MOLECULAR CHARACTERIZATION OF THYROID HORMONE TRANSPORTERS

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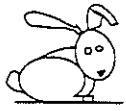
Cover: White Rabbit Photo / Gert-Jan van den Bemd 

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OF
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MOLECULAIRE KARAKTERISERING
VAN
SCHILDKLIERHORMOON TRANSPORTERS

PROEFSCHRIFT

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The investigations described in this thesis were performed at the Department of Internal Medicine of the Erasmus University Medical Center Rotterdam, The Netherlands.

Voor mijn ouders

Contents

Chapter 1

INTRODUCTION

INTRODUCTION

The thyroid gland is the largest endocrine organ in humans, weighing about 20 grams in normal healthy adults (1). The thyroid secretes predominantly the prohormone 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) and a smaller amount of the biologically active hormone 3,3',5-triiodothyronine (T_3). T_3 is mainly formed in peripheral tissues, especially in the liver, by enzymatic elimination of an iodine from the phenolic ring of T_4, also termed outer ring deiodination (2). Both T_4 and T_3 are inactivated by deiodination of the tyrosyl ring (inner ring deiodination) to the inactive metabolites 3,3',5'-triiodothyronine (rT_3) and 3,3'-diiodothyronine ($3,3'\text{-}T_2$), respectively (Fig. 1).

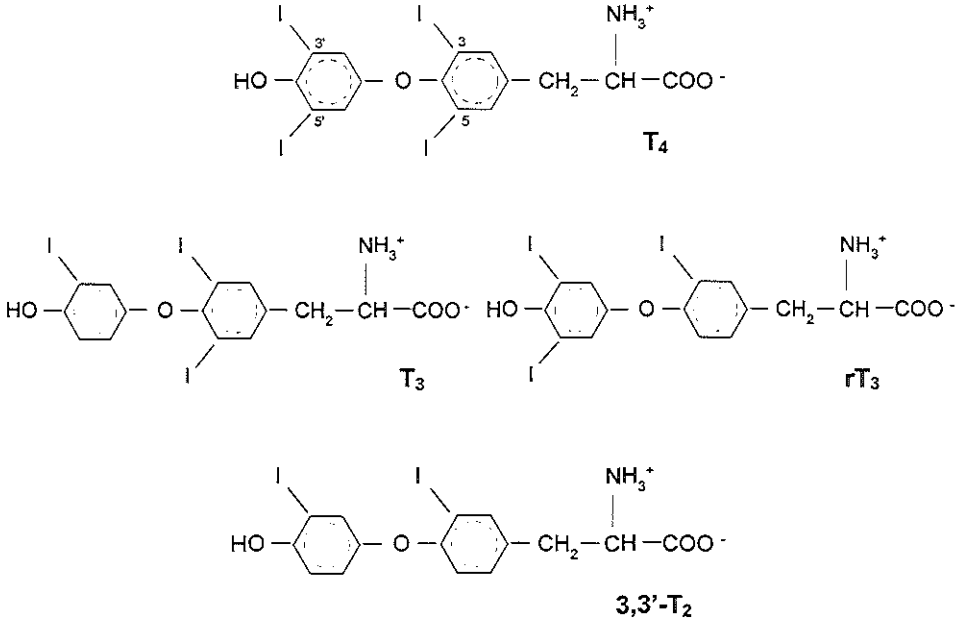

Fig. 1. Structures of thyroid hormones

Thyroid hormones are essential for growth and development of different organs, notably the brain, and for the regulation of energy metabolism of all tissues (3). Action of thyroid hormone is initiated by binding of T_3 to the specific nuclear thyroid hormone receptors, TRα and TRβ. The last two decades substantial evidence

has accumulated indicating the involvement of active and specific plasma membrane transporters in tissue-specific uptake of thyroid hormones (ref 4; see also Chapter 7 for a comprehensive review).

SYNTHESIS OF THYROID HORMONES

The thyroid is made up of multiple follicles of varying size. A single layer of epithelial follicular cells or thyrocytes surrounds a protein-rich lumen called colloid. The synthesis of thyroid hormones involves several steps (5) (Fig. 2):

Active transport of iodide (I^-) from the plasma into the thyroid cell (trapping of iodide). This transport is mediated by the recently cloned and characterized Na^+/I^- symporter (NIS) which is an intrinsic membrane protein located in the basolateral membrane of the thyroid follicular cell (6,7). The rat NIS contains 13 putative membrane spanning domains and consists of 618 amino acids (relative molecular mass ~65 kDa) (8). The uptake of I^- can be inhibited by perchlorate (ClO_4^-) and a number of other anions. The activity of the NIS allows the human gland to maintain a concentration of free I^- 30-40 times higher than in plasma (9).

Synthesis of thyroglobulin. The human thyroglobulin (Tg) is a homodimeric glycoprotein with a molecular weight of ~660 kDa, including about 140 tyrosyl residues. The follicle cells synthesize Tg, which is secreted into the colloid by exocytosis of granules.

Oxidation of I^- and iodination of tyrosyl residues in Tg. At the apical side of the thyrocyte, I^- is probably transported into the lumen by pendrin, a highly hydrophobic transmembrane protein composed of 780 amino acids, and containing 12 putative transmembrane domains (10-12). In the follicular lumen, I^- is rapidly oxidized by hydrogen peroxide (H_2O_2), catalyzed by thyroid peroxidase (TPO). TPO is a membrane-linked heme-containing protein also located at the apical membrane of the thyroid cell. After oxidation, I^- is incorporated into tyrosyl residues in Tg to form 3-monoiodotyrosine (MIT) and subsequently 3,5-diiodotyrosine (DIT).

Coupling of iodotyrosine molecules within Tg to form T_3 and T_4. TPO catalyzes also the coupling of iodotyrosyl residues in Tg to form iodothyronines. Within the Tg

molecule, two DIT residues may couple to form T_4, and a MIT and a DIT residue may couple to form T_3. After synthesis of T_4 and T_3 residues, the Tg molecules are stored in the lumen of the thyroid follicles until needed.

Proteolysis of Tg and thyroid hormone secretion. At the cell-colloid interface, colloid is engulfed into colloid vesicles pinocytosed into the thyroid cell. Lysosomes present in the thyroid cell containing proteolytic enzymes, fuse with the colloid vesicle and hydrolysis of thyroglobulin occurs releasing T_4, T_3, MIT and DIT. T_3 and T_4 are released into the circulation, while DIT and MIT are deiodinated and the I^- is re-utilized for Tg iodination.

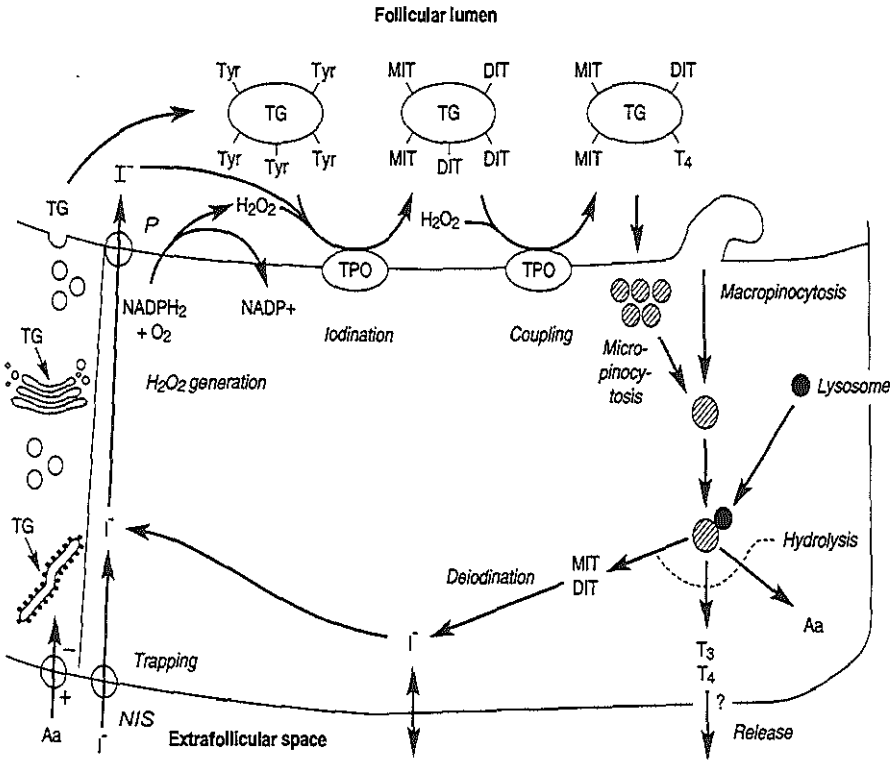

Fig. 2. Synthesis of thyroid hormones by the thyrocyte
(P, pendrin; Aa, amino acids; ?, unknown mechanism of release)

REGULATION OF THYROID HORMONE PRODUCTION

The growth and function of the thyroid gland is largely regulated by thyroid-stimulating hormone (TSH), a glycoprotein released by the thyrotropic cells of the anterior pituitary gland. Thyrotropin-releasing hormone (TRH), a pyroglutamyl-histidyl-prolinamide tripeptide, is synthesized in the hypothalamus which is a specific region at the base of the central nervous system (CNS). TRH is transported via the pituitary portal venous system down towards the anterior pituitary gland, where it stimulates synthesis and release of TSH after binding to specific membrane receptors on the thyrotropic cells. The production and secretion of TSH is, on the other hand, down-regulated by thyroid hormones (negative feedback) after binding of T_3 to the thyroid hormone nuclear receptor type β_2 which is mainly expressed in these specific thyrotropic cells (Fig. 3).

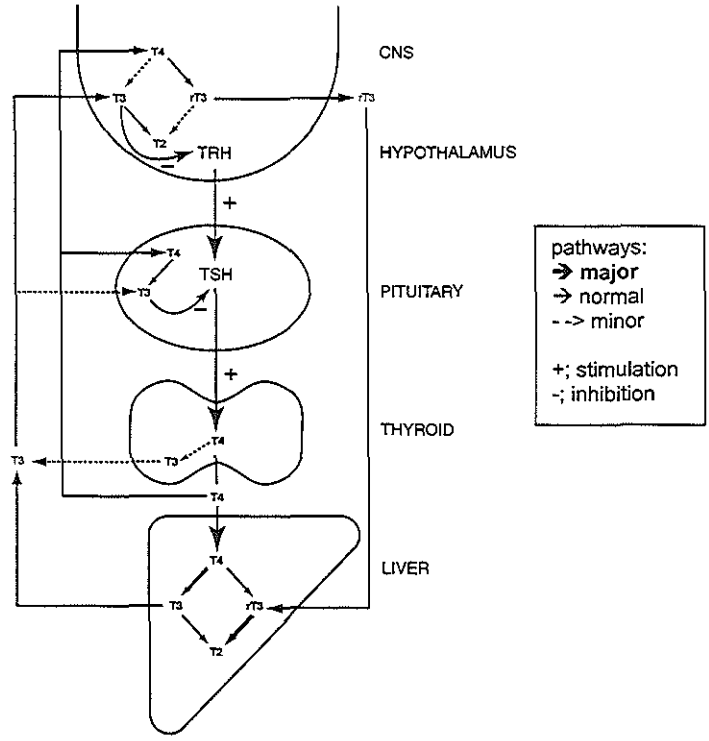

Fig. 3. Hypothalamo-pituitary-thyroid axis

Besides the regulation by TSH, thyroid hormone synthesis is subjected to autoregulation of the thyroid gland depending on the iodine supply. Administration of a high dose of iodine results in an acute, but transient decrease in thyroid hormone secretion and synthesis. A high dose of iodine also lowers the sensitivity towards TSH, while a shortage of iodine leads to a higher sensitivity for TSH. During iodine deficiency the production of T_4 is lowered and that of T_3 increased (5).

METABOLISM OF THYROID HORMONES

Iodothyronines are mainly metabolized via deiodination and conjugation (13). Deiodination is quantitatively and qualitatively the most important metabolic route. The three deiodinases involved in these conversions are homologous selenoproteins with different catalytic profiles, tissue distribution and physiological function (14). The type I iodothyronine deiodinase (D1) in liver and kidney appears important for plasma T_3 production, D2 in brain and pituitary for local T_3 production, and D3 in brain and other tissues for T_4 and T_3 degradation. All three deiodinases are transmembrane proteins with their active site exposed to the cytoplasm (2,13,14).

Conjugation is a so-called phase II detoxification reaction that transforms lipophilic substances into more water-soluble derivatives and, thus, facilitates their excretion in bile and urine (15). Conjugation of iodothyronines includes sulfation and glucuronidation of the phenolic hydroxyl group by transferases located in the cytoplasm and endoplasmic reticulum of different tissues (16,17). Sulfation is an important metabolic pathway facilitating the degradation of thyroid hormone by D1. Fig. 4 shows two types of sulfonation of the T_3 molecule; one at the 4'-OH group yielding the naturally occurring T_3 sulfate (T_3S), and another at the α-NH_2 group of the alanine side chain yielding the artificial derivative T_3 sulfamate (T_3NS) which may be regarded as an organic anion.

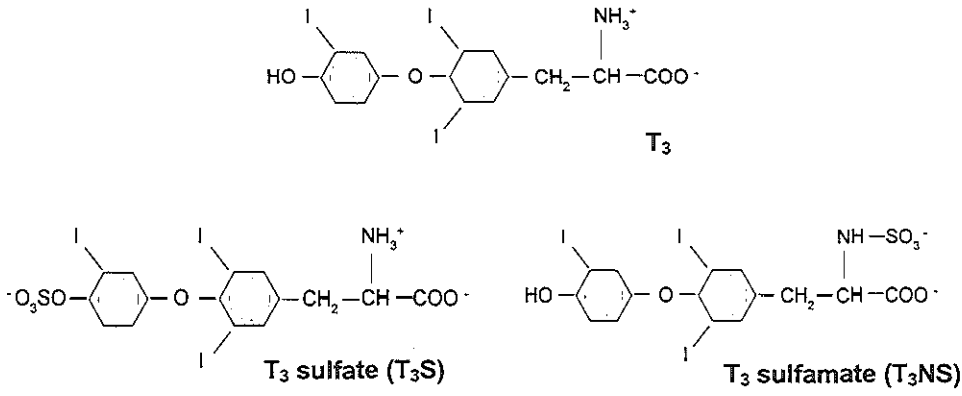

Fig. 4. Structure of T_3 and derivatives

TRANSPORT OF THYROID HORMONES

It has long been thought that thyroid hormones cross the cell membrane by simple diffusion. However, diffusion is effectively limited by the highly polar nature of the alanine side chain, which is an obstacle for simple passage of iodothyronines through the partly highly hydrophobic cell membrane. Many laboratories using different cell types from various species have now established that thyroid hormones are transported across the plasma membrane of target cells (see Chapter 7 for review). 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, that inhibited thyroid hormone transport into the cells (18). Immunoprecipitation using this monoclonal antibody resulted in the isolation of a plasma membrane protein from rat liver with a M_r of about 55 kDa. In another study, preincubation of rat hepatocytes with the covalent affinity label *N*-bromoacetyl-T_3 resulted in a decreased transmembrane transport of T_3 (19). These studies also indicated that transport of thyroid hormone is rate-limiting for subsequent metabolism of thyroid hormone (20). These transport processes have often been found to be temperature and energy dependent, and inhibited by hormone analogs and compounds that disturb the sodium-gradient across the plasma membrane (21).

