage dose/mL against time. In all experiments this medium disappearance can be described as the sum of two exponentials, indicating that the use of a two-exponential disappearance curve is adequate to describe the changes in tracer T_4 and T_3 in the medium.

In Table 1A and 1B, several transport and metabolic parameters obtained after 60 min perfusion of livers with T_4 or T_3 are shown. The medium flow through the liver was 40 mL/min. As calculated by the two-pool model, the mean total T_4 and T_3 concentrations in the medium over the 60 min perfusion were 6.7 nmol/L and 0.5 nmol/L, respectively, after addition of 10 nmol/L T_4 and 2 nmol/L T_3 to the medium. % FT_4 in the perfusion medium was 0.28 ± 0.01 % (n=12, mean ± SD)) and % FT_3 was 1.76 ± 0.04 % (n=12) as measured by equilibrium dialysis, whereas with ultrafiltration 0.29 ± 0.02 % and

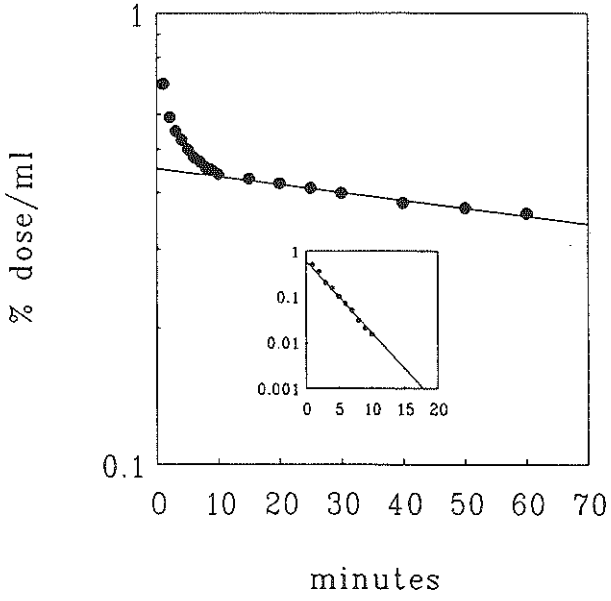

Figure 1A:
Typical example of the curve fitting of a ^{125}I-T_4 disappearance curve, which represents the sum of 2 exponentials, by a two-exponential model. Plot of log % dose per mL versus time, with the least squares regression line on the final straight part of the curve (slow component). Inset: log plot of the fast component, values obtained after subtracting the slow component, using the least squares regression line.

1.73 ± 0.05 % was found for T_4 and T_3, respectively. Percentage free T_4 in the medium is much lower than % free T_3, as T_4 has a higher affinity for albumin in the medium than T_3. The resulting mean free hormone concentrations in the medium were 19.4 pmol/L for T_4 and 8.7 pmol/L for T_3.

It can be seen that great differences exist between the model parameters for T_4 and T_3 (Tables 1A and 1B). Per pmol/L free hormone, more T_4 is transported into the liver than T_3, but disposal of T_4 per pmol/L free hormone is smaller than that of T_3. The half times of the two parts of the biphasic medium disappearance are longer for T_4 than T_3.

In the diluted cytosol of control livers (liver piece 6 x diluted), the free fractions of T_4 and T_3 were 0.0276 ± 0.0012 and 0.0516 ± 0.0024, respectively. The calculated mean free clearances of T_4, were 9.27 and 0.51

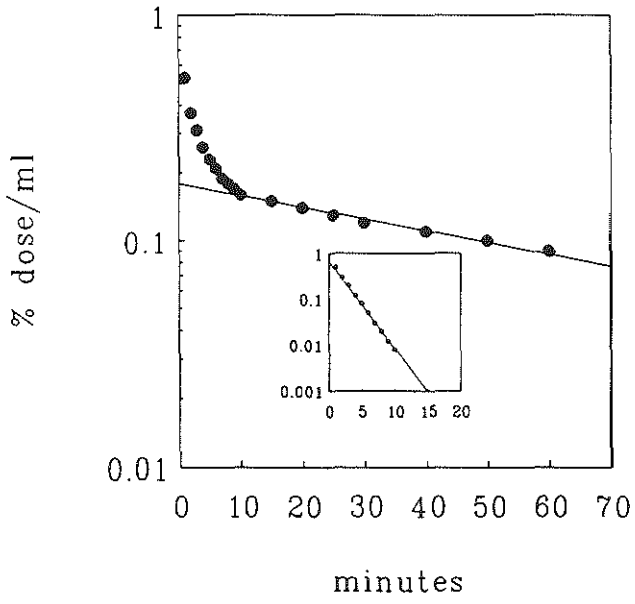

Figure 1B:
Typical example of the curve fitting of a ^{125}I-T_3 disappearance curve, which represents the sum of 2 exponentials, by a two-exponential model. Plot of log % dose per mL versus time, with the least squares regression line on the final straight part of the curve (slow component). Inset: log plot of the fast component, values obtained after subtracting the slow component, using the least squares regression line.

$mL.s^{-1}.g^{-1}$ for influx and efflux, respectively (Table 1B). The average free clearances of T_3, were 5.07 and 0.27 $mL.s^{-1}.g^{-1}$ for influx and efflux, respectively (Table 1B). From these values, gradients of 9.27 / 0.51 = 18.18 for T_4 and 5.07 / 0.27 = 18.78 for T_3 can be calculated, consistent with active, concentrative transport across the plasma membrane of both T_4 and T_3.

Influence of medium flow rate: The perfusion pressure necessary to obtain a medium flow of 30, 40 and 50 mL/min was 8.4 ± 0.9 (n=8), 12.1 ± 1.5 (n=8) and 16.0 ± 1.4 (n=8) cm (water), respectively.

In Table 2, the results of perfusion with different medium flow rates (30, 40 and 50 mL/min) on transport and metabolism of T_4 are shown. It appears that none of the transport and metabolic parameters of T_4 as measured with

Table 1A
Transport and metabolic parameters of T_4 and T_3 after 60 min perfusion with a medium flow of 40 mL/min.

	T_4	T_3
dose (pmol/150 mL)	1500	300
mean TH medium (pmol/L)	6695 ± 176	504.4 ± 59.8
FTH fraction medium (ultrafiltr)	0.0029 ± 0.0001	0.0173 ± 0.0001
mean FTH medium (pmol/L)	19.43 ± 0.01	8.65 ± 0.09
$t_{1/2}$ fast component (min)	2.40 ± 0.38	1.42 ± 0.07
$t_{1/2}$ slow component (min)	395.1 ± 33.1	76.3 ± 6.59
TMT (pmol)	5864 ± 324.3	1466 ± 8.5
disposal (pmol)	233.3 ± 19.2	135.5 ± 7.56
TMT/FTH (pmol per pmol/L free)	301.8 ± 16.7	169.5 ± 1.0
disposal/FTH (pmol per pmol/L free)	12.01 ± 0.28	15.7 ± 0.9

Both groups n=4, mean ± SEM. F = free fraction, TH = thyroid hormone, $t_{1/2}$ FC = $t_{1/2}$ of the fast component, $t_{1/2}$ SC = $t_{1/2}$ of the slow component, TMT = total mass transfer.

Table 1B
Transport and metabolic parameters of T_4 and T_3 after 60 min perfusion with a medium flow of 40 mL/min.

	T_4	T_3
k_{21} (s^{-1})	0.0018 ± 0.0002	0.0056 ± 0.0005[@]
k_{12} (s^{-1})	0.0023 ± 0.0002	0.0023 ± 0.0002
FTH medium (ultrafiltr)	0.0029 ± 0.0001	0.0173 ± 0.0001[@]
FTH in diluted cytosol	0.0276 ± 0.001	0.0516 ± 0.002[@]
medium volume (mL)	150	150
dilution factor liver	6	6
liver weight (g)	10.2 ± 0.72	9.7 ± 0.61
F Cl Infl ($mL.s^{-1}.g^{-1}$)	9.3 ± 1.3	5.1 ± 0.49[@]
F Cl Effl ($mL.s^{-1}.g^{-1}$)	0.51 ± 0.07	0.27 ± 0.02[@]
gradient	18.2 ± 1.5	18.8 ± 2.2

[@]: $p<0.001$ versus the value for T_4. Both groups n=4, mean ± SEM. F Cl Infl = free clearance for influx, F Cl Effl = free clearance for efflux, gradient = ratio of free clearance for influx versus free clearance for efflux.

the recirculating rat liver perfusion is dependent on the perfusate flow rate in

the range tested, as no signifant differences were found. As for T_3 (Table 3), the lowest perfusate flow rate gave significant lower values for some parameters, compared to those obtained with the flow rate of 40 mL/min. This holds for the fractional influx rate constant (k_{21}), total mass transfer and total disposal. In agreement with these findings, $t_{1/2}$ of the fast component of the

Table 2
Transport and disposal parameters of T_4 after 60 min perfusion with medium flow rates of 30, 40 or 50 mL/min.