As the liver plays an important role with regard to transport of T_4 and subsequent production of plasma T_3, we decided to characterize the structure of iodothyronine transporters from this target tissue. Uptake studies in rat and human hepatocytes have suggested multiple transporters for uptake of T_4, T_3 and rT_3 (4,21,22). The specific transport proteins have high affinity for thyroid hormones, with K_m values of 61 nM for T_3, 1.4 nM for T_4, and 6 nM for rT_3 measured in rat hepatocytes at 37 °C (23). Cloning and characterization of these transporters would allow to answer the questions about their role in thyroid hormone metabolism and bioavailability, especially of T_3.

Sofar, no information has been available about the structure of the thyroid hormone transport proteins. But during the last ten years, a large number of different plasma membrane transporters have been cloned following functional expression of mRNA in *Xenopus laevis* oocytes. This includes, for instance, transporters for amino acids (24-28), organic anions (29-32), bile acids (33), sulfate (34,35), iodide (6,36), and glucose (37,38). All cloned transporters consist of 6 to 13 membrane-spanning domains, but despite this common feature there is no sequence homology between these different types of transporters.

XENOPUS LAEVIS EXPRESSION SYSTEM

X. laevis frogs originally come from South Africa, and can easily be kept in large water-filled tanks at an optimum temperature of about 19-22 °C. A 12-h light, 12-h dark cycle has to be maintained to reduce seasonal variations in oocyte quality. Frogs are fed twice a week, and water must be changed a few hours after feeding (39-41). Ovarian lobes are removed from female frogs by anaesthesia with an ethyl *m*-aminobenzoate (MS 222) and hypothermia. As oogenesis in *X. laevis* is a continuous process, oocytes are present in the ovary in all stages from I to VI of development at all times during adult life (42). After collagenase treatment and selection on morphological criteria, such as size, polarization, pigmentation, and absence of follicular layer debris, the healthy looking stage V-VI oocytes (42; see also Fig. 5) are individually injected the next day with mRNA and kept in Modified Barths Solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM

$Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.4) containing 20 IU/ml penicillin, and 20 µg/ml streptomycin) at 18 °C.

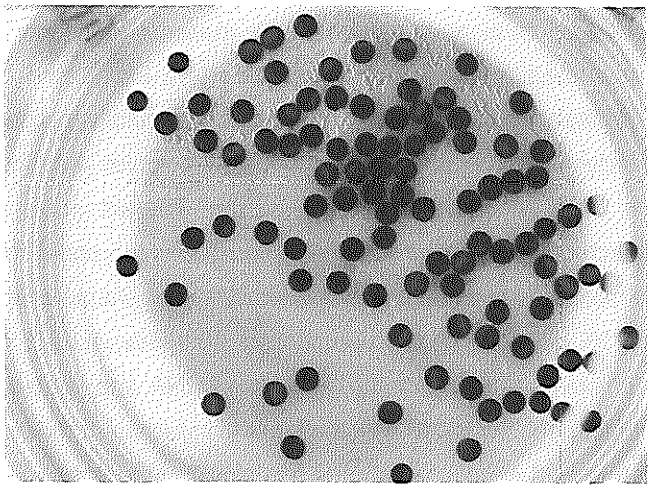

Fig. 5. Healthy looking oocytes after selection

The advantage of using *X. laevis* oocytes is that the translation of mRNA is undertaken in a normal living cell. In contrast to a cell-free system, the normal cell can correctly process protein precursors, phosphorylate, glycosylate, route and incorporate proteins into appropriate membranes or assemble multimeric proteins (43). Secondly, injected oocytes are capable of translating foreign mRNA with very little species specificity and a high efficiency for long periods. For maximum translation efficiency, only high-quality poly A^+ -containing RNA extracted from a tissue or from cultured cells and selected over an oligo-dT affinity column, or *in vitro* synthesized and capped RNA is injected (44). The capped structure on the 5' end and the poly A^+ at the 3' end efficiently protects the injected mRNA against degradation by exonucleases present in the oocyte. Also, the large size and the robustness of the cells towards microinjection of volumes up to 50 nl and easy availability of the *X. laevis* oocytes make this system very useful for mRNA translation from other animal species (45).

After selection of the target organ, intact mRNA is isolated and injected into *X. laevis* oocytes (Fig. 6). An experimental assay in which transporters can be tested by

monitoring labeled substrate uptake has to be explored for the encoded proteins to be cloned. For expression cloning, it is necessary to size-fractionate the mRNA of interest to enrich the desired mRNA coding for relevant transporter(s). When, for example, mRNA size-fraction d is found with maximal induction of the desired transport characteristics, a cDNA library is created. In Fig. 6, the cDNA library is divided into 10 different pools of 1,000-100,000 bacterial colonies representing 1,000-100,000 independent cDNAs per plate. The bacterial colonies are harvested, DNA is isolated and *in vitro* transcribed into cRNA. The different pools of cRNAs are subsequently injected into *X. laevis* oocytes. When one of the pools, for example pool 3, is found with maximal induction of the desired transport characteristics, this pool is subdivided into 10 different pools representing 200 independent cDNAs. Again, bacterial DNA is isolated, transcribed *in vitro* into cRNA and injected into oocytes. When, for example, pool 7 is found with the highest induction of the desired transport characteristics, this pool is subdivided until one single clone is isolated that contains the cDNA of interest (46).

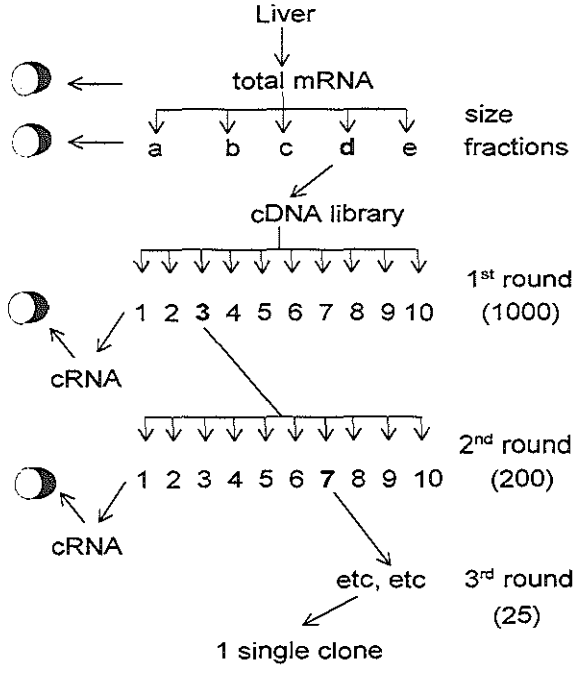

Fig. 6. Flow-chart of expression cloning using *X. laevis* oocytes

OUTLINE OF THE THESIS

The following chapters describe the use of the *X. laevis* oocytes expression system to characterize different cell membrane transporters for iodothyronines. As the liver plays a dominant role in the production of T_3 from T_4, molecular characterization of thyroid hormone transporters from liver is the main subject of this thesis.

The initial investigations were performed to express liver mRNA from male Wistar rats in *X. laevis* oocytes (Chapter 2). Oocytes were injected with 23 ng mRNA or size-fractions thereof and after 3-4 days, uptake of T_3, T_4 and T_3 sulfate (T_3S) was tested. To check for true internalization of hormone by oocytes, they were also injected with cRNA for rat type I deiodinase (D1), which is expressed intracellularly and can only act on intracellular iodothyronines. Oocytes injected with D1 cRNA and/or liver mRNA were investigated for uptake and metabolism of T_3S.

During expression of plasma membrane transporters for thyroid hormone, we found uptake and metabolism of iodothyronines by native *X. laevis* oocytes (Chapter 3). After 20 h incubation at 18 °C, we investigated uptake and sulfation of T_4, T_3, rT_3, and 3,3'-T_2 by native oocytes as well as sulfation of these compounds by isolated oocyte cytosol.

In order to minimize the endogenous uptake of iodothyronines by native *X. laevis* oocytes, and thus to find a relatively higher induced uptake signal in injected oocytes, transport studies were performed with iodothyronine derivatives, especially thyroid hormone sulfamates (synthetic *N*-sulfonated iodothyronines; for structure, see Fig. 4). As those thyroid hormone sulfamates are organic anions, we tested already cloned organic anion transporters from rat liver for transport of iodothyronine derivatives (Chapter 4). Oocytes were injected with cRNA coding for the rat Na^+/taurocholate cotransporting polypeptide (rNTCP) (33) or the Na^+ -independent organic anion transporting polypeptide 1 (rOATP1) (29). After 2-3 days, oocytes were incubated with taurocholate, iodothyronines, iodothyronine sulfates and iodothyronine sulfamates.

As thyroid hormones are iodinated amino acid derivatives built of two tyrosine molecules and indications were found in literature for competition between amino

acids (*i.e.* leucine) and thyroid hormone uptake (47-51), we tested a heterodimeric human system L amino acid transporter for uptake of thyroid hormones (Chapter 5). This heterodimeric transporter consists of a human 4F2 heavy chain (*h*4F2hc) combined with the human light chain of system L amino acid transport type 1 (*h*LAT1) (52,53). This transporter mediates the Na^+ -independent transport of large neutral (branched chain and aromatic) amino acids such as leucine, tyrosine, tryptophan and phenylalanine. After injection of cRNA coding for *h*4F2hc and/or *h*LAT1 into *X. laevis* oocytes, uptake and efflux of different amino acids and iodothyronines was measured.

In Chapter 6, studies are performed to further characterize different already cloned rat and human hepatic organic anion transporters in mediating thyroid hormone transport and also to focus on the specific plasma membrane transporters present in rat liver mRNA. Oocytes were injected with cRNA coding for rat and human NTCP or OATPs, rat liver mRNA or size-fractions thereof. After 2-4 days, oocytes were tested with taurocholate, iodothyronines and iodothyronine sulfamates to measure transport of these substrates.

Chapter 7 contains a review presenting the results of two and a half decades work on thyroid hormone transport and its role in thyroid hormone metabolism and bioavailability. A discussion of current ideas on transport of thyroid hormone and its derivatives is presented in Chapter 8.

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Chapter 2

EXPRESSION OF RAT LIVER CELL MEMBRANE TRANSPORTERS FOR THYROID HORMONE IN *XENOPUS LAEVIS* OOCYTES

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ABSTRACT

The present study was conducted to explore the possible use of *Xenopus laevis* oocytes for the expression cloning of cell membrane transporters for iodothyronines. Injection of stage V-VI *X. laevis* oocytes with 23 ng Wistar rat liver polyadenylated RNA (mRNA) resulted after 3-4 days in a highly significant increase in [^{125}I]T_3 (5 nM) uptake from 6.4 ± 0.8 fmol/oocyte·h in water-injected oocytes to 9.2 ± 0.65 fmol/oocyte·h (mean ± SEM, n=19). In contrast, [^{125}I]T_4 (4 nM) uptake was not significantly stimulated by injection of total liver mRNA. T_3 uptake induced by liver mRNA was significantly inhibited by replacement of Na^+ in the incubation medium by choline$^+$ or by simultaneous incubation with 1 µM unlabeled T_3. In contrast, T_3 uptake by water-injected oocytes was not Na^+ dependent. Fractionation of liver mRNA on a 6-20% sucrose gradient showed that maximal stimulation of T_3 uptake was obtained with mRNA of 0.8-2.1 kilobases (kb). In contrast to unfractionated mRNA, the 0.7- to 2.1-kb fraction also significantly stimulated transport of T_4, and it was found to induce uptake of T_3 sulfate (T_3S). Because T_3S is a good substrate for type I deiodinase (D1), 2.3 ng rat D1 complementary RNA (cRNA) were injected either alone or together with 23 ng of the 0.8- to 2.1-kb fraction of rat liver mRNA. Compared with water-injected oocytes, injection of D1 cRNA alone did not stimulate uptake of [^{125}I]T_3S (1.25 nM). T_3S uptake in liver mRNA and D1 cRNA-injected oocytes was similar to that in oocytes injected with mRNA alone, showing that transport of T_3S is independent of the metabolic capacity of the oocyte. Furthermore, coinjection of liver mRNA and D1 cRNA strongly increased the production of $^{125}I^-$, showing that the T_3S taken up by the oocyte is indeed transported to the cell interior.

In conclusion, injection of rat liver mRNA into *X. laevis* oocytes resulted in a stimulation of saturable, Na^+-dependent T_4, T_3 and T_3S transport, indicating that rat liver contains mRNA(s) coding for plasma membrane transporters for these iodothyronine derivatives.