	30 mL	40 mL	50 mL
$t_{1/2}$ FC (min)	2.46 ± 0.12	2.40 ± 0.38	2.47 ± 0.24
$t_{1/2}$ SC (min)	337.8 ± 27.7	395.1 ± 33.1	327.8 ± 32.2
k_{21} (min^{-1})	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
k_{12} (min^{-1})	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
k_{02} (min^{-1})	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.001
TMT (pmol)	5912 ± 444	5864 ± 324	5886 ± 218
disposal (pmol)	244.8 ± 20.9	233.3 ± 19.2	240.7 ± 13.3

All groups n=4, mean ± SEM. $t_{1/2}$ FC = $t_{1/2}$ of the fast component, $t_{1/2}$ SC = $t_{1/2}$ of the slow component, TMT = total mass transfer.

biphasic medium T_3 disappearance and $t_{1/2}$ of the slow component are both lower at a medium flow rate of 30 mL/min than at 40 mL/min. The fractional disposal rate constant (k_{02}) is not dependent on an increase in the flow rate, which is not surprising, as the quality of the livers is the same under all experimental conditions. When the medium flow rate is further increased to 50 mL/min, no further increase of the T_3 transport and disposal parameters is found, showing that at the medium flow rate of 40 mL/min, which is normally used in our perfusion studies, uptake of T_3 is optimal under the conditions used.

DISCUSSION

Gradient: An important finding in these studies is the presence of a gradient of free T_4 and T_3 across the liver cell membrane from medium to liver, using a recirculating rat liver perfusion system. In this system, the biphasic

Table 3
Transport and disposal parameters of T_3 after 60 min perfusion with medium flow rates of 30, 40 or 50 mL/min.

	30 mL	40 mL	50 mL
$t_{1/2}$ FC (min)	2.23 ± 0.12$^{@@@@}$	1.42 ± 0.07	1.60 ± 0.18
$t_{1/2}$ SC (min)	99.0 ± 2.27$^{@}$	76.3 ± 6.59	81.3 ± 8.60
k_{21} (min^{-1})	0.22 ± 0.01$^{@@}$	0.34 ± 0.03	0.31 ± 0.02
k_{12} (min^{-1})	0.120 ± 0.006	0.135 ± 0.01	0.125 ± 0.006
k_{02} (min^{-1})	0.011 ± 0.001	0.013 ± 0.002	0.013 ± 0.002
TMT (pmol)	1175 ± 56.5$^{@@@@}$	1466 ± 8.5	1351 ± 27.4
disposal (pmol)	113.4 ± 1.73$^{@}$	135.5 ± 7.56	132.0 ± 10.43

$^{@}$: $p<0.025$, $^{@@}$: $p<0.01$, $^{@@@@}$: $p<0.001$ versus flow of 40 mL/min. All groups n=4, mean ± SEM. $t_{1/2}$ FC = $t_{1/2}$ of the fast component, $t_{1/2}$ SC = $t_{1/2}$ of the slow component, TMT = total mass transfer.

disappearance of T_4 and T_3 from the medium is described by a two-pool model (13), which can be used to calculate fractional transfer rate constants of influx (k_{21}), efflux (k_{12}) and disposal (k_{02}), total mass transfer and total disposal.

It appeared that per pmol/L free hormone more T_4 than T_3 was transported to the liver. This is in accordance with our earlier findings in humans on the effects of caloric deprivation and propranolol treatment on transport of thyroid hormones (18,19). We found, using a three-pool model to describe thyroid hormone kinetics, that per pmol/L free hormone also more T_4 than T_3 was transported to the fast pool (mainly composed of the liver). Also, in these studies, we found a smaller k_{21} value for T_4 than for T_3, in spite of the greater value of total mass transfer. This is caused by the fact that the T_4 pool in the (perfusion medium/)plasma is much larger than the T_3 pool, whereas the free fraction of T_4 is smaller than that of T_3.

Although total mass transfer of T_4 per pmol/L free T_4 is 80 % higher than that of T_3 (Table 1A), disposal of T_4 per pmol/L free T_4, however, is 20 % less than that of T_3. This is in accordance with our findings that of the amount of T_3 that is transported into the metabolizing compartment of the liver (which is about 50 % of total mass transfer from medium with 1 % albumin), about 20

% is metabolized (13). For T_4 this is only 9 % (10).

The free hormone clearances of influx and efflux, which are the volumes of medium and cytosol, respectively, cleared of free thyroid hormone per second per gram of liver, are useful measures of the efficiency of transport steps driven by the free hormone concentration (12). From the hormone clearances for influx and efflux transport gradients of 18.18 and 18.78 could be calculated for T_4 and T_3, respectively (Table 1B). Therefore, this study gives further evidence that transport of T_4 and T_3 over the liver cell membrane is an active process, and is in agreement with the findings of a transport gradient in the liver by Oppenheimer et al. (20). However, the findings of Weisiger et al. (12) that T_3 uptake is equilibrative rather than actively driven by cellular energy metabolism are in contradiction with our findings of active transport (4-7,9-11). Apart from our studies, different groups have shown active, sodium dependent, transport of thyroid hormones into different tissues in several species (8,21-23).

Weisiger et al. used the multiple-indicator dilution (MID) method, which can also measure the peak influx rate constant, like the recirculating liver perfusion technique combined with a simple compartmental model. The difference between the findings of the latter authors and ours lies in the different results obtained for the influx rate constant and in the different calculation methods. As for the first reason, Weisiger et al. calculated k_{21} as the fraction of the sinusoidal volume cleared of T_3 per sec and found a value of 0.126 s^{-1}. We can calculate this parameter for T_3 with the results obtained in our perfusion system. The volume of the sinusoids is 16 % of the total liver volume (12) = 1.55 mL. The mean medium T_3 concentration during the perfusion is 504.4 pmol/L (Table 1A), so in 1.55 mL 0.78 pmol T_3 is present. Total mass transfer per sec is 0.41 pmol (Table 1A). This means that k_{21} in our system calculated according to the method of Weisiger, would be 0.41 / 0.78 = 0.525 s^{-1}. As for the second cause for the differences between the findings of Weisiger et al. and ours, we have directly measured the free fraction in the (6x) diluted cytosol (FFDC) (Table 1B) and also the amount of cytosol/g liver (CV/g), i.e. about 0.2 mL/g. Therefore, the free fraction in the cytosol is 0.0516 x (1/6) x 0.2 = 0.00172, in good agreement with the findings of Weisiger (0.00114, ref. 12) and Oppenheimer (0.00145, ref. 20). However, as the ratio CV/g is present in both the numerator and the denominator of the

basic formula describing the free clearance of efflux, it is absent in the final one (see Methods). Weisiger et al., however, measured the free fraction in the cytosol by ultrafiltration (0.00114), but used for calculation of CV/g in the numerator an approach different from ours, namely (1 minus the albumin space = 0.839). To our opinion this is not correct, as the cellular volume minus the albumin space does not represent the cytosol, but cytosol plus volume of intracellular organelles. As the value of CV/g (0.2) in our experiments is 4 times smaller than that of Weisiger et al. (0.839), we find a much smaller free clearance for the efflux and therefore a greater gradient. Our value of CV/g (0.2) is however in good agreement with the value of 0.28 of Oppenheimer et al. (20).

Recently, we have shown that total mass transfer of thyroid hormones to the liver from medium with 1 % albumin was composed for about 50 % of transport to an intracellular, metabolizing compartment and for 50 % of transport into an extracellular compartment (10,13,14). Transport to the intracellular pool was dependent on the free hormone concentration in the medium, whereas transport to the extracellular pool was dependent on the medium free hormone concentration and albumin concentration. It was further shown that 48 h fasting or addition of fructose to the medium inhibited transport to the intracellular pool, but not transport to the extracellular pool (10,14). This means that the k_{21}-value, as used in the calculation of the transport gradient, does not only represent transport into the cytosol, but also transport to the extracellular compartment. Correction for this overestimation of the k_{21}-value would decrease the found transport gradient. However, in the same way, k_{12} does not only represent efflux from the cytosol, but also efflux from the extracellular compartment. Correction for this overestimation of k_{12} would increase the transport gradient and might neutralize the effect of overestimation of k_{21}. Therefore, to our opinion, discrimination between transport to the intracellular and extracellular pool is for calculation of the transmembrane gradient not of great interest.

As experiments by Weisiger et al. (12) using the MID method last less than 1 min, this method seems less suitable for estimation of the size of the liver pool and the amount of hormone metabolized per time unit; parameters that can only be estimated after equilibrium is reached between medium and

liver. These parameters are very important for our understanding of the fate of the hormone under physiological conditions. Furthermore, under physiological conditions the liver is never challenged with a concentration gradient as large as in the MID technique, but only with slowly changing thyroid hormone concentrations in plasma (medium). As in vivo transport differences between inflow and outflow of T_4 or T_3 over the liver are very small and not possible to quantitate by the present techniques (24), we think it justified at least for thyroid hormones to consider the concentration in the sinusoids of the liver nearly constant and comparable with the medium concentration.

A critique on simple lumped models, like our liver perfusion model, is that in these models efflux to the medium may be underestimated as a result of re-uptake into adjacent liver cells (12). However, we may estimate the magnitude of this according to (12). If we assume a uniform distribution of cellular T_3 along the length of the sinusoid, then the mean time required for T_3 to exit the sinusoid (t) after efflux is half the sinusoidal transit time. The fraction that reenters the liver in this time is given by $(1 - \exp(-k_{21}t))$, according to the sinusoidal model. K_{21} in the sinusoidal model is 0.53 s^{-1} (see above), the sinusoidal transit time t can be calculated as the ratio of the sinusoidal volume (= 1.55 (see above)) and the medium flow rate (=40 mL/min), = 2.3 sec. Thus, half the sinusoidal transit time is 1.2 sec. Using these values, the reuptake fraction should be 0.53. Thus, the actual efflux constant may be estimated by dividing the apparent efflux constant by 1 - 0.53 = 0.47. In this case we find a ratio for the free clearance of influx versus efflux of 0.47 x 18.78 = 8.8, still pointing to active transport.