INTRODUCTION

T_4 is the main secretory product of the thyroid gland, which is enzymatically converted in peripheral tissues to the biologically active hormone T_3. About 80% of the plasma T_3 production in man results from this extrathyroidal pathway, in which the liver plays a dominant role (1). The conversion of T_4 to T_3 as well as the further deiodination of iodothyronines is effected by different types of deiodinases in tissues (2). Transport across the plasma membrane is required for intracellular deiodination. During the last 15 yr evidence has accumulated that the plasma membranes of different tissues contain one or more specific transport proteins for T_3 and T_4. The transport process appears to be temperature and energy dependent, and is inhibited by hormone analogs and compounds that disturb the Na^+ gradient across the plasma membrane (see Refs. 3 and 4 for comprehensive reviews). Although much is known about the physiology of transmembrane T_4 and T_3 transport, little is known about the molecular mechanisms of these processes. Studies with a monoclonal antibody that inhibits T_4 and T_3 transport into rat hepatocytes showed immunoprecipitation of a plasma membrane protein from rat liver with a M_r of about 55 kDa (5). In another study, photoaffinity labeling of rat erythrocyte membranes with [^{125}I]T_3 resulted in the identification of a 45 kDa protein (6). Additional studies using the above monoclonal antibody and other inhibitors of plasma membrane transport also indicated that this transport process is rate limiting for subsequent metabolism of thyroid hormone (7). Other workers have shown that this transport process is a determinant for the nuclear occupancy of thyroid hormone (8,9). Thus, plasma membrane transport may play an important role in the overall regulation of thyroid hormone bioactivity.

No information is as yet available about the structure of this transport protein(s). During the last years a large number of plasma membrane transporters (for instance, for amino acids, organic anions, bile acid, sulfate, and water) have been cloned following their expression in *Xenopus laevis* oocytes after microinjection of messenger RNA (mRNA) coding for these proteins (10-16). Therefore, we have adopted this technique to express the thyroid hormone transport protein(s) using total rat liver polyadenylated [poly(A)$^+$] RNA (mRNA) and fractions thereof as a first step in the cloning process.

MATERIALS AND METHODS

Materials

T_3 was purchased from Sigma Chemical Co. (St. Louis, MO), [3',5'-^{125}I]T_4 (>19 MBq/nmol), [3'-^{125}I]T_3 (>29 MBq/nmol) and L-[^{3}H]arginine (>2.22 MBq/nmol) were obtained from RCC Amersham (Aylesbury, UK). [3'-^{125}I]T_3 sulfate ([^{125}I]T_3S) was prepared by reaction of [^{125}I]T_3 with chlorosulfonic acid in dimethylformamide (both from Merck, Darmstadt, Germany) and purified by Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography as previously described (17). [^{125}I]T_4 was purified before use with the same method. All other chemicals were of reagent grade.

Animals

Two- to 3-yr-old adult *Xenopus laevis* females were obtained from the Hubrecht Laboratory (Utrecht, The Netherlands). They were maintained in a water-filled tank with three dark sides at a temperature of 18-22 C. A 12-h light, 12-h dark cycle was maintained to reduce seasonal variations in oocyte quality. Frogs were fed twice a week. The water was changed about 2 h after feeding.

Livers of male Wistar rats, female Sprague-Dawley rats, and female Fisher rats were used to prepare mRNA. Animals had free access to food and water and were kept in a controled environment (21 C) with constant day length (12 h).

Oocyte isolation and RNA-injections

Oocytes were prepared as described previously (18), with some modifications. Ovarian fragments were removed from *X. laevis* females under MS-222 anaesthesia (Sigma; 1 g/liter 3-aminobenzoic acid ethyl ester, in tap water) and hypothermia. Small lumps containing 20-50 oocytes were washed in calcium-free ORII (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). To remove follicular layers, the lumps were incubated twice for 90 min each time at room temperature in ORII with 2 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany) on a rotator. The oocytes were washed thoroughly five times with ORII and subsequently five times with MBS [modified Barth's solution, 88 mM NaCl, 1 mM KCl, 0.82 mM

$MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.4), containing 10 IU/ml penicillin and 10 µg/ml streptomycin]. The oocytes were sorted manually on morphological criteria, such as size, polarisation, pigmentation, and absence of follicular layer debris. Healthy-looking stage V-VI oocytes (19) were transferred to six-well tissue culture plates and incubated in MBS at 18 C in the dark.

The next day, oocytes were injected with 0.23-23 ng RNA in 23 nl water or with water alone (control) using a Nanoject system (Drummond Scientific, Broomall, PA). Injected oocytes were maintained in MBS at 18 C for 3-4 days, with a daily change of medium.

mRNA isolation

A commercial kit (Stratagene, La Jolla, CA) was used for the isolation of mRNA from rat liver tissue according to the manufacturer's protocol. Tissue was homogenized in guanidinium isothiocyanate buffer with ß-mercaptoethanol. After dilution, precipitated proteins were removed by centrifugation, and the mRNA was bound to oligo(deoxythymidine)cellulose. After several wash steps, mRNA was eluted with elution buffer at 65 C. For size-fractionation, rat liver mRNA (150 µg) in water was heated to 65 C for 5 min and then loaded on a lineair 6-20% (wt/vol) sucrose gradient containing 15 mM piperazine-*N,N'*-[2-ethanesulfonic acid] (PIPES) (pH 6.4), 5 mM Na_2-EDTA, and 0.25% (wt/vol) Sarkosyl. The gradient was centrifuged for 19 h at 4 C at 25,000 rpm (80,000 x g_{av}) in a Beckman SW 41 rotor (Beckman, Palo Alto, CA). Subsequently, 0.7-ml fractions were collected from the bottom of the tubes. Total and size-fractionated mRNA were precipitated with 0.3 M sodium acetate in ethanol (20), resuspended in water at a concentration of 1 µg/µl, and stored at -80 C. mRNA concentrations were estimated by measuring the absorption at 260 nm (20). The size ranges of mRNAs in each fraction were estimated by electrophoresis of the fractions on 1% agarose gel and staining with ethidium bromide. Each fraction contains a maximum concentration of one size of mRNAs ($mRNA_{max}$) with gradually lower concentrations of smaller and larger species of mRNA, extending about 0.6 kilobase (kb) on each side of the $mRNA_{max}$. To combine data from different gradient experiments, results were grouped according to the $mRNA_{max}$ in each fraction, *i.e.* less than 0.5 kb, 0.5-1.5, 1.5-2.5, and more than 2.5 kb, respectively.

In vitro transcription

Complementary RNA (cRNA) coding for the arginine (Arg) transporter rBAT (16) or for rat type I deiodinase (D1) (21) was prepared by *in vitro* transcription using the AmpliScribe T3 transcription kit (Epicentre Technologies, Madison, WI) according to the protocol for synthesis of capped cRNA. For capping, the m7G[5']ppp[5']G cap analog was used (Epicentre Technologies). pBluescript DNAs containing the respective complementary DNAs (cDNAs) as insert were used as template after linearization with *Xho*I (Boehringer Mannheim). After transcription, the DNA template was digested using ribonuclease-free deoxyribonuclease I, and the incubation mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (Life Technologies, Breda, The Netherlands) and once with chloroform. The cRNA in the final water phase was precipitated with an equal volume of 5 M ammonium acetate, incubated on ice for 30 min, and centrifuged for 10 min at 4 C. cRNA pellets were dissolved in water and stored at -80 C.

Uptake assays

Groups of 10 oocytes were washed for 1 min at 18 C in choline$^+$-containing incubation buffer (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). Subsequently, the oocytes were incubated for 1 h at 25 C in 0.1 ml of the same buffer containing 50 µM Arg and 370 kBq/ml [^{3}H]Arg or with 4 nM [^{125}I]T_4 (60 kBq/ml), 5 nM [^{125}I]T_3 (60 kBq/ml), or 1.25 nM [^{125}I]T_3sulfate ([^{125}I]T_3S; 90 kBq/ml). Uptake of these labeled compounds was also tested in Na^+ buffer (same buffer with 100 mM NaCl instead of choline chloride) to assess Na^+-dependent uptake. After 1 h, incubation buffer was removed, and the oocytes were washed four times with 2.5 ml ice-cold Na^+ buffer containing 0.1% BSA. Oocytes were transferred to new tubes or scintillation vials and counted individually.

Metabolism assays

Groups of 10 oocytes were transferred to a 96-well tissue culture plate. Subsequently, the oocytes were incubated at 18 C in the dark in 0.1 ml MBS containing 1.25 nM [^{125}I]T_3S (90 kBq/ml). After 18 hours, the incubation medium was removed, and the oocytes were transferred to tubes and washed four times with 2.5 ml ice-cold Na^+

buffer containing 0.1% BSA. Each group of 10 oocytes was divided into 2 groups of 5 oocytes, transferred to new tubes, counted, homogenized in 0.1 ml 0.1 M NaOH, and centrifuged. The supernatants were analyzed for $^{125}I^-$, [^{125}I]T_3S and [^{125}I]T_3 by Sephadex LH-20 chromatography as previously described (22).

Statistics

Data are presented as the mean ± SEM. Statistical significance was evaluated by Student's *t* test for unpaired observations. Data from the gradient experiments were analyzed by ANOVA, using the Studentized range for comparison of group means.

RESULTS

Injection of 0.23 ng rBAT cRNA, which expresses Na^+-independent transport of L-arginine, L-cysteine and L-leucine (16), resulted in a rise in [^{3}H]Arg uptake from 10 (water-injected) to 205 pmol/oocyte·h in choline$^+$-containing medium (Table 1) without a change in T_3 uptake both in the presence or absence of Na^+. Injection of 23 ng total liver mRNA resulted in a moderate, but significant (P<0.001), increase in T_3 uptake from 6.4 ± 0.8 (water-injected) to 9.2 ± 0.65 fmol/oocyte·h (Table 1). This increase was completely blocked by replacing Na^+ in the uptake medium by choline$^+$, indicating that the increase in T_3 uptake is Na^+ dependent. From the data in Table 1, it is also clear that water-injected oocytes exhibited an endogenous T_3 uptake that was not significantly inhibited by replacement of Na^+ by choline$^+$.

Table 1. Uptake of T_3 and arginine by *X. laevis* oocytes

	Uptake[a]		
Injected material	T_3 (Na^+) (fmol/oocyte)	T_3 (choline$^+$) (fmol/oocyte)	Arg (choline$^+$) (pmol/oocyte)
Water	6.4 ± 0.8	5.0 ± 0.5	10 ± 1.8
0.23 ng rBAT cRNA	6.5 ± 0.7	5.1 ± 0.4	205 ± 18.7[b]
23 ng liver mRNA	9.2 ± 0.65[c]	5.5 ± 0.5	

Results represent the mean ± SEM of 19 (T_3) or 14 (Arg) experiments.
[a]Ten oocytes injected 4 days previously with water, rBAT cRNA, or Wistar rat liver mRNA were incubated for 1 h with 5 nM [^{125}I]T_3 or 50 µM [^{3}H]Arg in 0.1 ml medium with Na^+ or choline$^+$.
[b] P<0.001 *vs.* water-injected oocytes.
[c] P<0.01 *vs.* water-injected oocytes.

Furthermore, it appeared that this endogenous uptake was highly dependent on the batch of oocytes used (range 1.7-13.9 fmol T_3/oocyte·h). To eliminate this variable endogenous uptake, we have taken the difference in uptake between mRNA-injected and water-injected oocytes in each further experiment as a measure of mRNA-induced T_3 uptake.

Figure 1 shows the induction of T_3 uptake by oocytes injected with 23 ng of mRNA prepared from livers of three different rat strains and sexes. No significant difference was found between the preparations. mRNA-induced T_3 uptake in choline$^+$-containing medium was not significantly different from zero, indicating that the expressed transport system is completely Na^+ dependent. Furthermore, addition of 1 µM unlabeled T_3 inhibited the uptake of [^{125}I]T_3 by more than 50%.

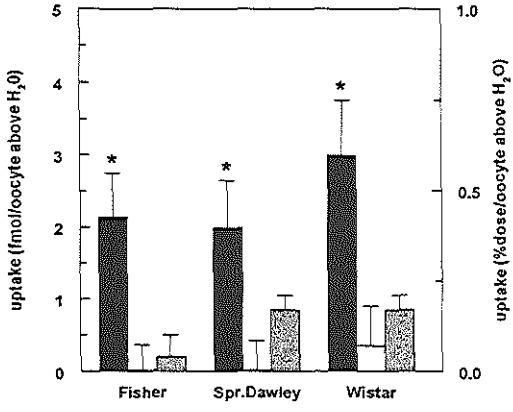

Fig. 1. Induction of T_3 uptake by oocytes after injection of total liver mRNA from different rat strains. Groups of 10 oocytes were injected with 23 ng/oocyte liver mRNA or with water, and after 4 days they were incubated for 1 h at 25 C with 5 nM [^{125}I]T_3 in 0.1 medium containing Na^+ (■), choline$^+$ (□), or Na^+ and 1 µM unlabeled T_3 (▒). The results show the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes, and are presented as the mean ± SEM of 10 oocytes. *Left y*-axis, Uptake of [^{125}I]T_3 in femtomoles per oocyte/h; *right y*-axis, uptake as a percentage of the added [^{125}I]T_3. *, P<0.001 *vs.* zero.

Total Sprague-Dawley rat liver mRNA was size-fractionated on a 6-20% sucrose gradient, and the mRNA size ranges were determined by agarose gel electrophoresis. Of each fraction, 23 ng mRNA were injected into oocytes. The fractions containing mRNA of 0.8-2.1 kb showed the largest stimulation of Na^+-dependent T_3 uptake. A typical experiment is depicted in Fig. 2.

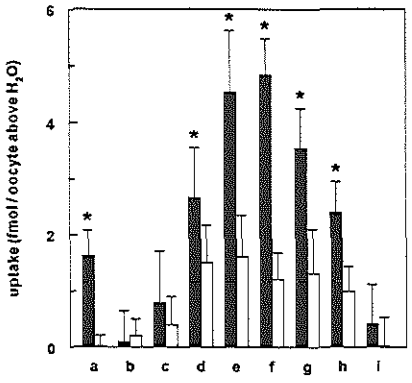

Fig. 2. Induction of T_3 uptake by oocytes after injection of various size fractions of rat liver mRNA. Groups of 10 oocytes were injected with 23 ng/oocyte liver mRNA or with water, and after 4 days they were incubated for 1 h at 25 C with 5 nM [^{125}I]T_3 in 0.1 ml medium containing Na^+ (■) or choline$^+$ (□). The results show the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes, and are presented as the mean ± SEM of 10 oocytes. Size ranges of RNA fractions: a, unfractionated mRNA; b, 3.0-4.5 kb; c, 2.2-3.5 kb; d, 1.5-3 kb; e, 1.25-2.5 kb; f, 0.8-2.1 kb; g, 0.5-1.4 kb; h, 0.3-1.25 kb; and i, 0.2-0.9 kb. *, P<0.001 *vs.* zero.