Using our perfusion system, transport parameters of T_4 to the liver have been shown to correlate very nicely with liver ATP concentrations (10). In these studies livers were perfused with or without fructose in the medium, which is known to decrease the intracellular ATP concentration. The results point to ATP-dependence of thyroid hormone transport. Similar results were obtained in humans in whom initial T_4 uptake into the liver was decreased after i.v. administration of fructose (10). Furthermore, in perfused livers of 48 h starved rats, we showed an inhibition of transport of T_3 into the intracellular liver compartment (14). It was hypothesized that decreased transport after fasting was caused by a decrease in intracellular ATP levels. Indeed it has been

shown that during fasting intracellular ATP is decreased in vivo (25).

Influence of medium flow rate: Our k_{21} of T_3 (0.34 min^{-1}) using a medium flow rate of 40 mL/min agrees well with the theoretically maximal rate constant of 40/130 (= flow/medium volume) = 0.31 min^{-1}, pointing to the fact that under these conditions all T_3 delivered to the liver per min is indeed being removed from the medium. Therefore, in order to study to what extent thyroid hormone availability in the liver is dependent on perfusate flow rate, we have performed studies to measure the transport parameters at different medium flow rates. In the flow-experiments in this study using T_3, it appeared that after an increase in the flow rate from 30 to 40 mL/min a proportional increase was seen in T_3 transport parameters. However, no further effect on T_3 transport was seen when the flow rate was increased to 50 mL/min. This indicated that below 40 mL/min flow rate was rate-limiting on transport.

An important finding is the fact that when transport is limited at a flow rate of 30 mL/min, also disposal is decreased, pointing to a regulatory role of the transport process on further intracellular metabolism. This study is therefore in accordance with our earlier in vitro studies that also showed that the carrier-mediated uptake process of thyroid hormones is rate-limiting on intracellular metabolism (11).

It is important to observe that the lower total disposal of T_3 at a flow rate of 30 mL/min, can not be explained by a decrease of liver quality due to e.g. insufficient liver supply of oxygen at this lower perfusion rate. It has been published that adequate oxygen supply to the liver can be reached in aqueous solutions at high pO_2, requiring a perfusate flow of at least 2 mL/min/g liver (26), which means at least 20 mL/min in our experiments. In accordance with this is the fact that the fractional disposal rate constant (k_{02}) of T_3 is not significantly different at flow rates of 30, 40 and 50 mL/min (Table 3). Furthermore, perfusion with T_4 shows at all medium flow rates the same total mass transfer and disposal, underlining that a medium flow rate of 30 mL/min does not impair liver quality. This while it has been shown that T_4 transport is much more sensitive to a decrease in intracellular ATP concentration of the liver than is transport of T_3 (5). So, these arguments strongly militate against a decrease of liver quality during perfusion at a medium flow rate of 30

mL/min.

We may try to translate the meaning of these in vitro results with regard to (partial) flow dependence of T_3 transport to the in vivo situation. It is of interest to note that in the rat no TBG is present in the blood, being the most important thyroid hormone binding protein in plasma in man. Thyroid hormones in the circulation of rats are for the greatest part bound to TBPA and to a lesser extent to albumin (27). We have performed rat liver perfusions with T_3 in the presence of 4 % albumin in the medium (flow rate 40 mL/min, % FT_3 = 0.5 % (13)), and found a fractional influx rate constant (k_{21}) of 0.28 ± 0.03 (mean ± SD, n=4, unpublished results), which is not significantly different from the value that is obtained with 1 % albumin in the perfusion medium. The % FT_3 in vivo in rats is about 0.35 % (28), which is slightly lower than our free fraction in medium with 4 % albumin. The physiological liver flow rate in vivo, however, is only about 1 mL/min/gram in several species (29,30), which is 3 times lower than the medium flow rate at which we found flow dependence of T_3 transport, therefore, it is very likely that T_3 transport into the liver in the rat is (at least partially) flow dependent. The inhibitory influence of e.g. fasting is much more pronounced on transport of T_4 than on transport of T_3 (18) into human livers, suggesting that flow dependence of T_3 transport may also play a role in man in vivo.

In summary, the present study shows the existence of a concentration gradient for free T_4 and T_3 across the liver cell membrane. Furthermore, it shows that uptake of T_3, but not of T_4, is flow dependent at a medium flow rate of 30 mL/min, which phenomenon may also play a role in vivo. Of great interest is the finding that a decrease of transport into the cells also causes a decrease in total metabolism of thyroid hormone, indicating a regulatory role of transport on metabolism, which is in agreement with earlier findings (11).

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REFERENCES

1 Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Thyroid hormone metabolism. G. Hennemann. editor. Marcel Dekker, New York and Basel. p189-229

2 Kaplan MM, Utiger RD 1978 Iodothyronine metabolism in rat liver homogenates. J. Clin. Invest. 61:459-471

3 Van Doorn J, Van der Heide D, Roelfsema F 1983 Sources and quantity of 3,5,3'-triiodothyronine in several tissues in the rat. J Clin Invest 72:1778-1972

4 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314-320

5 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1982 Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229-233

6 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett. 91:113-116

7 Everts ME, Docter R, Van Buuren JCJ, Van Koetsveld PM, Hofland LJ, De Jong M, Krenning EP, Hennemann G 1993 Evidence for carrier-mediated uptake of triiodothyronine in cultured anterior pituitary cells of euthyroid rats. Endocrinology, in press.

8 Holm AC, Wong KY, Pliam NB, Jorgensen EC, Goldfine ID 1980 Uptake of L-triiodothyronine into human cultured lympfocytes. Acta Endocrinol (Copenh) 95:30-33

9 Docter R, Krenning EP, Bernard HF, Hennemann G 1987 Active transport of iodothyronines into human cultured fibroblasts. J Clin Endocrinol Metab 65:624-628

10 De Jong M, Docter R, Bernard HF, Van der Heijden JTM, Van Toor H, Krenning EP, Hennemann G 1993 T_4 transport into the perfused rat liver and computed liver T_4 uptake in humans is inhibited by fructose. Accepted for publication (Am J Physiol)

11 Hennemann G, Krenning EP, Polhuis M, Mol JA, Bernard HF, Visser TJ, Docter R 1986 Carrier-mediated transport of thyroid hormones into rat hepatocytes is rate limiting in total cellular uptake. Endocrinology 119:1870-1872

12 Weisiger RA, Luxon BA, Cavalieri RR 1992 Hepatic uptake of 3,5,3'-triiodothyronine: electrochemical driving forces. Am J Physiol 262:G1104-G1112

13 Docter R, De Jong M, Van der Hoek HJ, Krenning EP, Hennemann G 1990 Development and use of a mathematical two pool model of distribution and metabolism of 3,3',5-triiodothyronine in a recirculating rat liver perfusion system: albumin does not play a role in cellular transport. Endocrinology 126:451-459

14 De Jong M, Docter R, Van der Hoek HJ, Vos RA, Krenning EP, Hennemann G 1992 Transport of T_3 into the perfused rat liver and subsequent metabolism are inhibited by

fasting. Endocrinology 131:463-470

15 Meijer KF, Keulemans K, Mulder GJ 1981 Isolated perfused rat liver technique. Methods in Enzymology 77:81-94

16 Sterling K, Brenner MA 1966 Free thyroxine in human serum: simplified measurement with the aid of magnesium precipitation. J Clin Invest 45:153-163

17 Snedecor GW, Cochran WG 1967 One-way classifications. Analysis of variance. In: Statistical Methods, 6th ed. 258-298. The Iowa Univ. Press, Ames, Iowa USA

18 Van der Heijden JTM, Docter R, Van Toor H, Wilson JHP, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251:E156-E163

19 Van der Heijden JTM, Krenning EP, Van Toor H, Hennemann G, Docter R 1988 Three-compartmental analysis of effects of D-propranolol on thyroid hormone kinetics. Am J Physiol 255:E80-E86

20 Oppenheimer JH, Schwartz HL 1985 Stereospecific transport of triiodothyronine from plasma to cytosol and from cytosol to nucleus in rat liver, kidney, brain and heart. J Clin Invest 75:147-154

21 Blondeau JP, Osty J, Francon J 1988 Characterization of the thyroid hormone transport system of isolated hepatocytes. J Biol Chem 263:2685-2692

22 Pontecorvi A, Robbins J 1986 Energy-dependent uptake of 3,3,3'-triiodo-L-thyronine in rat skeletal muscle. Endocrinology 119:2755-2761

23 Centanni M, Mancini G, Andreoli M 1989 Carrier-mediated [^{125}I]-T_3 uptake by mouse thymocytes. Endocrinology 124:2443-2448

24 Bauer AGC, Wilson JHP, Lamberts SWJ, Docter R, Hennemann G, Visser TJ 1987 Handling of iodothyronines by liver and kidney in patients with chronic liver disease. Acta Enocrinol 116:339-346

25 Cunningham CC, Malloy CR, Radda GK 1986 Effect of fasting and acute ethanol administration on the energy state of in vivo liver as measured by 31P-NMR spectroscopy. Biochim Biophys Acta 885:12-22

26 Bartosek I, Guaitani A, Miller LL 1983 In: Isolated liver perfusion and its applications (Bartosek I, Guaitani A, Miller LL, Eds.) Raven press, New York. pp 1-276

27 Sutherland RL, Simpson-Morgan MW 1975 The thyroxine binding properties of serum proteins. A competitive binding technique employing Sephadex G-25. J Endocrinol 65:319-332

28 Rutgers M, Bonthuis F, De Herder WW, Visser TJ 1987 Accumulation of plasma triiodothyronine sulfate in rats treated with propylthiouracil. J Clin Invest 80:758-762

29 Greenway CV, Stark RD 1971 Hepatic vascular bed. Physiol Rev 51:23-65

30 Ross BD 1972 Perfusion techniques in biochemistry: a laboratory manual. Clarendon Press, Oxford

CHAPTER 10

GENERAL DISCUSSION

In this thesis, studies on the (patho)physiological significance of the transmembrane thyroid hormone transport process into liver cells are described.