In 9 fractionation experiments, water-injected oocytes showed a T_4 uptake of 3.7 ± 0.7 fmol/oocyte·h, and a T_3 uptake of 7.0 ± 1.3 fmol/oocyte·h. Injection of 23 ng total liver mRNA did not stimulate the uptake of T_4 significantly. mRNA-induced uptake of T_4 amounted to only 0.12 ± 0.08 fmol/oocyte·h, whereas mRNA-induced T_3 uptake in the same experiments was 0.95 ± 0.16 fmol/oocyte·h (P<0.001; Fig. 3a). However, after fractionation of rat liver mRNA on a 6-20% sucrose gradient, uptake of T_4 was also significantly induced, in particular with mRNA of 0.5-2.5 kb, similar to T_3 uptake (Fig. 3, c and d).

Figure 4 shows that uptake of T_3S during 1 h was stimulated by injection of oocytes with a 0.8- to 2.1-kb fraction of rat liver mRNA, and that this uptake was Na^+ dependent. mRNA-induced T_3S uptake was inhibited by 50% in the presence of 1 µM unlabeled T_3S (data not shown). As basal uptake of T_3S by water-injected oocytes was low, injection of rat liver mRNA produced a relatively much larger signal compared with the effect on T_3 uptake. Injection of 2.3 ng D1 cRNA did not stimulate T_3S uptake, whereas injection of a mixture of 0.8-2.1 kb liver mRNA and D1 cRNA stimulated T_3S uptake to a similar extent as liver mRNA alone.

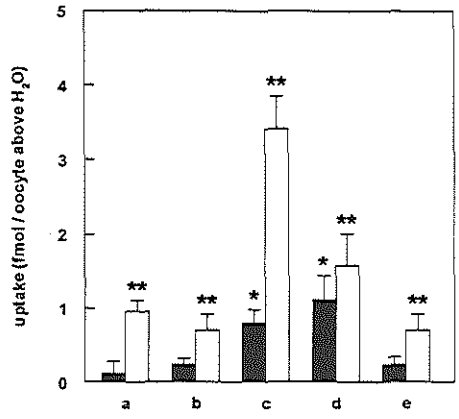

Fig. 3. Induction of T_4 (■) and T_3 (□) uptake by oocytes after injection of various size fractions of rat liver mRNA. Groups of 10 oocytes were injected with 23 ng/oocyte liver mRNA or with water, and after 4 days they were incubated for 1 h at 25 C with 5 nM [^{125}I]T_3 or 4 nM [^{125}I]T_4 in 0.1 ml Na^+ medium. The results show the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes and are presented as the mean ± SEM of nine experiments. Size ranges of RNA fractions: a, unfractionated mRNA; b, $mRNA_{max}$ less than 0.5 kb; c, $mRNA_{max}$ 0.5-1.5 kb; d, $mRNA_{max}$ 1.5-2.5 kb; e, $mRNA_{max}$ more than 2.5 kb. c *vs.* b: T_4, P<0.05; T_3, P<0.001; c *vs.* d: T_4, P = NS, T_3, P<0.01; d *vs.* e: T_4, P<0.01; T_3, P = NS. **, P<0.001; *, P<0.01 (*vs.* zero).

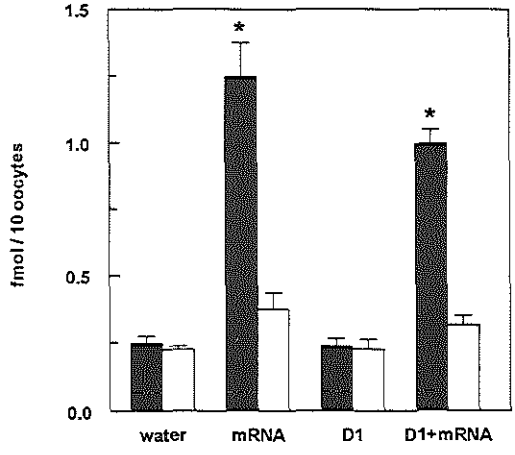

Fig. 4. T_3S uptake by oocytes injected with water, 0.8-2.1 kb rat liver mRNA, D1 cRNA, or the combination of liver mRNA and D1 cRNA. Groups of 10 oocytes were injected with 23 ng/oocyte mRNA, 2.3 ng/oocyte D1 cRNA, or both, and after 4 days they were incubated for 1 h at 25 C with 1.25 nM [^{125}I]T_3S in 0.1 ml medium containing Na^+ (■) or $choline^+$ (□). Data are presented as the mean ± SEM of 10 oocytes. *, P< 0.001 *vs.* water-injected oocytes.

Figure 5 shows the results of prolonged incubations (18 h) of similarly injected oocytes with [^{125}I]T_3S. Total T_3S uptake after 18 h was obviously higher than with 1-h incubations (Fig. 4). Total T_3S uptake was low in water- or D1 cRNA-injected oocytes, but was strongly stimulated in oocytes injected with 0.8-2.1 kb liver mRNA. Total T_3S uptake by oocytes injected with liver mRNA together with D1 cRNA was similar to uptake of oocytes injected with rat liver mRNA alone. In water-injected oocytes deiodination of T_3S and deconjugation of T_3S to T_3 were very low. Deconjugation was similarly low in D1 cRNA-injected oocytes, but deiodination of T_3S was stimulated by expression of D1 (P<0.01). In oocytes injected with liver mRNA, more T_3S was hydrolyzed than in water- or D1 cRNA-injected oocytes (P<0.001), and T_3S was also deiodinated to some extent (P<0.001). Finally, injection of oocytes with the combination of liver mRNA and D1 cRNA led to a much larger production of iodide from T_3S than injection of either RNA alone (P<0.001).

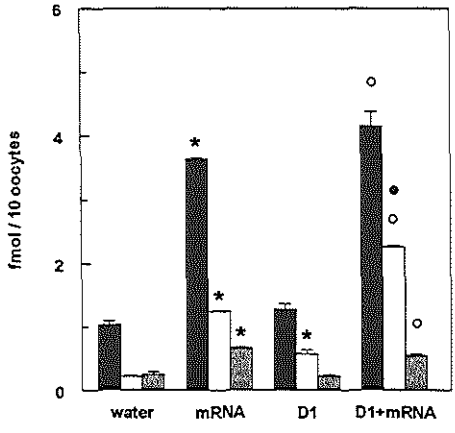

Fig. 5. Metabolism of T_3S in oocytes injected with water, 0.8-2.1 kb rat liver mRNA, D1 cRNA, or the combination of liver mRNA and D1 cRNA. Groups of 10 oocytes were injected with water, 23 ng/oocyte mRNA, 2.3 ng/oocyte D1 cRNA, or both, and after 4 days they were incubated for 18 h at 18 C with 1.25 nM [^{125}I]T_3S in 0.1 ml Na^+ medium. Results show the total amount of radioactivity (■), I^- (□) and T_3 (▒) in the oocytes at the end of the incubation and are presented as the mean ± SEM of four pools of five oocytes. *, P<0.001 *vs.* water-injected oocytes, °, P<0.001 *vs.* D1 cRNA- or water-injected oocytes; •, P<0.001 *vs.* liver mRNA-injected oocytes.

DISCUSSION

Our present findings that Na^+-dependent uptake of T_3, T_4, and T_3S is induced in *X. laevis* oocytes by the injection of rat liver mRNA are in agreement with our previous suggestion that transport of iodothyronines into rat hepatocytes proceeds via a plasma membrane transporter, as it is a saturable process dependent on temperature, the intracellular ATP concentration, and the Na^+ gradient over the cell membrane (3,23). Because the oocytes exhibit an endogenous uptake system for T_3 which is independent of the Na^+ gradient, we have defined mRNA-induced T_3 uptake as the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes in the same medium. Our finding that mRNA-induced T_3 uptake is completely abolished by replacement of Na^+ in the uptake medium by $choline^+$ strongly suggests that this uptake is tightly coupled to the Na^+ gradient. This may also explain the ATP dependence of uptake of T_4 and T_3 by hepatocytes, because ATP is necessary to maintain the Na^+ gradient over the plasma membrane (24,25). Livers from different rat strains contain similar amounts of mRNA coding for the T_3 transporter, showing the same Na^+ dependence and similar saturability with unlabeled T_3.

Uptake of T_4, T_3, and T_3S by oocytes is induced by the injection of certain mRNA species, but not by others. Thus, T_3 uptake by oocytes injected with rBAT cRNA is not different from that by water-injected oocytes. Similarly, injection of D1 cRNA does not induce T_3S uptake. Furthermore, not all rat liver mRNA fractions tested stimulate T_3 uptake; mRNAs of 0.2-0.9 kb and of 2.2-4.5 have no effect, but mRNA of 0.8-2.1 kb induces a 2.5-fold greater stimulation of T_3 uptake than crude rat liver mRNA. Taken together, our results suggest that the size of the mRNA coding for the T_3 transporter is 1.5 ± 0.5 kb, a size large enough to code for a protein of 45-55 kDa, previously estimated by immunoprecipitation and photoaffinity labeling of putative cellular T_3 transport proteins (5,6).

Induction of T_4 uptake in oocytes is undetectable after the injection of total rat liver mRNA. However, when liver mRNA is fractionated on a 6-20% sucrose gradient, a highly significant stimulation is found in fractions with $mRNA_{max}$ ranging between 0.5-2.5 kb. This stimulation is significantly higher than that by fractions with $mRNA_{max}$ smaller than 0.5 kb or larger than 2.5 kb. Although the stimulation of T_4 uptake seems

somewhat larger in fractions in which $mRNA_{max}$ ranges between 1.5 and 2.5 kb, there is no significant difference from that in the fractions in the 0.5-1.5 kb size range. The largest stimulation of T_3 uptake was found in fractions containing $mRNA_{max}$ of 0.5-1.5, significantly higher than the smaller and larger mRNA size fractions. Therefore, stimulation of T_4 and T_3 uptake seems to peak in different mRNA fractions, suggesting that T_4 and T_3 transporters are translated from different mRNAs. This is in agreement with previous indirect evidence, suggesting different mechanisms for T_3 and T_4 uptake in liver cell membranes. Uptake of T_4 is lower than that of T_3, which is also in accordance with previous findings using rat hepatocytes (26), indicating that the V_{max} for the specific uptake of T_4 was 3.5-fold lower than that for T_3 uptake.

Uptake of T_3 and T_3S by oocytes is linear during the first hour (data not shown), suggesting that the 1-h uptake data represent binding and/or transport at the level of the cell membrane. Although the Na^+ dependence of the initial T_3 uptake process strongly suggests that this represents transmembrane transport, subsequent metabolism of T_3 would be an unequivocal indication that the hormone indeed enters the oocytes. Unfortunately, T_3 is not metabolized by oocytes. Induction of D1 by injection of its cRNA (21) does not change the situation, because T_3 is a poor substrate for this enzyme. On the other hand, [3'-^{125}I]T_3S is rapidly deiodinated by D1, initially in the inner ring and subsequently in the outer ring with liberation of $^{125}I^-$ (27). Our results indicate that the relative increase of T_3S uptake by *X. laevis* oocytes after the injection of rat liver mRNA is much greater than that in T_3, although the absolute rate of T_3S uptake is less than that of T_3 even in mRNA-injected oocytes. A similar difference was found between T_3 and T_3S uptake in rat hepatocytes (28). Like T_3 uptake, liver mRNA-induced T_3S uptake by oocytes is Na^+ dependent, whereas endogenous T_3S uptake by water-injected oocytes is Na^+-independent. The finding that deiodination of T_3S is highest in oocytes coinjected with D1 cRNA and rat liver mRNA indicates that T_3S is indeed transported to the cell interior as D1 is an intracellular membrane protein (29). Furthermore, it is clear that uptake of T_3S in mRNA plus D1 cRNA-injected oocytes is similar to that in oocytes injected with mRNA alone. This indicates that plasma membrane transport is independent of the metabolic capacity of the oocyte, underlining the rate-limiting potential of the transport process for entry and subsequent metabolism of thyroid hormone (7).

In conclusion, we present a system for expression cloning of cDNA coding for rat liver T_4, T_3, and T_3S transporter(s) based on the 0.8- to 2.1-kb mRNA fraction. This technique may lead to the molecular characterization of thyroid hormone plasma membrane transport proteins and to a better understanding of the molecular mechanism of translocation of thyroid hormone across the plasma membrane of target cells.

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Chapter 3

RAPID SULFATION OF 3,3',5'-TRIIODOTHYRONINE IN NATIVE *XENOPUS LAEVIS* OOCYTES

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ABSTRACT

Sulfation is an important metabolic pathway facilitating the degradation of thyroid hormone by the type I iodothyronine deiodinase. Different human and rat tissues contain cytoplasmic sulfotransferases that show a substrate preference for 3,3'-diiodothyronine (3,3'-T_2) > T_3 > rT_3 > T_4. During investigation of the expression of plasma membrane transporters for thyroid hormone by injection of rat liver RNA in *Xenopus laevis* oocytes, we found uptake and metabolism of iodothyronines by native oocytes. Groups of 10 oocytes were incubated for 20 h at 18 C in 0.1 ml medium containing 500,000 cpm (1-5 nM) of [^{125}I]T_4, [^{125}I]T_3, [^{125}I]rT_3, or [^{125}I]3,3'-T_2. In addition, cytosol prepared from oocytes was tested for iodothyronine sulfotransferase activity by incubation of 1 mg cytosolic protein/ml for 30 min at 21 C with 1 μM [^{125}I]T_4, [^{125}I]T_3, [^{125}I]rT_3, or [^{125}I]3,3'-T_2 and 50 μM 3'-phosphoadenosine-5'-phosphosulfate. Incubation media, oocyte extracts, and assay mixtures were analyzed by Sephadex LH-20 chromatography for production of conjugates and iodide. After 20-h incubation, the percentage of added radioactivity present as conjugates in the media and the oocytes amounted to 0.9 ± 0.2 and 1.0 ± 0.1 for T_4, less than 0.1 and less than 0.1 for T_3, 32.5 ± 0.4 and 29.3 ± 0.2 for rT_3, and 3.8 ± 0.3 and 2.3 ± 0.2 for 3,3'-T_2, respectively (mean ± SEM; n=3). The conjugate produced from rT_3 was identified as rT_3 sulfate, as it was hydrolyzed by acid treatment. After injection of oocytes with copy RNA coding for rat type I iodothyronine deiodinase, we found an increase in iodide production from rT_3 from 2.3 % (water-injected oocytes) to 46.2 % accompanied by a reciprocal decrease in rT_3 sulfate accumulation from 53.7% to 7.1%. After 30-min incubation with cytosol and 3'-phosphoadenosine-5'-phosphosulfate, sulfate formation amounted to 1.8% for T_4, less than 0.1% for T_3, 77.9% for rT_3, and 2.9% for 3,3'-T_2. These results show that rT_3 is rapidly metabolized in native oocytes by sulfation. The substrate preference of the sulfotransferase activity in oocytes is rT_3 >> 3,3'-T_2 > T_4 > T_3. The physiological significance of the high activity for rT_3 sulfation in *X. laevis* oocytes remains to be established.

INTRODUCTION

The main secretory product of the thyroid gland, T_4, is enzymatically converted in peripheral tissues to the biologically active hormone T_3 (1). This transformation concerns the elimination of an iodine from the phenolic ring of T_4, also termed outer ring deiodination (ORD). Deiodination of the tyrosyl ring [inner ring deiodination (IRD)] is an inactivation step by which T_4 and T_3 are converted to the inactive metabolites rT_3 and 3,3'-diiodothyronine (3,3'-T_2), respectively. The latter is also produced by ORD of rT_3 (2). These reactions are catalyzed by different iodothyronine deiodinases with distinct tissue distributions. The type I iodothyronine deiodinase (D1) is found predominantly in liver, kidney, and thyroid. It is a nonselective enzyme capable of ORD as well as IRD of different iodothyronines (1-3).

Besides deiodination, conjugation of the phenolic hydroxyl group with glucuronic acid or sulfate is another important step in thyroid hormone metabolism (2). In general, the purpose of these so-called phase II detoxification reactions is to increase the water solubility of lipophilic substances and, thus, to facilitate their excretion in bile and/or urine (4). The iodothyronine glucuronides are stable conjugates that are rapidly excreted in the bile (2). However, sulfate conjugation has been shown to facilitate the subsequent deiodination of iodothyronines by D1 in the liver (5,6). Although sulfation blocks the ORD of T_4, it strongly facilitates the IRD of both T_4 and T_3, suggesting that sulfation is an important step in the irreversible inactivation of thyroid hormone (5,6).

Sulfate conjugation of various compounds is catalyzed by sulfotransferases, which represent a group of homologous enzymes with overlapping substrate specificities that occur in the cytosolic fraction of different tissues, in particular liver (7). For all these enzymes 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acts as the sulfate donor. Iodothyronine sulfotransferase activities in rat and human tissues show a substrate preference for 3,3'-T_2 >> T_3 > rT_3 > T_4 (8-12).

In experiments to express plasma membrane transport proteins for thyroid hormone by injection of RNA from rat tissues in *Xenopus laevis* oocytes (13), we found that native oocytes transport and sulfate iodothyronines, in particular rT_3. We have, therefore, investigated the uptake and metabolism of T_4, T_3, rT_3 and 3,3'-T_2 by *X. laevis* oocytes as well as the sulfation of these compounds by isolated oocyte cytosol.

MATERIALS AND METHODS

Materials

T_4, T_3, PAPS and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). rT_3, 3,3'-T_2 and 3-iodothyronine (3-T_1) were obtained from Henning Berlin (Berlin, Germany). [3',5'-^{125}I]T_4 [43 megabecquerels (MBq)/nmol], [3'-^{125}I]T_3 (>66 MBq/nmol), and carrier-free $Na^{125}I$ (80 MBq/nmol) were purchased from Amersham (Aylesburg, UK). [3',5'-^{125}I]rT_3 and 3,[3'-^{125}I]T_2 were prepared from $Na^{125}I$ and 3,3'-T_2 or 3-T_1, respectively, using the chloramine-T method followed by purification on Sephadex LH-20 (12). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

Capped rat D1 copy RNA (cRNA) was prepared from the complementary DNA in pBluescript (14) (provided by Dr. P. Reed Larsen), linearized with *Xho*I (Boehringer Mannheim, Mannheim, Germany), using the AmpliScribe T3 transcription kit (Epicentre Technologies, Madison, WI) according to the protocol of the supplier. For capping, the m7G[5']ppp[5']G cap analog was used. cRNA pellets were dissolved in water (0.04 µg/µl) and stored at -80 C.

Animals

Two- to 3-yr-old adult *X. laevis* females were obtained from the Hubrecht Laboratory (Utrecht, The Netherlands). Frogs were maintained in a water-filled tank with three dark sides at a temperature of 18 - 22 C. A 12-h light, 12-h dark cycle was maintained to reduce seasonal variations in oocyte quality. Frogs were fed twice a week, and water was changed immediately after feeding.

Oocyte isolation and RNA injection

Oocytes were prepared as described previously (13). After isolation, the oocytes were sorted manually on morphological criteria, such as size, polarization, pigmentation, and absence of follicular layer debris. Healthy-looking stage V-VI oocytes (15) were transferred to six-well tissue culture plates and incubated in the dark at 18 C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.4), containing 10 IU/ml penicillin and

10 µg/ml streptomycin]. The next day, oocytes were injected with 23 nl water containing 0.92 ng D1 cRNA using the Nanoject system (Drummond Scientific, Broomall, PA). Injected and uninjected oocytes were maintained for 3-4 days at 18 C in modified Barth's solution, with daily change of medium.

Uptake and metabolism assays

Groups of 10 oocytes were transferred to a 96-well tissue culture plate and subsequently incubated in the dark at 18 C with 4 nM [^{125}I]T_4, 5 nM [^{125}I]T_3, 2 nM [^{125}I]rT_3, or 2 nM [^{125}I]T_2 in 0.1 ml sodium-containing incubation medium (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5) After 20 h, incubation medium was collected, and the oocytes were transferred to tubes and washed four times with 2.5 ml ice-cold sodium buffer containing 0.1% BSA. Two groups of 5 oocytes from each group of 10 were transferred to new tubes, counted, and lysed with 0.1 ml 0.1 M NaOH. Lysates were cleared by centrifugation. Lysates (in duplicate) and incubation media were acidified with 0.1 M HCl and analyzed by Sephadex LH-20 chromatography (16). The products were separated by successive elution with 1 ml 0.1 M HCl (iodide) twice, 1 ml water (conjugates) 6 times, and 1 ml 1 % NH_4OH in ethanol (iodothyronines) three times.

rT_3 conjugate hydrolysis

Acid hydrolysis of rT_3 conjugate was tested by incubation for 1 h at 80 C in 1 M HCl (17). The reaction was stopped by placing the mixtures on ice, and the products were analyzed by Sephadex LH-20 chromatography as described above.

Sulfotransferase assay

Oocytes were homogenized on ice in 2-3 volumes 0.1 M phosphate buffer (pH 7.2), 2 mM EDTA, and 1 mM dithiothreitol, and cytosol was isolated after centrifugation for 1 h at 100,000x*g*. The protein concentration was determined by the method of Bradford (18), using BSA as the standard.

Iodothyronine sulfotransferase activities were measured by incubation of 1 µM T_4, T_3, rT_3, or 3,3'-T_2 and 100,000 cpm of the ^{125}I-labeled compound for 30 min at 37 C (optimal temperature for warm-blooded animals) or 21 C (optimal temperature for cold-

blooded animals) with 0.1 or 1 mg cytosolic protein/ml in the presence (in triplicate) or absence (blank) of 50 μM PAPS in 0.2 ml 0.1 M phosphate buffer (pH 7.2) and 2 mM EDTA, as described previously (11,12). The reaction was started by the addition of diluted cytosol and stopped by the addition of 0.8 ml 0.1 M HCl. Iodothyronine sulfate formation was analyzed by Sephadex LH-20 chromatography as described above.

Statistics

Uptake and metabolism studies were performed with groups of 10 oocytes. Data are expressed as the percent uptake of total radioactivity per 10 oocytes and are presented as mean ± SEM. Statistical significance was evaluated by Student's *t* test for unpaired observations.

RESULTS

Uptake and metabolism of iodothyronines in oocytes

The percentage of added radioactivity present in the media and, after washing, in the oocytes after 20-h incubation amounted to 19.4 ± 3.7 and 67.1 ± 7.0 for T_4, 3.3 ± 0.3 and 51.5 ± 0.7 for T_3, 50.9 ± 1.4 and 35.0 ± 1.4 for rT_3 and 24.2 ± 0.5 and 68.1 ± 0.7 for 3,3'-T_2 (Fig. 1). The incomplete recovery of radioactivity in particular with [^{125}I]T_3, is primarily due to adsorption of label to the tube as the incubation medium did not contain protein. For T_4, T_3, and 3,3'-T_2, almost all radioactivity present in the oocytes was in the form of the added iodothyronine. However, only 15.4 ± 0.5% and 4.9 ± 0.1% of added [^{125}I]rT_3 was recovered as intact iodothyronine from the medium and oocytes, respectively. After incubation with [^{125}I]rT_3, most of the added radioactivity was recovered as conjugate, *i.e.* 32.3 ± 0.4% in the medium and 29.3 ± 0.2% in the oocytes, indicating active conjugation of rT_3 in the oocytes. With none of the iodothyronines was significant iodide production observed in the oocytes.

Native iodothyronines are regenerated from their sulfate conjugates by acid-catalyzed hydrolysis (17). The conjugate formed after incubation of rT_3 with oocytes was completely hydrolyzed after treatment for 1 h at 80 C with 1 M HCl (Fig. 2), indicating that rT_3 was indeed sulfated by the oocytes.

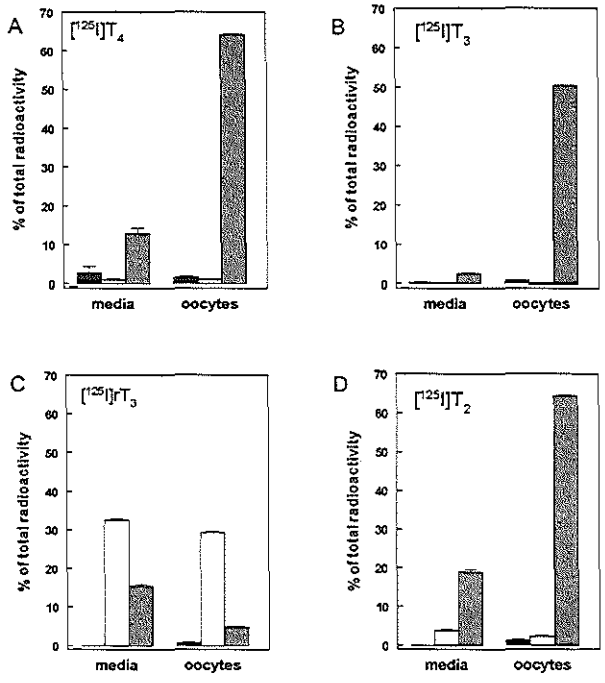

Fig. 1. Uptake and metabolism of [^{125}I]T_4 (A), [^{125}I]T_3 (B), [^{125}I]rT_3 (C), and [^{125}I]3,3'-T_2 (D) during incubation for 20 h at 18 C with 10 oocytes. Percentages of iodide (■), conjugates (□), and iodothyronines (▨) in the incubation buffer and in the homogenized oocytes, analyzed by Sephadex LH-20 chromatography. *Bars* represent the mean ± SEM (n=3).

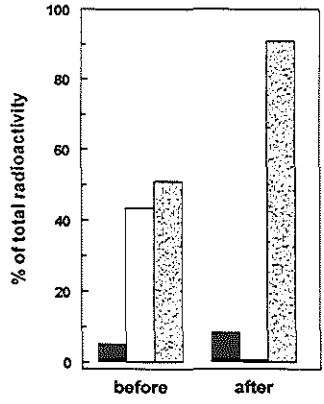

Fig. 2. Acid-catalyzed hydrolysis of rT_3 conjugate produced after incubation of [^{125}I]rT_3 for 20 h at 18 C with 10 oocytes. The percentages iodide (■), conjugate (□), and rT_3 (▨) in the incubation medium and oocytes before and after the acid-catalyzed hydrolysis were determined by Sephadex LH-20 chromatography. Results are derived from a duplicate incubation in a representative experiment.

Injection of cRNA coding for rat D1 resulted, after incubation of the oocytes for 20 h with [^{125}I]rT_3, in a large increase in iodide formation from 2.3 % to 46.2 % of the added radioactivity, which was accompanied by a reciprocal decrease in rT_3 sulfate (rT_3S) accumulation from 53.7% to 7.1% compared to water-injected oocytes (Fig. 3). Water-injected oocytes showed the same rate of rT_3 sulfation as native (uninjected) oocytes (Figs. 1C and 3). Figure 3 also shows that the amount of iodide in the D1 cRNA-injected oocytes incubated with [^{125}I]rT_3 was 3 times higher than that in the medium.

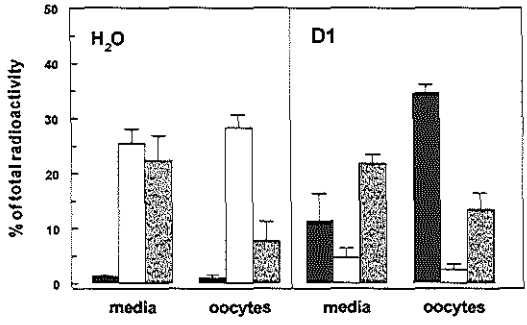

Fig. 3. Metabolism of rT_3 in D1 cRNA-injected and water-injected oocytes during incubation of [^{125}I]rT_3 for 20 h at 18 C with 10 oocytes. Percentages of iodide (■), conjugates (□), and rT_3 (▒) in the incubation medium and oocytes were determined by Sephadex LH-20 chromatography. *Bars* represent the mean ± SEM (n=3).

Sulfotransferase assay

Iodothyronine sulfotransferase activities were measured in cytosol from native oocytes at incubation temperatures of 37 C and 21 C, producing the same results (not shown). Figure 4 presents the results of the experiments performed at 21 C. Although sulfation rates were higher at 1 than at 0.1 mg cytosolic protein/ml, the pattern of sulfation of the different iodothyronines was independent of the protein concentration. The sulfotransferase activity in oocyte cytosol showed a preference for rT_3 >> 3,3'-T_2 > T_4 > T_3.