Figure 1 shows the role of this transport process in (peripheral) thyroid hormone synthesis and metabolism. T_4 is exclusively formed in the thyroid (1). T_3, the only bioactive thyroid hormone, and rT_3, which is an inactive metabolite, are also synthetized in this gland, but their secretion amounts to only 20 and 9 % of their total daily production, respectively (1). The remainder is formed by extrathyroidal deiodination of T_4. For the synthesis of thyroid hormones, iodide is taken up from the circulation via an energy-dependent iodide pump, oxidized and bound to tyrosyl residues of thyroglobulin. At the end of this process, iodothyronines are secreted into the blood stream (2). The major factor stimulating the function of the thyroid (step A in Figure 1) is TSH (3). Step A can be inhibited by metabolic inhibitors, competitive anions, and inhibitors of the organification process (4-6).

Production of plasma T_3 and clearance of rT_3 takes place predominantly in tissues with PTU-sensitive, type I deiodinase activity. In these processes the liver plays quantitatively the most important role, the contribution of the kidneys is less important (7,8).

Originally, it was thought that thyroid hormones, by way of their lipophilic nature, would pass the lipid bilayer of the cell membrane by diffusion to become intracellularly handled. However, this appeared not to be the case as will be outlined below. Thyroid hormones are transported into the liver by two different energy-dependent, active uptake systems, one for T_4 and rT_3 (depicted as a square, step B in Figure 1) and one for T_3 (depicted as a circle, step C in Figure 1), both in rat and in man (9-13). Step B and C are suggested to occur via receptor-mediated endocytosis (14). From the latter study (14) and studies with monoclonal antibodies (13,15), it appeared that diffusion plays no role in transmembrane transport of thyroid hormones. Step B and C are saturable (10) and can be inhibited by various compounds (16), including CMPF (17), indoxyl sulfate (17), bilirubin (18) and non-esterified fatty acids (18) and by low temperature (16). Step B and C are also impaired during e.g. illness (19,20), fasting (21,22), hyperthyroidism (23), and by fructose treatment in vivo (24), showing that also in vivo the transport process is energy

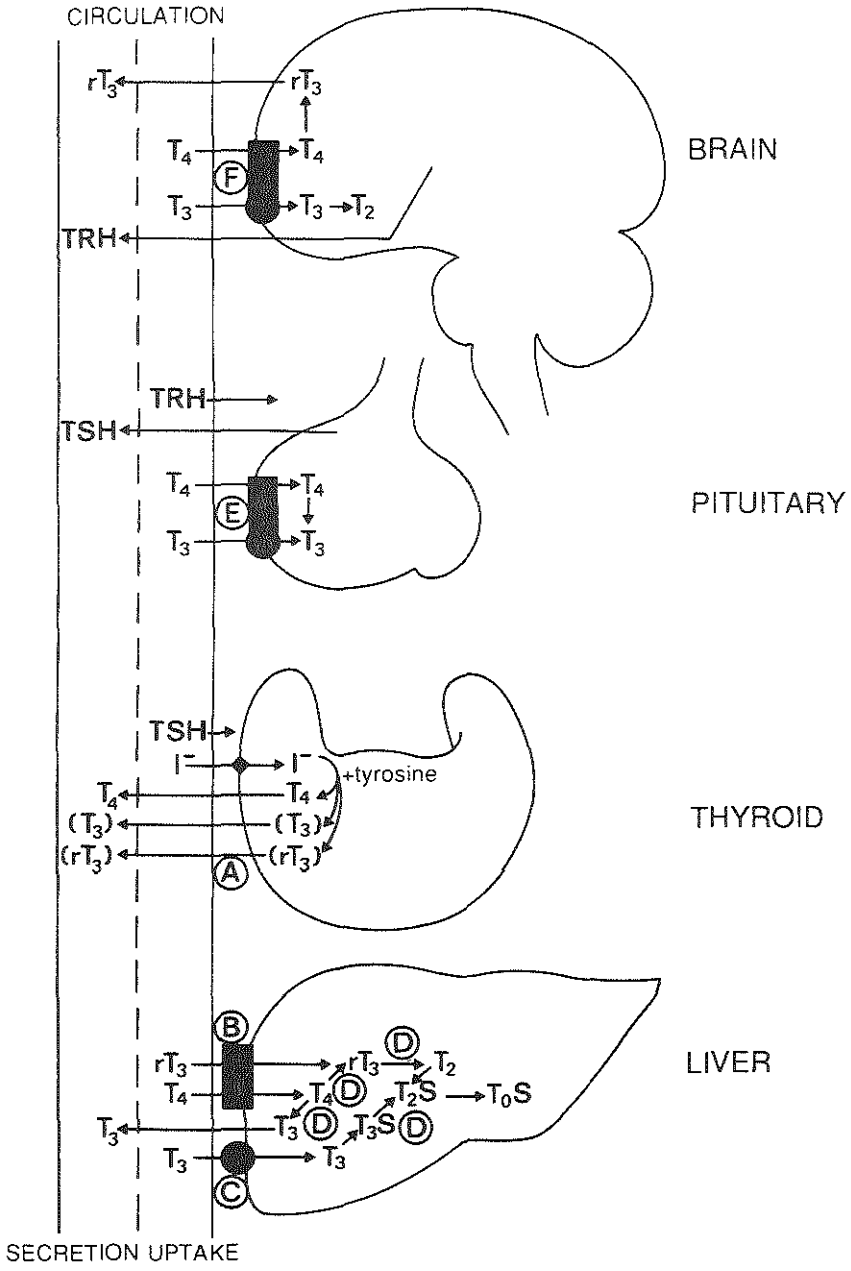

Figure 1
Model of thyroid hormone synthesis and metabolism.

dependent. It has been described that after fasting intracellular ATP is decreased (25), suggesting that transport inhibition after caloric deprivation could be caused by a decrease of intracellular ATP. CMPF and indoxyl sulfate, both present in high concentrations in uremia, caused T_4 transport inhibition into isolated rat hepatocytes (17). Therefore, it was suggested that these substances may account for the low T_3 in uremic patients by inhibiting T_4 transport into the T_3-producing tissues. In non-uremic NTI, serum non-esterified fatty acids and bilirubin are frequently elevated and may be the putative inhibitors of thyroid hormone transport in this group of NTI patients (18), as these substances caused transport inhibition into rat hepatocytes, that was significantly correlated with the molar ratios of bilirubin:albumin and non-esterified fatty acid:albumin. In addition to these mentioned compounds, a lowered ATP may as well play a role in transmembrane transport inhibition of thyroid hormones in NTI, as in this condition a negative energy balance almost invariably exists. The decrease of transport into the cells during fasting and illness, resulting in the "low T_3 syndrome", may play a regulatory role in subsequent intracellular thyroid hormone metabolism and action. The induction of the low T_3 syndrome is considered as an energy and protein saving, adaptive mechanism in situations of stress (26).

Amiodarone treatment of rats inhibits step B, but not step C in liver perfusion using livers of treated animals, suggesting that also in the intact organ T_4 and T_3 are transported via separate transport systems (27). Recently, we also reported evidence for 2 different transport systems for T_4 and T_3 in vivo in a human subject, suffering from a T_4 and rT_3 transport inhibition syndrome in the liver, whereas T_3 transport was not affected at all (28). Step B and C are not identical to amino acid transport system A, in spite of several common characteristics (29). As shown in rat liver perfusion studies, albumin does not play a role in cellular thyroid hormone uptake, it only stimulates transport to the non-metabolizing, presumably extracellular, liver compartment (22-24,27,30). Transport to this non-metabolizing pool was not influenced by 48-h fasting (22) or addition of fructose to the medium (24), whereas transport into the metabolizing pool (independent of the albumin concentration) and subsequent intracellular metabolism were severely affected during these conditions. This indicates that thyroid hormone transport to the non-

metabolizing pool is not energy dependent and that hormone in this pool is not available for metabolism. Thus, transport to this pool might represent binding to the outside of the cells, in agreement with data observed in isolated rat hepatocytes (10).

After uptake, T_4 is degraded to either rT_3 (step D in Figure 1), which is rapidly further deiodinated (31), or T_2 (step D), which is mainly released into the circulation in favour of T_3-dependent tissues, that do not or to a lesser extent deiodinate T_4 (32). T_3 taken up into the liver is not a good substrate for the type I deiodinase, unless T_3 is sulfated first to T_3S (31). In a similar way, deiodination of T_2 occurs only after sulfation, because T_2S is a much better substrate for the type I deiodinase than T_2 itself. Step D is inactivated by PTU, is decreased in the hypothyroid and increased in the hyperthyroid state (31,33).