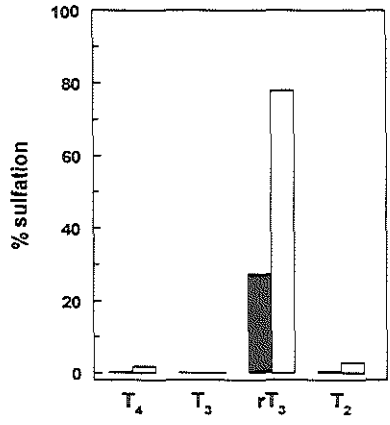

Fig. 4. Sulfation of 1 µM iodothyronines during incubation for 30 min at 21 C with oocyte cytosol in the presence of 50 µM PAPS. The cytosolic protein concentration amounted to 0.1 mg/ml (■) and 1.0 mg/ml (□). Results are the means of two closely agreeing experiments.

DISCUSSION

In experiments using *X. laevis* oocytes to express rat liver cell membrane transporters for thyroid hormones (13), we found that native *X. laevis* oocytes transport iodothyronines and sulfate rT_3. We also found that transport and sulfation of rT_3 in oocytes injected with rat liver messenger RNA (mRNA) were similar to those in uninjected oocytes (data not shown). Thus, the possible induction of an exogenous transport protein for rT_3 by the injection of rat liver mRNA was completely masked by the endogenous transport system for rT_3 in the oocytes. This is in contrast to previous findings concerning the expression of hepatic cell membrane transporters for T_3 and T_4 (13), showing that injection of rat liver mRNA or partially purified fractions thereof induce a significant 2- to 3-fold increase in T_3 and T_4 transport into oocytes.

After 20-h incubation with ^{125}I-labeled T_4, T_3, or 3,3'-T_2, most radioactivity was associated with the oocytes as unmetabolized iodothyronines. As after incubation the oocytes were extensively washed in BSA-containing medium, cell-associated radioactivity most likely represents internalized iodothyronine. This conclusion is supported by the observation that uptake of iodothyronines by *X. laevis* oocytes is a Na^+-dependent process (13). On the contrary, after 20-h incubation with [^{125}I]rT_3, most

radioactivity was recovered from the medium, largely as rT_3S. Taking medium and oocytes together, more than 50% of added rT_3 was sulfated. Sulfation of iodothyronines is catalyzed by cytoplasmic sulfotransferases (7-12), which enzymes are apparently also present in oocytes. These results, therefore, support the conclusion that rT_3 was internalized by the oocytes and sulfated intracellularly. Subsequently, (part of) the rT_3S formed is transported back to the medium. The finding that after 20-h incubation of oocytes with rT_3 and other iodothyronines almost no iodide is formed indicates that *X. laevis* oocytes possess little or no endogenous D1 activity, in agreement with St. Germain *et al.* (19). In fact, D1 activity in not expressed at any stage of development in amphibians (20). On the other hand, injection of cRNA coding for rat D1 leads to a large increase in iodide production from rT_3 at the expense of rT_3S accumulation. As, like native D1 in mammalian cells, exogenous D1 in oocytes is probably located intracellularly (1-3), these results again strongly support the presence of endogenous transporter(s) for iodothyronines in *X. laevis* oocytes.

D1 is a nonselective enzyme capable of both ORD and IRD of iodothyronines (1-3). The enzyme is most effective in the ORD of rT_3, but the deiodination of different iodothyronines is dramatically affected by the sulfation of these compounds (5,6). Thus, IRD of both T_4 and T_3 by rat D1 is accelerated 40-200 times after their sulfation, suggesting that sulfation is an important step, leading to the irreversible inactivation of thyroid hormone (5,6). In contrast, ORD of T_4 is completely blocked by sulfation. This is not a general phenomenon, as ORD of rT_3 by rat D1 is not affected by sulfation of this substrate, whereas ORD of 3,3'-T_2 is facilitated 50-fold by sulfation (5,6). In contrast to the facilitated deiodination of iodothyronine sulfates by D1, deiodination of iodothyronines by the type II (D2) and type III (D3) iodothyronine deiodinases is inhibited by sulfation (21) (Visser TJ, unpublished observations). As rT_3 and rT_3S are deiodinated equally well by rat D1, it is uncertain to what extent iodide production from rT_3 in rat D1 cRNA-injected oocytes proceeds via rT_3S or represents direct ORD of rT_3. The iodide formed remains trapped inside the oocytes, indicating the absence of transporters for both influx and efflux of iodide in native oocytes, in accordance with the studies of Dai *et al.* (22).

Our experiments have not directly addressed the possible IRD of the different iodothyronines in the oocytes. However, St. Germain *et al.* (23) have shown that native

X. laevis oocytes kept in Barth's medium do not express D3. Moreover, we did not find conjugate formation after incubation of T_4 with oocytes, which would have been expected if T_4 underwent IRD to rT_3. Therefore, it is also unlikely that T_3 was metabolized by IRD in the oocytes.

Sulfate conjugation of iodothyronines is catalyzed by phenol sulfotransferases located in the cytosolic fraction of different tissues (7). In both rats and humans, iodothyronine sulfotransferase activities show a substrate preference for 3,3'-T_2 >> T_3 > rT_3 > T_4, with rT_3 being a relatively poor substrate (8-12). Our results show that sulfotransferase activity in *X. laevis* oocyte cytosol has a clear substrate preference for rT_3, whereas 3,3'-T_2, T_3, and T_4 are hardly sulfated at all. The physiological importance of thyroid hormone sulfation is still unknown. As discussed above, sulfation is an irreversible pathway of thyroid hormone metabolism when D1 activity is high, as sulfated iodothyronines are rapidly degraded by this enzyme (5,6). However, sulfation is a reversible pathway of thyroid hormone inactivation when D1 activity is low because of the regeneration of free iodothyronines by sulfatases produced in different tissues and by intestinal bacteria (5,6). It has been speculated that due to the low D1 activity during fetal development in mammals, sulfation/desulfation of T_3 is an important mechanism for the tissue-specific and time-dependent regulation of thyroid hormone bioactivity (6,21). Indeed, high concentrations not only of T_3S, but also of T_4S, rT_3S, and 3,3'-T_2S have been detected in fetal sheep serum and human cord serum (24,25).

It is remarkable that during embryonic and fetal development in different organisms, two active pathways exist for the inactivation of thyroid hormone: IRD of T_4 to rT_3 and of T_3 to 3,3'-T_2 by D3, and sulfation (1-3,24,25). That these are true inactivation pathways is suggested by the findings that rT_3, 3,3'-T_2, and T_3S have little or no affinity for the T_3 receptor (26,27). Thyroid hormone plays an important role not only in the embryonic development of tadpoles, but also in the metamorphosis of the tadpole to the froglet (20). This requires the tissue-specific and development stage-dependent regulation of the balance between thyroid hormone activation by D2 and inactivation by D3 (20). We hypothesize that sulfation/desulfation contributes to the regulation of thyroid hormone bioactivity during embryonic development and metamorphosis in frogs. Although direct evidence is lacking, this hypothesis is supported by the absence of D1 in frogs (20). Why, then, the sulfotransferase(s) involved shows a profound substrate

preference for rT_3 remains unknown. The possibility that rT_3S exerts a biological function by binding to a transcription factor other than the T_3 receptor deserves investigation.

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Chapter 4

IDENTIFICATION OF THYROID HORMONE TRANSPORTERS

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ABSTRACT

Thyroid hormone action and metabolism are intracellular events that require transport of the hormone across the plasma membrane. We tested the possible involvement of the Na^+/taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide (oatp1) in the hepatic uptake of the prohormone T_4, the active hormone T_3, and the metabolites rT_3 and 3,3'-T_2. *Xenopus laevis* oocytes were injected with 2.3 ng Ntcp or oatp1 cRNA and, after 2-3 days, incubated for 1 h at 25 °C with usually 0.1 μM ^{125}I-labeled ligand. Uninjected oocytes showed marked uptake of iodothyronines and this was further increased by Ntcp and oatp1 cRNA, *i.e.*, 1.9- and 2.8-fold for T_4, 1.7- and 1.7-fold for T_3, 1.8- and 6.0-fold for rT_3, and 1.3 and 1.4-fold for 3,3'-T_2, respectively. Mostly due to much lower uptake by uninjected oocytes, Ntcp and oatp1 cRNA induced larger, 12- to 76-fold increases in uptake of iodothyronine sulfates. The Ntcp cRNA-induced iodothyronine uptake was completely inhibited in Na^+-depleted medium, whereas the oatp1 cRNA-induced uptake was not affected. These results suggest that hepatic uptake of thyroid hormones and their metabolites is mediated at least in part by Ntcp and oatp1.

INTRODUCTION

The thyroid gland produces predominantly the prohormone thyroxine (T_4) from which the active hormone 3,3',5-triiodothyronine (T_3) is produced by enzymatic outer ring deiodination (1-4). Both T_4 and T_3 are inactivated by enzymatic inner ring deiodination to the metabolites 3,3',5'-triiodothyronine (rT_3) and 3,3'-diiodothyronine (3,3'-T_2), respectively (1-4). The three deiodinases involved have recently been identified as homologous transmembrane selenoproteins with their active site exposed to the cytoplasm in the various tissues (1-4). Additional pathways of thyroid hormone metabolism include sulfation and glucuronidation of the phenolic hydroxyl group by transferases located in the cytoplasm and endoplasmic reticulum of different tissues (2,4).

Thyroid hormone is essential for the development of different organs, notably the brain, and for the regulation of energy metabolism of all tissues throughout life (5). Most of the actions of thyroid hormone are initiated by binding of T_3 to nuclear receptors, which changes the interaction of these receptors with regulatory elements of thyroid hormone-responsive genes (5). Access of plasma thyroid hormone to intracellular receptors and enzymes requires transport across the cell membrane. On the basis of the lipophilic nature of iodothyronines, it was assumed for a long time that they cross the cell membrane by simple diffusion. However, this ignored the highly polar nature of the alanine side chain which is a formidable obstacle for membrane passage of iodothyronines. During the last two decades overwhelming evidence has accumulated indicating the involvement of plasma membrane transporters in tissue uptake of thyroid hormone (4,6).

Studies using isolated rat hepatocytes have suggested multiple transporters for uptake of T_4, T_3 and rT_3 (4,6). Uptake of the different iodothyronines is energy (ATP)-dependent, but T_4 and rT_3 uptake appear to be more sensitive to decreases in cellular ATP levels than T_3 uptake (7). Treatment of hepatocytes with the Na/K-ATPase inhibitor ouabain results in a marked inhibition of the uptake of thyroid hormone, suggesting that this is a Na^+-dependent process (8).

In recent years, we have explored the possibility to clone iodothyronine transporters from rat liver using the *Xenopus laevis* oocytes expression system (9-12).

We observed modest increases in T_4 and T_3 uptake by oocytes 3-4 days after injection with rat liver mRNA, in particular the 0.8- to 2.1-kb size fraction, compared to the endogenous uptake of iodothyronines by native oocytes (9). A much lower background uptake was observed with T_3 sulfate (T_3S), resulting in a larger relative increase after injection of rat liver mRNA (9). We also found that T_4 sulfamate (T_4NS) and T_3 sulfamate (T_3NS) are useful water-soluble ligands, showing negligible uptake by native oocytes and high uptake by oocytes injected with rat liver mRNA (11,12). This uptake is competitively inhibited by T_4 and T_3, suggesting the involvement of common transporters (11,12). T_4NS and T_3NS are organic anions, and different multispecific transporters for such compounds have been cloned from rat liver, including the Na^+/taurocholate cotransporting polypeptide (Ntcp) and the Na^+-indepedent organic anion transporting polypeptide (oatp1) (13-16). This study was therefore conducted to test the possible involvement of Ntcp and oatp1 in liver uptake of iodothyronine derivatives.

MATERIALS AND METHODS

Materials

T_4 and T_3 were obtained from Sigma Chemical Co. (St. Louis, MO). rT_3 and 3,3'-T_2 were obtained from Henning Berlin GmbH (Berlin, Germany). [^{125}I]T_4 and [^{125}I]T_3 were purchased from Amersham (Amersham, UK). All other ^{125}I-labeled compounds were prepared as previously described (17). [^{125}I]T_4 and [^{125}I]rT_3 as well as their derivatives were purified on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) immediately before use (17).

RNA preparation

Rat liver poly(A)$^+$ RNA was isolated as described previously (9). mRNA was stored in water at -80 °C at a concentration of 1 µg/µl. Capped Ntcp and oatp1 cRNA were prepared from the cDNA clones (13,14) linearized with *Pvu*I or *Xba*I (Boehringer Mannheim, Mannheim, Germany), using the Ampliscribe T3 or T7 RNA transcription kit (Epicentre Technologies, Madison, WI), respectively. For capping, the m7G[5']ppp[5']G

cap analog was used (Epicentre Technologies). cRNA was stored in water at -80 °C at a concentration of 0.1 µg/µl.

Oocyte isolation and RNA injection

Oocytes were prepared as described previously (9). After isolation, the oocytes were sorted on morphological criteria and defolliculated manually. Healthy-looking stage V-VI oocytes (18) were transferred to six-well tissue culture plates and incubated overnight at 18 °C in modified Barth's solution, containing 20 IU/ml pencillin and 20 µg/ml streptomycin (9). The next day, oocytes were injected with 23 nl water containing 23 ng mRNA, 2.3 ng Ntcp cRNA or 2.3 ng oatp1 cRNA using the Nanoject system (Drummond Scientific, Broomall, PA). Injected and uninjected oocytes were maintained for 2-3 days at 18 °C in modified Barth's solution.

Uptake assay

Uptake assays were performed as described previously (9). Eight to 10 oocytes were incubated for 1 h at 25 °C with usually 0.1 µM [^{125}I]iodothyronine derivative in 0.1 ml incubation buffer (100 mM NaCl or choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). After 1 h, incubation buffer was removed and the oocytes were washed four times with 2.5 ml ice-cold Na^+-containing incubation buffer supplemented with 0.1% BSA. Oocytes were transferred to new tubes and counted individually. Similar results were obtained in uninjected and water-injected oocytes. Uptake of [^{3}H]taurocholate by oocytes was studied in parallel, showing similar stimulation of Na^+-dependent transport after injection of Ntcp cRNA and Na^+-independent transport after injection of oatp1 cRNA as previously reported (13,14).