At the level of the pituitary gland, with type II deiodinase activity, only one transport system is depicted, as there exists (be it preliminary) evidence for one active transport system (step E in Figure 1) (34). After uptake, T_4 is deiodinated to T_3 as a local source of intracellular T_3, but also as a producer of plasma T_3 during hypothyroidism (33). The latter is important in all type II tissues, including brain and brown adipose tissue. Step E is saturable and inhibited by various drugs (34), but not by CMPF and indoxyl sulfate (35), in contrast with the findings in the liver.

Clearance of plasma T_3 and production of plasma rT_3 is located mainly in tissues such as brain, with PTU-insensitive, type III deiodinase activity. No studies have been reported examining whether also at this level thyroid hormones are taken up via two different transport systems; therefore, only one transport system (step F in Figure 1) is depicted. In the hypothalamus TRH is produced, which stimulates TSH secretion of the pituitary.

Although active transport systems for thyroid hormones have now been shown in many cell types of different species (9-11,36-50), not all findings with regard to thyroid hormone transport in vitro and in vivo are entirely uniform. Some workers did not find saturability of T_3 transport in isolated rat hepatocytes (51) or perfused rat liver (52) or they did not find a dependence on cellular energy (44). Weisiger et al. (53) concluded that in the perfused rat

liver T_3 uptake was equilibrative rather than actively driven by cellular energy metabolism. Furthermore, some studies did not show energy-dependent uptake of T_4 in the same systems in which energy-dependent T_3 uptake was demonstrated (42,54-57). There are several possible explanations for these controversial findings. Some results with regard to the demonstration of passive, instead of active, transport of thyroid hormones could be accounted for by the fact that studies were performed in freshly isolated cells (44,55). In our hands, in these conditions cellular ATP is low, thereby decreasing thyroid hormone transport in general, and T_4 transport in particular, as it is strongly dependent on intracellular ATP (11). It is possible that mainly the low affinity system has been measured in these cell suspensions, which system is not energy dependent. In studies with rat skeletal muscle (54,58), isolated intact muscles were incubated in medium with a conceivably less than optimal O_2 supply, probably leading to a less than optimal ATP concentration in the tissues. In studies with rat liver slices (59), tissue was incubated with micromolar concentrations of T_4, orders of magnitude above the K_m of the high-affinity (energy-dependent) uptake system. In this case the uptake of thyroid hormones will follow diffusion kinetics, and active transport will not be demonstrated. As for the findings of Weisiger et al. in the perfused rat liver (53), they are extensively dealt with in Chapter 9. It was shown that the difference between the findings of the latter authors and ours was caused by the different values obtained for the influx rate constant and by differences in experimental and calculation methods.

It can be concluded that discrepancy in the litterature with regard to active transmembrane transport systems for thyroid hormones can mostly be explained by the fact that less than optimal experimental conditions prevented the demonstration of active transport.

The studies described in this thesis further clarify and underscore the regulatory role of the active thyroid hormone transport processes in subsequent intracellular metabolism and action in (patho)physiological conditions.

REFERENCES

1 Hennemann G 1986 Thyroid hormone deiodination in healthy man. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 277-296

2 Björkman U, Ekholm R 1990 Biochemistry of thyroid hormone formation and secretion. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 83-125

3 Wilber JF, Yamada M 1990 Thyrotropin-releasing hormone: current concepts. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 127-145

4 Ohta M, Field JB 1966 Pyridine nucleotides in the thyroid gland. VII. Effect of thyroid stimulating hormone on ATP concentration in dog thyroid slices. Endocrinology 79:732-736

5 Wolff J, Maurey JR 1961 Thyroidal iodide transport. II. Comparison with non-thyroid iodide-concentrating tissues. Biochim Biophys Acta 47:467-474

6 Wolff J 1964 Transport of iodide and other anions in the thyroid gland. Physiol Rev 44:45-90

7 Bauer AGC, Wilson JHP, Lamberts SWJ, Docter R, Hennemann G, Visser TJ 1987 Handling of iodothyronines by liver and kidney in patients with chronic liver disease. Acta Endocrinol (Copenh) 116:339-346

8 DiStefano III JJ, Jang M, Malone TK, Broutman M 1982 Comprehensive kinetics of triiodothyronine production, distribution, and metabolism in blood and tissue pools of the rat using optimized blood-sampling protocols. Endocrinology 110:198-213

9 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1978 Active transport of triiodothyronine (T_3) into isolated rat liver cells. FEBS Lett 91:113-116

10 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314-320

11 Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G 1982 Decreased transport of thyroxine (T_4), 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229-233

12 De Jong M, Bernard HF, Van Toor H, Visser TJ, Docter R, Krenning EP, Hennemann G 1993 The presence of a gradient of free T_4 and T_3 over the rat liver cell membrane. Influence of medium flow rate on handling of T_4 and T_3 in perfused rat livers. Submitted

13 De Jong M, Visser TJ, Bernard HF, Docter R, Vos RA, Hennemann G, Krenning EP 1993 Transport and metabolism of iodothyronines in cultured human hepatocytes. J Clin Endocrinol Metab 77:139-143

14 Cheng SY, Maxfield FR, Robbins J, Willingham MC, Pastan IH 1980 Receptor-mediated uptake of 3,3',5-triiodo-L-thyronine by cultured fibroblasts. Proc Natl Acad Sci USA

77:3425-3429

15 Mol JA, Krenning EP, Docter R, Rozing J, Bernard HF, Hennemann G 1986 Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. J Biol Chem 261:7640-7643

16 Docter R, Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 233-254

17 Lim CF, Bernard HF, De Jong M, Docter R, Krenning EP, Hennemann G 1993 A furan fatty acid and indoxyl sulfate are the putative inhibitors of thyroxine metabolism in uremia. J Clin Endocrinol Metab 76:318-324

18 Lim CF, Docter R, Visser TJ, Krenning EP, Bernard HF, Van Toor H, De Jong M, Hennemann G 1993 Inhibitors of thyroxine hepatocytes transport in non-uremic critical illness. Effects of bilirubin and non-esterified fatty acids. J Clin Endocrinol Metab (in press)

19 Vagenakis AG 1981 Non-thyroid diseases affecting the thyroid hormone metabolism. In: Hesch RD (ed) The low T_3 syndrome, Academic press, New York. 40:128-139

20 Vos RA, de Jong M, Docter R, van Toor H, Bernard HF, Krenning EP, Hennemann G 1991 Morbidity-dependent thyroid hormone transport inhibition by serum of patients with non-thyroidal illness (NTI) in rat hepatocytes and in the perfused rat liver. In: Gordon, Gross and Hennemann (eds) Progress in Thyroid Research. Balkema Rotterdam. pp 693-696

21 Van der Heijden JTM, Docter R, Van Toor H, Wilson JPH, Hennemann G, Krenning EP 1986 Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T_3 syndrome. Am J Physiol 251:E156-E163

22 De Jong M, Docter R, Van der Hoek JH, Vos RA, Krenning EP, Hennemann G 1992 Transport of T_3 into the perfused rat liver and subsequent metabolism are inhibited by fasting. Endocrinology 131:463-470

23 De Jong M, Docter R, Van der Hoek JH, Vos RA, Krenning EP, Hennemann G 1993 Adaptive changes in transmembrane transport and metabolism of T_3 in perfused livers of fed and fasted hypo- and hyperthyroid rats. Accepted for publication in Metabolism.

24 De Jong M, Docter R, Bernard HF, Van der Heijden JTM, Van Toor H, Krenning EP, Hennemann G 1993 T_4 transport into the perfused rat liver and computed liver T_4 uptake in humans are inhibited by fructose. Accepted for publication in Am J Physiol

25 Cunningham CC, Malloy CR, Radda GK 1986 Effect of fasting and acute ethanol administration on the energy state of in vivo liver as measured by ^{31}P-NMR spectroscopy. Biochim Biophys Acta 885:12-22

26 Gardner DF, Kaplan MM, Stanley CA, Utiger RD 1979 Effect of triiodothyronine replacement on the metabolic and pituitary responses to starvation. New Engl J Med 300:579-584

27 De Jong M, Docter R, Van der Hoek HJ, Krenning EP, Van der Heide D, Quero C, Plaisier P, Vos RA, Hennemann G 1993 Different effects of amiodarone on transport of T_4 and T_3 into the perfused rat liver. Submitted.

28 Hennemann G, Vos RA, De Jong M, Krenning EP, Docter R 1993 Decreased peripheral T_3 production from T_4; A syndrome of impaired thyroid hormone activation due to transport inhibition of T_4 into T_3 producing tissues. J Clin Endocrinol Metab. In press.

29 De Jong M, Bernard HF, Docter R, Krenning EP, Vos RA, Hennemann G 1991 T_4 and T_3 are not transported into rat liver cells via amino acid transport system A. In: Gordon, Gross, Hennemann (eds) Progress in Thyroid Research, Balkema, Rotterdam, p 713-715

30 Docter R, De Jong M, Van der Hoek JH, Krenning EP, Hennemann G 1990 Development and use of a mathematical two pool model of distribution and metabolism of T_3 in a recirculating rat liver perfusion system: albumin does not play a role in cellular transport. Endocrinology 126:451-459

31 Visser TJ 1990 Importance of deiodination and conjugation in the hepatic metabolism of thyroid hormone. In: Greer MA (ed) The Thyroid Gland, Raven Press, New York, p255-283

32 Van Doorn J, Van der Heide D, Roelfsema F 1984 Sources and quantity of 3,5,3'-triiodothyronine in several tissues of the rat. J Clin Invest 72:1778-1792

33 Leonard JL, Visser TJ 1986 Biochemistry of deiodination. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 189-229

34 Everts ME, Docter R, Van Buuren JCJ, Van Koetsveld PM, Hofland LJ, De Jong M, Krenning EP, Hennemann G 1993 Evidence for carrier-mediated uptake of triiodothyronine in cultured anterior pituitary cells of euthyroid rats. Endocrinology 132:1278-1285

35 Hennemann G, Lim CF, Everts ME 1993 Effects of a furan fatty acid and indoxyl sulfate on ^{125}I-T_3 uptake and TSH secretion in cultured rat anterior pituitary cells. J Endocrinol Invest 16, suppl 2 to no. 6, p10

36 Rao GS, Eckel J, Rao ML, Breuer H 1976 Uptake of thyroid hormone by isolated rat liver cells. Biochem Biophys Res Commun 73:98-104

37 Parl F, Korcek L, Siegel JS, Tabachnick M 1977 Uptake of triiodothyronine and thyroxine by isolated rabbit adipocytes. FEBS Lett 83:145-147

38 Holm AC, Wong KY, Pliam NB, Jorgensen EC, Goldfine ID 1980 Uptake of L-triiodothyronine into human cultured lympfocytes. Acta Endocrinol (Copenh) 95:350-358

39 Krenning EP, Docter R, Visser TJ, Hennemann G 1983 Plasma membrane transport of thyroid hormone: its possible pathophysiological significance. J Endocrinol Invest 6:59-66

40 Horiuchi R, Cheng SY, Willingham MC, Pastan IH 1982 Inhibition of nuclear entry of 3,3',5-triiodo-L-thyronine by monodansylcadaverine in GH_3 cells. J Biol Chem 257:3139-

3144

41 Halpern J, Hinkle PM 1982 Evidence for an active step in thyroid hormone transport to nuclei: drug inhibition of L-^{125}I-triiodothyronine binding to nuclear receptors in rat pituitary tumor cells. Endocrinology 110:1070-1072

42 Pontecorvi A, Robbins J 1986 Energy-dependent uptake of 3,5,3'-triiodo-L-thyronine in rat skeletal muscle. Endocrinology 119:2755-2761

43 Docter R, Krenning EP, Bernard HF, Hennemann G 1987 Active transport of iodothyronines into human cultured fibroblasts. J Clin Endocrinol Metab 65:624-628

44 Blondeau JP, Osty J, Francon J 1988 Characterization of the thyroid hormone transport system of isolated hepatocytes. J Biol Chem 263:2685-2692

45 Movius EG, Phyillaier MM, Robbins J 1989 Phloretin inhibits cellular uptake and nuclear receptor binding of triiodothyronine in human HEPG2 hepatocarcinoma cells. Endocrinology 124:1988-1997

46 Topliss DJ, Kolliniatis E, Barlow JW, Lim CF, Stockigt JR 1989 Uptake of 3,5,3'-triiodothyronine by cultured rat hepatoma cells is inhibitable by nonbile acid cholephils, diphenylhydantoin, and nonsteroidal antiinflammatory drugs. Endocrinology 124:980-986

47 Centanni M, Mancini G, Andreoli M 1989 Carrier-mediated [^{125}I]-T_3 uptake by mouse thymocytes. Endocrinology 124:2443-2448

48 Krenning EP, Docter R 1986 Plasma membrane transport of thyroid hormone. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York. pp 107-131

49 Docter R, Krenning EP 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Monte A Greer (ed), The Thyroid Gland. Raven Press, New York. pp 233-254

50 Pontecorvi A, Robbins J 1989 The plasma membrane and thyroid hormone entry into cells. Trends Endocrinol Metab 1:36-40

51 Mooradian AD, Schwartz HL, Mariash CN, Oppenheimer JH 1985 Transcellular and transnuclear transport of 3,5,3'-triiodothyronine in isolated hepatocytes. Endocrinology 117:2449-2456

52 Terasaki T, Pardridge WM 1987 Stereospecifity of triiodothyronine transport into brain, liver and salivary gland: role of carrier- and plasma protein-mediated transport. Endocrinology 121:1185-1191

53 Weisiger RA, Luxon BA, Cavalieri RR 1992 Hepatic uptake of 3,5,3'-triiodothyronine: electrochemical driving forces. Am J Physiol 262:G1104-G1112

54 Centanni M, Robbins J 1987 Role of sodium in thyroid hormone uptake by rat skeletal muscle. J Clin Invest 80:1068-1072

55 Rao GS, Rao ML 1983 L-thyroxine enters the rat liver cell by simple diffusion. J Endocrinol 97:277-284

56 Pontecorvi A, Di Cerbo A, Gonçalves E, Antonozzi I, Andreoli M, Robbins J 1988 Thyroxine entry by simple diffusion in rat myoblasts. Presented at the 8th International Congress of Endocrinology, Kyoto, Japan.

57 Galton VA, St. Germain DL, Whittemore S 1986 Cellular uptake of 3,5,3'-triiodothyronine and thyroxine by red blood and thymus cells. Endocrinology 118:1918-1923

58 Centanni M, Pontecorvi A, Robbins J 1988 Insulin effect on thyroid hormone uptake in rat skeletal muscle. Metabolism 37:626-630

59 Nicoloff JT, Warren DW, Mizuno L, Spencer CA, Kaptein EM 1981 Hepatic thyroxine uptake as a mechanism for regulation of triiodothyronine generation in rat liver slices. Life Sci 28:1713-1718

SUMMARY

In this thesis the role of the transmembrane transport process of thyroid hormones in the bioavailability of thyroid hormones in (patho)fysiological conditions is described.

Chapter 1 is an introduction to the studies described in the Chapters 3-9. It opens with a short description of thyroid hormone synthesis, transport in blood to the different organs, transport into cells, nuclear binding and the different routes of intracellular metabolism. The isolated rat liver perfusion system was used to study the transport process in most of our studies. Therefore, this perfusion system is also described in this chapter. Furthermore, the two-compartment model, which describes thyroid hormone kinetics in the liver perfusion system, is dealt with. The chapter ends with the scope of the thesis subdivided in several questions that were studied, the results of which are described in each following chapter.

In *Chapter 2* the Materials and Methods of the chapters 3-9 are described. It deals with the human subjects of the fructose study, the rats used for most other studies, and the materials used. Furthermore, it describes the procedure of the human studies, the rat liver perfusions, the human and rat hepatocytes studies, the data analysis, the calculation of the hormone gradient over the plasma membrane, and the methods of statistical evaluation.

As there exists discrepancy in the litterature about the effects of fasting on transmembrane transport and deiodination of thyroid hormones, the effects of 48-h fasting on transport of T_3 and subsequent metabolism in the isolated perfused rat liver are investigated. Tracer T_3 disappearance curves from the recirculating medium consisted of a fast and a slow component. Using a two-compartment model, both transport (expressed as the fractional transport rate constant from medium to liver (k_{21})) and disposal of T_3 were calculated.

The results are described in *Chapter 3*. It appeared that after fasting, k_{21}, total metabolism and metabolism corrected for differences in mass transfer were diminished, pointing to both decreased transport and metabolism, presumably caused by depletion of liver ATP. Concerning transport, it was shown that only transport into the intracellular liver compartment and not transport to the extracellular liver compartment was decreased after fasting. As for metabolism, T_3-glucuronidation was diminished, whereas T_3-sulfation and subsequent deiodination were not affected. All mentioned decreased

parameters normalized after addition of a combination of insulin, cortisol and/or glucose to the medium, possibly by (partially) restoration of cellular energy stores.

In *Chapter 4* a study on the role of the transport process in altered thyroid and nutritional states is described. Transport and subsequent metabolism of T_3 were investigated in isolated perfused livers of eu-, hypo-, and hyperthyroid rats, both fed and 48-h fasted.

In fed hypothyroid livers transport parameters were not significantly changed compared to euthyroid livers, whereas metabolism was decreased. In fed hyperthyroid livers fractional rate constants for influx and efflux were decreased, while metabolism, corrected for differences in intracellular mass transfer, was increased. Furthermore, concerning transport in hyperthyroid livers, it was shown that only total mass transfer into the intracellular, metabolizing, liver compartment and not into the non-metabolizing, presumably extracellular, liver compartment was decreased.

In fasted hypothyroid livers transport and metabolic parameters were decreased compared to euthyroid fed livers. In fasted hyperthyroid livers transport and metabolism were not significantly different from euthyroid fed livers, so transport was increased versus hyperthyroid fed livers. It appeared therefore that fasting normalized the effects of hyperthyroidism on both transport and metabolic processes of T_3 in the liver.

The present findings might favour tissue euthyroidism, in spite of altered thyroid and nutritional states, and may therefore be seen as adaptation mechanisms to these altered states at the tissue level.