Statistics

Data are presented as means ± SEM. Statistical significance was evaluated by Student's *t* test for unpaired observations.

RESULTS

Figure 1 demonstrates that uptake of T_3NS by oocytes is stimulated after injection with increasing amounts of Ntcp or oatp1 cRNA. Uptake of T_3NS by uninjected oocytes was negligible. T_3NS uptake by Ntcp cRNA-injected oocytes was completely inhibited by replacement of Na^+ in the medium with choline (Ch^+), whereas T_3NS uptake by oatp1 cRNA-injected oocytes was not affected by Na^+ depletion. At all cRNA doses tested, T_3NS uptake was stimulated to a higher degree by Ntcp cRNA than by oatp1 cRNA. Both Ntcp and oatp1-mediated uptake of T_3NS reached a maximum after injection of ≈1.5 ng cRNA per oocyte, and in all further experiment 2.3 ng cRNA was injected per oocyte.

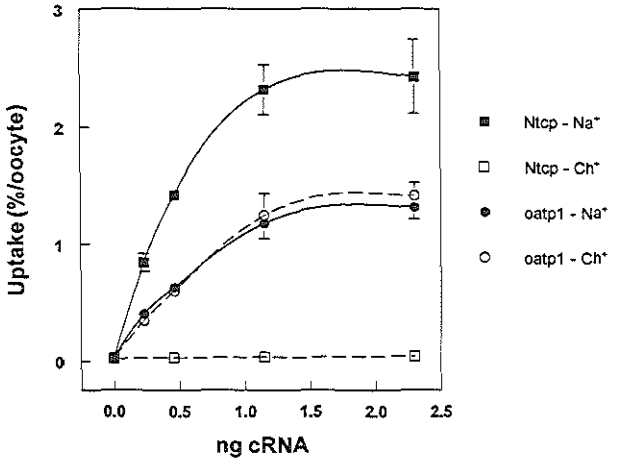

Fig. 1. Uptake of T_3NS by oocytes injected with 0-2.3 ng Ntcp or oatp1 cRNA. Two days after injection, oocytes were incubated for 1 h at 25 °C with 0.1 μM [^{125}I]T_3NS in Na^+ or Ch^+ medium. Data are presented as means ± SEM of 7-10 oocytes.

Figure 2 shows the effects of injection of 23 ng total rat liver mRNA or 2.3 ng Ntcp or oatp1 cRNA on the uptake of [^{125}I]T_4NS by oocytes. T_4NS uptake by uninjected oocytes was negligible. Injection of liver mRNA resulted in a significant increase in T_4NS uptake, and this increase was markedly reduced by substituting Ch^+ for Na^+ in the medium. A dramatic increase in T_4NS uptake was observed after injection with Ntcp cRNA or oatp1 cRNA. As with T_3NS, the increase induced by Ntcp cRNA was larger

than that elicited by oatp1 cRNA. Again, T_4NS uptake in Ntcp cRNA-injected oocytes was completely blocked in Na^+ depleted medium, whereas T_4NS uptake in oatp1 cRNA-injected oocytes was similar in Na^+ and Ch^+ medium. Ntcp and oatp1 mediated transport of T_4NS somewhat (16% and 44%) better than transport of T_3NS.

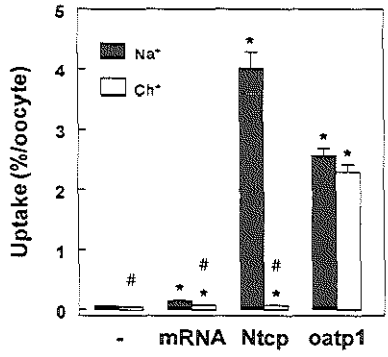

Fig. 2. Uptake of T_4NS by uninjected oocytes and oocytes injected with 23 ng rat liver mRNA or 2.3 ng Ntcp or oatp1 cRNA. Three days after injection, oocytes were incubated for 1 h at 25 °C with 14 nM [^{125}I]T_4NS in Na^+ or Ch^+ medium. Data are presented as means ± SEM of 18-20 oocytes. * $p<0.001$ *vs* uninjected; # $p<0.001$ *vs* Na^+.

Figure 3 presents the effects of injection of oocytes with Ntcp or oatp1 cRNA on the uptake of unconjugated iodothyronines. In contrast to the sulfamates, marked uptake of the different iodothyronines was observed with uninjected oocytes which was partially inhibited by replacement of Na^+ in the medium with Ch^+. On top of this high background, uptake of the different iodothyronines was significantly stimulated after injection of Ntcp or oatp1 cRNA. The Ntcp cRNA-induced uptake varied between 1.3- and 1.9-fold. Injection of oatp1 cRNA produced by far the largest increase in uptake of rT_3 (6.0-fold), followed by T_4 (2.8-fold), T_3 (1.7-fold) and 3,3'-T_2 (1.4-fold). Incubation in Na^+ depleted medium completely inhibited the Ntcp cRNA-stimulated uptake of the iodothyronines but did not affect the increase induced by oatp1 cRNA (not shown).

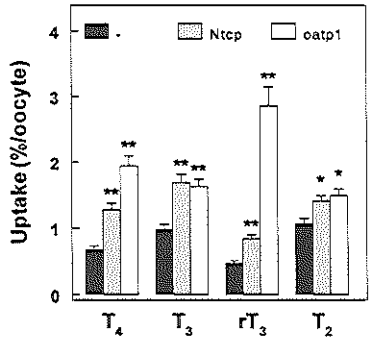

Fig. 3. Uptake of iodothyronines by uninjected oocytes and oocytes injected with 2.3 ng Ntcp or oatp1 cRNA. Two days after injection, oocytes were incubated for 1 h at 25 °C with 0.1 µM ^{125}I-labeled T_4, T_3, rT_3, or 3,3'-T_2 in Na^+ medium. Data are presented as means ± SEM of 16-38 oocytes. * $p<0.005$ *vs* uninjected; ** $p<0.001$ *vs* uninjected.

Figure 4 shows the effects of injection of oocytes with Ntcp or oatp1 cRNA on the uptake of iodothyronine sulfates. Uptake of the conjugates by native oocytes was very low compared with the uptake of the nonsulfated compounds. Injection of both Ntcp cRNA and oatp1 cRNA produced dramatic increases in the uptake of the different sulfoconjugates. Although these increases were similar in magnitude as those observed with the nonsulfated iodothyronines, because of the lower background uptake by uninjected oocytes the relative increases induced by Ntcp and oatp1 cRNA were much greater for the sulfates, *i.e.*, 16- and 25-fold for T_4S, 53- and 76-fold for T_3S, 12- and 26-fold for rT_3S, and 49- and 53-fold for 3,3'-T_2S, respectively. Uptake of the sulfates was more strongly stimulated after oatp1 cRNA than after Ntcp cRNA injection.

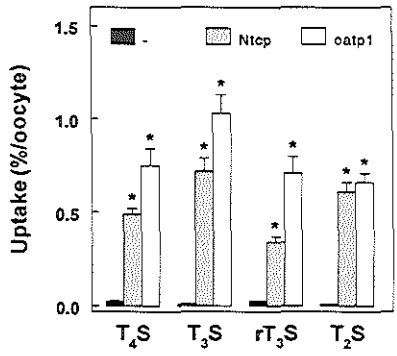

Fig. 4. Uptake of iodothyronine sulfates by uninjected oocytes and oocytes injected with 2.3 ng Ntcp or oatp1 cRNA. Two days after injection, oocytes were incubated for 1 h at 25 °C with 1-2 nM ^{125}I-labeled T_4S, T_3S, rT_3S or 3,3'-T_2S in Na^+ medium. Data are presented as means ± SEM of 7-8 oocytes. *p<0.001 *vs* uninjected.

DISCUSSION

Rat Ntcp is a 362-amino-acid protein containing 7 putative transmembrane domains and 2 glycosylation sites with an apparent molecular mass of 51 kDa (13,15,16). It is only expressed in differentiated mammalian hepatocytes, where it is localized selectively to the basolateral cell membrane (15,16). It is the major transporter of conjugated bile acids in liver but it also mediates uptake of unconjugated bile acids and a number of non-bile acid amphipathic compounds, including estrogen conjugates such as estrone 3-sulfate (15,16,19). A homologous bile acid transporter is expressed in rat ileum and kidney, where it is localized to the apical cell membrane (20,21). The human orthologs of these transporters have also been characterized (22-24).

Rat oatp1 is a 670-amino-acid protein with 12 putative transmembrane domains and 2 glycosylation sites with an apparent molecular mass of 80 kDa (14-16). Oatp1 is not only expressed in liver but also in kidney and brain. Like Ntcp, oatp1 is localized to the basolateral liver cell membrane. Oatp1 is a multispecific transporter mediating the uptake of a wide variety of amphipathic ligands (15,16,25-28), including conjugated and unconjugated bile acids, conjugated steroids (*e.g.*, DHEA sulfate) and other organic

anions (*e.g.*, the prototypic bromosulfophthalein), but also neutral steroids (*e.g.*, corticosterone), cardiac glycosides (*e.g.*, ouabain) and even organic cations (*e.g.*, ajmalinium). In contrast to Ntcp, transport through oatp1 is not coupled to Na^+. There is evidence suggesting that oatp1 mediates exchange of intra- and extracellular anions (29). Human OATP has recently also been characterized (30) as well as 2 strongly homologous transporters expressed in rat liver, kidney and brain (oatp2; 31) or exclusively in kidney (OAT-K1; 32). Less homologous proteins have recently been identified as prostaglandin transporters in rat and human liver (33,34).

Our findings demonstrate marked stimulation of the uptake of native iodothyronines as well as their N_α-sulfonated (sulfamate) and 4'-OH-sulfonated (sulfate) derivatives after injection of oocytes with cRNA coding for Ntcp or oatp1. The Na^+ dependence of Ntcp and the Na^+ independence of oatp1 were confirmed with all these ligands. The largest stimulation was observed with the sulfamates which showed higher transport rates in Ntcp cRNA than in oatp1 cRNA-injected oocytes, with a slight preference of T_4NS over T_3NS. Maximum uptake was observed for both Ntcp and oatp1 after injection of $\approx$1.5 ng cRNA per oocyte. Since the efficiency of the translation of the cRNAs and the processing of the proteins is unknown, differences in uptake rates induced by these messengers cannot be interpreted in terms of intrinsic transport rates.

Although smaller in magnitude, the stimulation of the uptake of the different iodothyronine sulfates in Ntcp or oatp1 cRNA-injected oocytes was as dramatic as that observed with the sulfamates. In general, uptake of the sulfates was induced to a larger extent by oatp1 cRNA than by Ntcp cRNA, with a preference for T_3S. In contrast to the sulfamates, iodothyronine sulfates are naturally occuring thyroid hormone metabolites. Sulfation plays an important role in the metabolism of thyroid hormone (4,36). It facilitates the type I deiodinase (D1)-catalyzed inner ring deiodination (degradation) of the prohormone T_4 and the active hormone T_3, whereas it blocks the outer ring deiodination (activation) of T_4 to T_3 by D1 (4,35). Our results suggest that both Ntcp and oatp1 contribute importantly to the uptake of T_4S and T_3S by rat hepatocytes, thus allowing their degradation by D1 located in the endoplasmic reticulum (1-4).

Perhaps the most important finding of our study is the stimulation of the uptake of the different native iodothyronines by injection of Ntcp or oatp1 cRNA. Although the relative increase in uptake of the iodothyronines by either cRNA was less than that

observed with the sulfamates and the sulfates, this was primarily due to the much higher endogenous uptake by uninjected oocytes. In fact, the increase in uptake of the different iodothyronines induced by Ntcp or oatp1 cRNA was similar in magnitude as that seen with the sulfates. The degree of stimulation of iodothyronine uptake by oatp1 cRNA was equal to or larger than the increase induced by Ntcp cRNA. By far the largest stimulation occured after injection of oatp1 cRNA using rT_3 as the ligand.

We have also found significant stimulation of thyroid hormone uptake in oocytes after injection with oatp2 cRNA, although the magnitude of this stimulation was less than that induced by oatp1 cRNA (ECH Friesema, R Docter, EPCM Moerings, B Stieger, B Hagenbuch, PJ Meier, EP Krenning, G Hennemann, TJ Visser, unpublished observations). After completion of our study, Abe *et al.* (36) reported on the transport of T_4 and T_3 by oatp2 and a newly characterized homologous transporter, oatp3, which has a more wide-spread tissue distribution than oatp1 and oatp2. Together, these data strongly suggest that families of homologous organic anion transporters are involved in uptake of thyroid hormone in different tissues. The identification of transporters mediating the uptake of thyroid hormone in different tissues is not only important in the study of the regulation of these processes in health and disease, but it may also open ways for the development of tissue-specific thyroid hormone agonists and antagonists which may be beneficial in the treatment of conditions such as obesity and cardiovascular diseases.

In conclusion, we found that Ntcp and oatp1 are capable of mediating uptake of thyroid hormone derivatives in oocytes. Further studies need to be done to establish the roles of these and homologous transporters in the regulation of thyroid hormone bioactivity.