Amiodarone, an anti-arrhythmic drug, has structural resemblance to thyroid hormones. Patients receiving amiodarone develop a low T_3 syndrome. It was, however, not clear whether this syndrome was due to decreased T4 deiodination, or to decreased transport of thyroid hormones into the liver. In *Chapter 5* the study on transport and metabolism of T_4 and T_3 in isolated perfused livers of control and amiodarone-treated (40 mg/kg BW/day, 22 days) rats is described.

In livers of the amiodarone-treated rats both transport and metabolism of T_4 were decreased. Furthermore, it was shown that only transport into the intracellular liver compartment and not transport to the non-metabolizing liver

compartment was decreased. In the livers of amiodarone-treated rats both transport and total metabolism of T_3 were unaffected. The results showed that the different transport systems for T_4 and T_3, described in isolated rat hepatocytes, may also be operative in the intact rat liver. Furthermore, it can be concluded that the low T_3 syndrome caused by treatment with amiodarone may be due to a combination of both impaired T_4 transport and 5'-deiodination.

In *Chapter 6* the results of some studies on the energy dependence of the transport process of thyroid hormones into the liver in vivo are described. These studies are performed in perfused rat livers during control and fructose perfusion, as it has been shown that intracellular ATP is decreased after fructose loading. Furthermore, we studied serum T_4 tracer disappearance curves and computed T_4 hepatic uptake in 4 human subjects before and after intravenous fructose loading.

In the perfused rat liver a decrease in liver ATP concentration and a decrease in medium T_4 disappearance and T_4 uptake in the liver pool after fructose was found. It was shown that when corrected for differences in the free hormone concentration only transport to the metabolizing liver pool was decreased after fructose perfusion, whereas transport to the non-metabolizing pool was unaffected. Disposal, corrected for differences in transport into the metabolizing pool, was also not affected after fructose. In the human studies intravenous fructose administration induced a rise in serum lactic acid and uric acid, suggesting a decrease in liver ATP. This was observed in concomitance with a decrease in serum tracer T_4 disappearance and computed T_4 uptake in the liver during the first hours after fructose administration. These results point to ATP dependence of transport of iodothyronines into the liver in vivo, and show that in the rat liver and in humans uptake of T_4 may be regulated by intracellular energy stores. In this way the tissue uptake process may affect intracellular metabolism and bioavailability of thyroid hormone.

We were able to investigate the presence of thyroid hormone transport systems in isolated human hepatocytes and their resemblance to the transport systems described in rat hepatocytes. The human liver cells were isolated from donor livers, partly used for auxiliary liver transplantation.

The results, described in *Chapter 7*, indicate the presence of saturable, active uptake systems for T_4, T_3 and rT_3, as addition of the unlabeled hormone

(1, 5 and 2 μmol/L, respectively) to the medium resulted in a decrease of cell associated radioactivity of 20-30 %. Inhibition was also achieved after 30 min pre-incubation with fructose (10 mmol/L), which induces a decrease of intracellular ATP, or ouabain (0.5 mmol/L), indicating energy dependence and necessity of a sodium gradient for at least part of the transport process, respectively. After 20 h incubation, iodide production was inhibited in the presence of ouabain (0.5 mmol/L), PTU (100 μmol/L) or a monoclonal antibody (81-1A1-10, ascites dilution 1:200) directed against thyroid hormone transport systems in rat hepatocytes.

These data indicate that there is a high degree of similarity between the properties of the uptake process and subsequent conversion of thyroid hormones in human and rat hepatocytes, although rates of uptake and conversion are lower in human hepatocytes. Furthermore, regulation of thyroid hormone uptake at the level of the plasma membrane may also be operative in human hepatocytes.

Transport system A of neutral amino acids and those of thyroid hormones in the rat liver have several characteristics in common, e.g. temperature and ATP dependence, and stimulation after fasting by insulin. We have studied if amino acid transport system A and the thyroid hormone transport system could be identical, as thyroid hormones are derivatives of the amino acid tyrosine. This was done using isolated rat hepatocytes in primary culture, the results are described in *Chapter 8*.

It appeared that both T_4 and T_3 did not inhibit amino acid (α-AIB) transport into the liver cells. Two monoclonal antibodies that inhibited thyroid hormone transport into the liver cells, did also not influence amino acid transport. Furthermore, an inhibitor of the amino acid transport system (Me-α-AIB) had no effects on T_3 uptake into the cells.

From these results it was concluded that thyroid hormone transport systems in rat hepatocytes are different from amino acid transport system A.

In *Chapter 9* is described how the possible existence of a concentration gradient of free T_4 and T_3 across the liver cell membrane, which is strongly suggestive for active transport, was studied in isolated perfused rat livers. Using the fractional transfer rate constants for influx (k_{21}) and efflux (k_{12}), free hormone clearances were calculated for influx and efflux.

For T_4 and T_3, it appeared that the ratio of these parameters was 18.2 and 18.8, respectively, pointing to a gradient of free T_4 and T_3 over the cell membrane and thus to active transport of these thyroid hormones over the liver cell membrane.

Furthermore, we tested the influence of the perfusate flow rate on liver handling of T_4 and T_3. It appeared that for T_4 both transport and metabolic parameters were not significantly different at medium flow rates of 30, 40 or 50 mL/min. For T_3, however, at 30 mL/min both transport and metabolism were lower than at 40 or 50 mL/min. Between the last 2 flow rates no significant differences were found, indicating that at a medium flow rate of 40 mL/min is optimal for T_3 transport. These results show flow dependence of T_3 transport, which may also play a role in vivo. Interesting is also the finding that a decrease of transport into the cells due to a decrease in flow from 40 to 30 mL/min, caused a decrease in total metabolism, while metabolic capacity (k_{02}) of the liver was unchanged. This indicates a regulatory role of transmembrane transport on hepatic T_3 metabolism, which is in agreement with our earlier findings.

Chapter 10 is a general discussion of the results described in the chapters 3-9. In this chapter these results are related to the earlier findings on thyroid hormone transport systems, described in Chapter 1. Furthermore, some explanations are given for the fact that some investigators were unable to demonstrate active thyroid hormone transport into cells.

It is concluded that the studies described in Chapter 3-9 further clarify the mechanism of the thyroid hormone transport systems and underscore the regulatory role that this transport process may play in the bioavailability of thyroid hormone in (patho)physiological conditions.

SAMENVATTING

De schildklier bevindt zich bij de mens in de hals, aan weerszijden van de luchtpijp. Hier wordt het hormoon thyroxine (T_4) geproduceerd, dat na vorming wordt afgegeven aan de bloedbaan en zo door het hele lichaam wordt verspreid. T_4 is nauwelijks biologisch actief, daarom wordt het beschouwd als een prohormoon. De schildklier maakt verder nog een kleine hoeveelheid van het biologisch actieve trijodothyronine (T_3) en de inactieve vorm: reverse-trijodothyronine (rT_3). Ongeveer 80 % van het T_3 en 90 % van het rT_3 worden echter gevormd door dejodering (zie verder) van T_4 elders in het lichaam.

Schildklierhormoon is van vitaal belang voor de groei en ontwikkeling van ieder individu; het bepaalt ook het energieverbruik van onze lichaamscellen. In Nederland lijden ongeveer een half miljoen mensen aan schildklieraandoeningen, veelal veroorzaakt door een ontregelde hormoonproduktie. Verder kunnen ook 'niet-schildklierziekten' en het gebruik van bepaalde medicijnen de schildklierfunctie verstoren. De produktie van schildklierhormoon door de schildklier staat onder invloed van thyroid stimulerend hormoon (TSH), dat geproduceerd wordt door de hypofyse. Dit orgaan wordt op zijn beurt gestimuleerd tot de synthese en afgifte van TSH door thyrotropine releasing hormoon (TRH), afkomstig uit de hypothalamus. T_4 en T_3 uit het bloedplasma remmen daarentegen de afgifte van TSH. Er bestaat hier dus een feed-back systeem dat bij normale personen de schildklierhormoonwaarden in het bloed constant houdt.

In het bloed is meer dan 99 % van schildklierhormoon gebonden aan eiwitten (waaronder albumine). Deze schildklierhormoon-bindende eiwitten zijn van groot belang voor het normale transport naar weefsels in het lichaam die T_4 of T_3 nodig hebben. Bij gezonde mensen vindt de omzetting van T_4 naar het actieve T_3 vooral plaats in de lever, die rijk is aan het dejoderende enzym: dejodase. Dit enzym splitst een jodiumatoom van T_4 af, waarbij T_3 of rT_3 gevormd wordt. Gevormd rT_3 wordt vervolgens snel afgebroken in de lever, terwijl het grootste deel van het gevormde T_3 weer aan de bloedbaan wordt afgegeven. Na opname in een lichaamscel wordt, door binding van T_3 aan de receptoren in de kern van de cel, een reeks van biochemische processen gestart, waaronder synthese van eiwitten.

Aangezien omzetting van T_4 in T_3 en binding van T_3 aan de

kernreceptoren intracellulair plaatsvinden, moeten de schildklierhormonen vanuit het plasma via het plasmamembraan worden opgenomen. Op grond van de hydrofobe eigenschappen van schildklierhormonen werd lange tijd aangenomen dat deze hormonen via diffusie de cel binnenkomen. In de laatste jaren is echter aangetoond dat transport van schildklierhormoon door het plasmamembraan van levercellen van de rat een energie-afhankelijk proces is, dat verloopt via specifieke transportsystemen in het plasmamembraan. T_4 en rT_3 worden getransporteerd via hetzelfde transportsysteem, terwijl T_3 door een ander transportsysteem (met andere biochemische eigenschappen) getransporteerd wordt.

Het in dit proefschrift beschreven onderzoek richt zich op de rol die deze actieve transportsystemen voor schildklierhormonen kunnen spelen met betrekking tot de intracellulaire beschikbaarheid onder verschillende (patho)fysiologische omstandigheden. Een beter inzicht in de werking van deze processen zal bijdragen aan begrip hoe bepaalde verstoringen in de schildklierhormoonhuishouding veroorzaakt of juist gereguleerd worden.

Hoofdstuk 1 van het proefschrift is een algemene inleiding. Hierin wordt veel van het bovenstaande beschreven.

In *Hoofdstuk 2* is beschreven welke materialen en methoden gebruikt zijn voor het onderzoek. Als proefdier is de rat gebruikt; bij bepaalde proeven zijn echter ook mensen betrokken geweest. De lever is een kwantitatief belangrijk orgaan voor de omzetting van T_4 naar T_3, daarom hebben wij bij onze proeven vooral gebruik gemaakt van hetzij geïsoleerde levercellen, hetzij geperfundeerde intacte rattelevers. Door middel van beide systemen kon zowel transport als metabolisme van schildklierhormoon onderzocht worden; in het laatstgenoemde systeem werd hierbij gebruik gemaakt van een (computer) twee-compartimentenmodel.

In de literatuur bestond onduidelijkheid over het effect van vasten op het transport over het plasmamembraan en de dejodering van schildklierhormoon. Daarom is het effect van 48 uur vasten op het transport en metabolisme van T_3 onderzocht in geperfundeerde rattelevers. De resultaten zijn beschreven in *Hoofdstuk 3*. Het bleek dat na 48 uur vasten zowel transport als metabolisme van T_3 verminderd waren, waarschijnlijk veroorzaakt door daling van het ATP-gehalte in de lever. Onlangs hebben

we in een studie met geperfundeerde rattelevers laten zien dat transport van schildklierhormoon naar de lever verdeeld kon worden in transport naar een metaboliserend levercompartiment en transport naar een niet-metaboliserend levercompartiment. Transport naar het eerstgenoemde compartiment was alleen afhankelijk van de vrije (niet-eiwitgebonden) schildklierhormoonconcentratie en niet van de albumineconcentratie in het medium. Transport naar het tweede compartiment bleek afhankelijk van zowel de vrije schildklierhormoon- als de albumineconcentratie. In de vasten-proeven bleek dat alleen het transport naar het metaboliserende compartiment geremd was, en niet het transport naar het albumine-afhankelijke, niet-metaboliserende compartiment. Transport en metabolisme van T_3 herstelden na perfusie met medium waaraan insuline, cortisol en/of glucose toegevoegd waren, waarschijnlijk door (gedeeltelijk) herstel van het intracellulaire ATP-gehalte.

In *Hoofdstuk 4* is een onderzoek naar de rol van het transportproces tijdens veranderde schildklier- en voedingsstatus beschreven. Transport en metabolisme van T_3 zijn onderzocht in geïsoleerde levers van eu-(normale hoeveelheid schildklierhormoon), hypo- (te weinig schildklierhormoon) en hyperthyreote (te veel schildklierhormoon) ratten, die ofwel gevoed ofwel 48 uur gevast waren.

In gevoede hypothyreote levers was het T_3 transport niet veranderd, terwijl het metabolisme verminderd was. In hyperthyreote levers was het T_3 transport geremd, terwijl het metabolisme juist gestimuleerd was. Ook hier bleek de remming van het totale transport veroorzaakt te zijn door remming van het transport naar de metaboliserende leverpool. Na vasten waren in de hypothyreote levers zowel transport als metabolisme geremd, terwijl in de hyperthyreote levers transport juist gestimuleerd was en het metabolisme geremd in vergelijking met de situatie voor het vasten. Deze effecten helpen de lever om euthyreoot te blijven, ondanks de veranderingen in de schildklier- en voedingsstatus, en kunnen daarom beschouwd worden als aanpassingsmechanismen op weefselniveau.

Amiodarone, een medicijn dat soms gegeven wordt bij hartritmesoornissen, lijkt qua structuur op schildklierhormoon. Patienten die amiodarone gebruiken, ontwikkelen een zogenaamd 'laag T_3-syndroom'. Het

was echter niet duidelijk of dit syndroom veroorzaakt werd door verminderde activiteit van T_4-dejoderende enzym of door verminderd transport van schildklierhormoon in de lever. In *Hoofdstuk 5* is een studie naar transport en metabolisme van T_4 en T_3 beschreven in levers van controle en amiodarone-behandelde ratten.

In levers van amiodarone-behandelde ratten was zowel transport naar de metaboliserende leverpool en metabolisme van T_4 verminderd, terwijl deze processen met betrekking tot T_3 onveranderd waren. De resultaten tonen dat de verschillende transportsystemen voor T_4 en T_3, zoals beschreven in de rattehepatocyten, ook aanwezig zijn in de intacte rattelever. Geconcludeerd werd verder dat het 'laag T_3-syndroom' door amiodaronegebruik veroorzaakt wordt door een combinatie van zowel geremd transport als geremde dejodering van T_4.

In *Hoofdstuk 6* zijn onderzoeken beschreven naar de energie-afhankelijkheid van het transportproces in intacte levers. Hiervoor is gekeken naar het effect van fructose, omdat aangetoond is dat het intracellulaire ATP-gehalte in de lever snel daalt als fructose gemetaboliseerd wordt. Dit effect werd onderzocht in geperfundeerde rattelevers en ook in mensen.

In de rattelevers werd een verminderde opname van T_4 in de metaboliserende leverpool gevonden tijdens perfusie met fructose-bevattend medium. Hetzelfde werd ook gevonden in de mensen. Hieruit blijkt dat ook in vivo transport van schildklierhormoon ATP-afhankelijk is.

In *Hoofdstuk 7* staat beschreven hoe de schildklierhormoon-transportsystemen onderzocht zijn in menselijke levercellen, afkomstig van donorlevers die gedeeltelijk voor levertransplantatie gebruikt werden. De resultaten laten zien dat er een grote overeenkomst bestaat tussen het opname- en conversieproces van schildklierhormoon in levercellen van de mens en de rat, hoewel beide processen langzamer verliepen in de menselijke cellen.

Aminozuren worden ook getransporteerd in de lever door middel van transportsystemen, zoals bijvoorbeeld transportsysteem A voor neutrale aminozuren. Dit systeem A heeft overeenkomsten met de transportsystemen voor schildklierhormoon, zoals temperatuur- en ATP-afhankelijkheid. In

Hoofdstuk 8 staat beschreven hoe we onderzocht hebben of deze transportsystemen voor aminozuren en schildklierhormoon, dat een derivaat is van het aminozuur tyrosine, identiek zouden kunnen zijn in rattelevercellen. Uit de resultaten, waaruit onder meer bleek dat schildklierhormonen en substraten voor systeem A elkaars transport niet beïnvloeden, kon echter geconcludeerd worden dat dit niet het geval is.

In *Hoofdstuk 9* is beschreven hoe het bestaan van een concentratiegradiënt van vrij T_4 en T_3 over het celmembraan in de rattelever onderzocht is. Zo'n concentratiegradiënt is een sterke aanwijzing voor het bestaan van actief transport. Voor zowel T_4 als T_3 werd een gradiënt gevonden, wat dus wijst op actief transport en in overeenstemming is met onze eerdere bevindingen in rattelevercellen.

Verder is in dit onderzoek gekeken naar de invloed van de mediumstroomsnelheid op T_4- en T_3-transport en metabolisme in de lever. Voor T_4 werden geen effecten gevonden van de stroomsnelheid op deze processen, terwijl met betrekking tot T_3 zowel transport als metabolisme verminderd waren bij vermindering van de normale mediumstroomsnelheid. Hieruit blijkt dat het T_3-transport afhankelijk is van de stroomsnelheid, hetgeen ook in vivo een rol zou kunnen spelen. Interessant is ook de bevinding dat vermindering van het transport van T_3 een vermindering in het metabolisme veroorzaakte, hoewel de metabole activiteit van de lever niet veranderd was. Hieruit blijkt dat het transport over het celmembraan een regulerende rol kan spelen met betrekking tot het T_3 metabolisme in de lever, hetgeen in overeenstemming is met onze eerdere bevindingen.

Hoofdstuk 10 is een algemene discussie van de resultaten beschreven in de hoofdstukken 3-9. In dit hoofdstuk zijn deze resultaten gerelateerd aan de eerdere bevindingen met betrekking tot schildklier-hormoontransportsystemen, beschreven in Hoofdstuk 1. Ook zijn enkele verklaringen gegeven waarom sommige onderzoekers geen actief transport voor schildklierhormoon aan hebben kunnen tonen.

Geconcludeerd is dat de onderzoeken beschreven in Hoofdstuk 3-9 het mechanisme van de transportsystemen voor schildklierhormoon in de lever verder verduidelijken en de regulerende rol van het transportproces met betrekking tot de intracellulaire beschikbaarheid van schildklierhormoon

onder (patho)fysiologische omstandigheden onderstrepen.

CURRICULUM VITAE

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1985-1989	Onderzoeksmedewerker Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) op de afdeling Interne III, Dijkzigt, Rotterdam.
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