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Chapter 5

THYROID HORMONE TRANSPORT BY THE HETERODIMERIC HUMAN SYSTEM L AMINO ACID TRANSPORTER

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ABSTRACT

Transport of thyroid hormone across the cell membrane is required for thyroid hormone action and metabolism. We have investigated the possible transport of iodothyronines by the human system L amino acid transporter, a protein consisting of the human 4F2 heavy chain (*h*4F2hc) and the human LAT1 light chain (*h*LAT1). *Xenopus* oocytes were injected with the cRNAs coding for *h*4F2hc and/or *h*LAT1, and after 2 days incubated at 25 C with 0.01-10 μM [^{125}I]T_4, [^{125}I]T_3, [^{125}I]rT_3 or [^{125}I]3,3'-T_2, or with 10-100 μM [^{3}H]Arg, [^{3}H]Leu, [^{3}H]Phe, [^{3}H]Tyr or [^{3}H]Trp. Injection of *h*4F2hc cRNA alone stimulated uptake of Leu and Arg, due to dimerization of *h*4F2hc with an endogenous *Xenopus* light chain, but did not affect uptake of other ligands. Injection of *h*LAT1 cRNA alone did not stimulate uptake of any ligand. Coinjection of cRNAs for *h*4F2hc and *h*LAT1 stimulated uptake of Phe > Tyr > Leu > Trp (100 μM) and of 3,3'-T_2 > rT_3 ≈ T_3 > T_4 (10 nM), which in all cases was Na^+-independent. Saturation analysis provided apparent Michaelis constant (K_m) values of 7.9 μM for T_4, 0.8 μM for T_3, 12.5 μM for rT_3, 7.9 μM for 3,3'-T_2, 46 μM for Leu, and 19 μM for Trp. Uptake of Leu, Tyr and Trp (10 μM) was inhibited by the different iodothyronines (10 μM), in particular T_3. *Vice versa*, uptake of 0.1 μM T_3 was almost completely blocked by coincubation with 100 μM Leu, Trp, Tyr or Phe.

Our results demonstrate stereospecific Na^+-independent transport of iodothyronines by the human heterodimeric system L amino acid transporter.

INTRODUCTION

Thyroid hormone is essential for the development of different organs, in particular the brain, and for the metabolic control of virtually all tissues throughout life (1-3). Its major effects include stimulation of oxygen consumption and thermogenesis, acceleration of carbohydrate, protein, lipid and bone mineral turnover, and increased contractility of skeletal muscles and the heart (2,3). Most actions of thyroid hormone are initiated by the binding of the active form of the hormone T_3 to nuclear receptors, which are associated with regulatory elements in the promoter region of target genes (3,4). Binding of T_3 to its receptor induces the release of corepressors and the recruitment of coactivators, usually resulting in the stimulation of gene transcription (4).

Although T_3 is the receptor-active form of thyroid hormone, its precursor T_4 is the predominant product secreted by the follicular cells of the thyroid gland (5,6). Although some T_3 is also secreted, most T_3 is produced by enzymatic outer ring deiodination (ORD) of T_4 in peripheral tissues (5,6). Both T_4 and T_3 are inactivated by inner ring deiodination (IRD) to the metabolites rT_3 and 3,3'-diiodothyronine (3,3'-T_2), respectively (5,6). The three deiodinases (D1-D3) involved in these conversions are homologous selenoproteins with different catalytic profiles, tissue distributions and physiological functions (7,8). D1 in liver and kidney appears important for systemic T_3 production, D2 in tissues such as brain and pituitary for local T_3 production, and D3 in brain and other tissues for T_4 and T_3 degradation. All three deiodinases are transmembrane proteins with their active site exposed to the cytoplasm (5-8).

Metabolism and action of thyroid hormone are intracellular events requiring uptake of extracellular hormone through the plasma membrane. Although iodothyronines are lipid-soluble compounds, they cannot readily cross the lipid bilayer of the cell membrane by simple diffusion. This is because the polar zwitter-ionic alanine side chain prevents passage of the iodothyronine molecule through the hydrophobic inner part of the cell membrane constituted of the aliphatic fatty acid chains. Evidence accumulated over the last two decades indicates that uptake of thyroid hormone in different tissues is mediated by transporters (6,9-12). Work in our laboratory has demonstrated the presence of multiple iodothyronine transporters in rat and human liver cells (6,11,12). Two energy- and Na^+-dependent transporters appear of particular

importance for hepatic uptake of T_4 and rT_3, and of T_3, respectively, showing nM affinities for their ligands but different dependencies on cellular ATP levels (6,11,12). However, iodothyronine transporters in other tissues show different characteristics. Isolated rat pituitary cells also show carrier-mediated uptake of different iodothyronines but this appears to be mediated by a single transporter (13). In contrast, cultured neonatal rat cardiomyocytes show specific uptake of T_3 but not of T_4 (14). A variety of cells, including rat pituitary cells (13), growth hormone-producing tumor cells (15), erythrocytes (16,17), cardiomyocytes (14) and astrocytes (18), mouse neuroblastoma (19) and thymocytes (20), and human choriocarcinoma cells (21,22) show competition between uptake of iodothyronines and neutral amino acids such as leucine (Leu) and tryptophan (Trp). This may not be surprising since iodothyronines are iodinated amino acid derivatives built from two tyrosine (Tyr) molecules. Based on the above observations it has been suggested that thyroid hormone may be taken up in different tissues at least in part through system L or system T amino acid transporters (15-19).

In particular through the pioneering work of Christensen, different classes of amino acid transporters have been distinguished on the basis of their preference for certain types of amino acids (*e.g.* neutral, acidic or basic), their specificity for natural or artificial prototypic ligands, as well as their mechanism of transport (*e.g.* Na^+-dependent or Na^+-independent) (23,24). A rapidly increasing number of amino acid transporters has been characterized in recent years, including the 4F2-related heterodimeric transporters (for reviews, see Refs. 25-27). The 4F2 or CD98 cell surface antigen has been known for some time to be expressed in many tissues, especially on activated lymphocytes and tumor cells, but only recently it has been identified as a family of amino acid transporters (25-28). These heterodimeric transporters consist each of a common 4F2 heavy chain (4F2hc) and a member of a family of homologous light chains, seven of which have now been cloned (25-41). 4F2hc is a glycosylated protein with a single transmembrane domain, whereas the light chains are not glycosylated and have 12 putative transmembrane domains; they are linked through a disulfide bond (25-28). One of the seven light chains mentioned above appears to dimerize preferentially with another heavy chain, termed rBAT (for 'related to basic amino acid transport'), which is homologous to 4F2hc, suggesting the existence of a superfamily of heterodimeric amino acid transporters consisting of multiple heavy and light chains (25-28,38-40).

In combination with 4F2hc, two 4F2 light chains mediate the Na^+-independent transport of large neutral (branched chain and aromatic) amino acids such as Leu, Tyr, Trp and phenylalanine (Phe). This is typical for the system L amino acid transporter, hence the name LAT1 and LAT2 for these light chains (25-27,29-33). Two other light chains forming heterodimers with 4F2hc mediate the Na^+-dependent uptake of neutral amino acids such as Leu as well as the Na^+-independent uptake of basic amino acids such as arginine (Arg). This is characteristic of the system y^+L amino acid transporter, which is why these light chains are named y^+LAT1 and y^+LAT2 (25-27,34-36). We have tested the possible involvement of these heterodimeric 4F2 transporters in the transport of thyroid hormone by studying the uptake of the iodothyronines T_4, T_3, rT_3 and 3,3'-T_2 by *Xenopus laevis* oocytes injected with cRNA coding for human 4F2hc (*h*4F2hc) alone or in combination with cRNA coding for human LAT1 (*h*LAT1), mouse LAT2 (*m*LAT2), *hy*$^+$LAT1 or *hy*$^+$LAT2. Whereas the system y^+L transporters did not mediate uptake of iodothyronines, effective thyroid hormone transport was observed with the system L transporters, in particular the *h*4F2hc/*h*LAT1 heterodimer, which is the subject of this report.

MATERIALS AND METHODS

Materials

Nonradioactive L-iodothyronines and 3,3',5-triiodothyroacetic acid (Triac) were obtained from Henning Berlin GmbH (Berlin, Germany). [3',5'-^{125}I]T_4, [3'-^{125}I]T_3 and carrier-free $Na^{125}I$ were purchased from Amersham Pharmacia (Uppsala, Sweden). [3',5'-^{125}I]rT_3 and 3,[3'-^{125}I]T_2 were prepared by radioiodination of 3,3'-T_2 and 3-T_1, respectively, using the chloramine-T method, followed by purification on Sephadex LH-20 (Amersham Pharmacia). [^{125}I]T_4 and [^{125}I]rT_3 were also purified on Sephadex LH-20 immediately before use (42). D-T_3, phenylalanine (Phe), tyrosine (Tyr) and 2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) were purchased from Sigma (St. Louis, MO), arginine (Arg) and leucine (Leu) from Merck (Darmstadt, Germany), and tryptophan (Trp) and bromosulfophthalein (BSP) from Fluka (Buchs, Switzerland). [^{3}H]-

labeled Arg, Leu, Phe, Tyr and Trp were purchased from Amersham Pharmacia. All other chemicals were of reagent grade.

RNA preparation

The plasmids containing cDNA coding for *h*4F2hc and *h*LAT1, pSPORT1-*h*4F2hc (43) and pcDNA1-E16 (29), were linearized with *Hind*III and *Eco*RV (Roche, Mannheim, Germany), respectively, and transcribed using the Ampliscribe High Yield T7 RNA transcription kit (Epicentre, Madison, WI). The copy RNAs (cRNAs) were capped with the m7G[5']ppp[5']G cap analog (Epicentre) and stored in sterile water at -80 C.

Oocyte isolation and cRNA injection

Oocytes were prepared as described previously (44). After isolation, oocytes were sorted on morphological criteria and defolliculated manually. Healthy looking stage V-VI oocytes were kept at 18 C in modified Barth's solution, containing 20 IU/ml penicillin and 20 µg/ml streptomycin (44). The next day, oocytes were injected with 2.3 ng *h*4F2hc cRNA and/or 2.3 ng *h*LAT1 cRNA in 23 nl water using the Nanoject system (Drummond Scientific, Broomall, PA). Uninjected oocytes were used as controls as similar results were obtained using water-injected oocytes. Injected and uninjected oocytes were kept for 2 days at 18 C in modified Barth's solution.

Uptake

Uptake assays were performed as reported previously (44). Groups of 8-10 oocytes were incubated for 2 to 60 min at 25 C with 0.01-10 µM [^{125}I]T_4, [^{125}I]T_3, [^{125}I]rT_3 or [^{125}I]3,3'-T_2, or with 10-100 µM [^{3}H]Arg, [^{3}H]Leu, [^{3}H]Phe, [^{3}H]Tyr or [^{3}H]Trp in 0.1 ml incubation medium (100 mM NaCl or choline chloride (ChCl), 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes and 10 mM Tris, pH 7.5). After incubation, oocytes were washed four times with 2.5 ml ice-cold Na^+-containing incubation medium containing 0.1% BSA. Oocytes were transferred to new tubes and counted individually.

Efflux

Oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1 were incubated in groups of 8-10 for 30 min at 25 C with 10 µM [^{3}H]Leu or 0.1 µM [^{125}I]T_3 or [^{125}I]T_2 in 0.1 ml Ch^+-containing incubation medium. One group of oocytes was processed to determine total uptake of each ligand as described above. Efflux of internalized ligand from other groups of oocytes was analyzed as follows. After removal of the medium, oocytes were rapidly washed with 0.5 ml of Ch^+-containing incubation medium at 25 C, and incubated for successive 2 min periods at 25 C with 0.5 ml of the same medium without or with 10 mM unlabeled Leu. After each interval, medium was rapidly replaced by fresh medium and counted for radioactivity. Radioactivity still associated with the oocytes at the end of the 20 min total efflux period was counted as well. Efflux was quantified by expressing the cumulative release of radioactivity as a percentage of that present in the oocytes at the start of the efflux period.

Statistics

Data are presented as means ± SEM. Differences were tested for statistical significance using Students *t* test. Kinetic parameters were determined by fitting the plot of uptake rate (v) *versus* ligand concentration (S) to the Michaelis-Menten equation: $v=V_{max}/(1+K_m/S)$, where V_{max} is the maximum uptake rate, and K_m the Michaelis constant.

RESULTS

Initial experiments were carried out to reproduce the induction of amino acid transport in *Xenopus* oocytes after injection of cRNAs coding for 4F2-related proteins as reported by others (25-41). Uninjected and water-injected oocytes showed negligible uptake of the basic amino acid Arg and different neutral amino acids, *i.e.*, the aliphatic amino acid Leu and the aromatic amino acids Phe, Tyr and Trp. Figure 1A shows the effects of injection of 2.3 ng *h*4F2hc cRNA alone or together with 2.3 ng *h*LAT1 cRNA on the uptake of Leu, Phe, Tyr, Trp and Arg. Incubations were done 2 days after cRNA injection at a ligand concentration of 50 µM using incubation medium containing Na^+ or choline (Ch^+). Injection of oocytes with cRNA coding for *h*LAT1 alone did not stimulate

transport of the different amino acids (data not shown). Injection of *h*4F2hc cRNA alone did not effect transport of Phe, Tyr or Trp, but induced Na^+-dependent transport of Leu and Na^+-independent transport of Arg. This is characteristic for the induction of a y^+L-type transporter which has been documented in different studies and is explained by the dimerization of exogenous *h*4F2hc with an endogenous y^+LAT-type light chain expressed in native oocytes (25-30). This assumption is supported by observations that injection of cRNA coding for *hy*$^+$LAT1 or *hy*$^+$LAT2 in addition to *h*4F2hc cRNA further markedly increased Na^+-dependent transport of Leu and Na^+-independent transport of Arg compared with oocytes injected with *h*4F2hc cRNA alone (data not shown). Coinjection of oocytes with *h*4F2hc cRNA and *h*LAT1 cRNA did not increase Arg transport above that observed after injection with *h*4F2hc cRNA alone. Compared with oocytes injected with *h*4F2hc cRNA alone, oocytes injected in addition with *h*LAT1 cRNA showed a further marked increase in Leu uptake which, however, became almost completely Na^+-independent. Coinjection of cRNA coding for *h*4F2hc and *h*LAT1 also resulted in a large induction of the transport of Phe and Tyr and a much smaller increase in the uptake of Trp which in all cases was Na^+-independent. This is in agreement with previous reports, and characteristic for the induction of an L-type amino acid transporter. Marked stimulation of the Na^+-independent uptake of 100 µM Leu was also observed after coinjection of *h*4F2hc and *m*LAT2 cRNA (data not shown).

Figure 1B shows the uptake of 10 µM Leu, Tyr and Trp in Ch^+-containing medium by oocytes coinjected with *h*4F2hc and *h*LAT1 cRNA as a function of incubation time. Data were corrected for transport in uninjected oocytes which was significant only for Trp because of the relatively small induction of Trp transport by *h*4F2hc plus *h*LAT1 cRNA injection. Trp uptake was linear with time of incubation for at least 60 min. Transport of Leu and Tyr was much faster than Trp uptake and linear with time for only approximately 15 min.

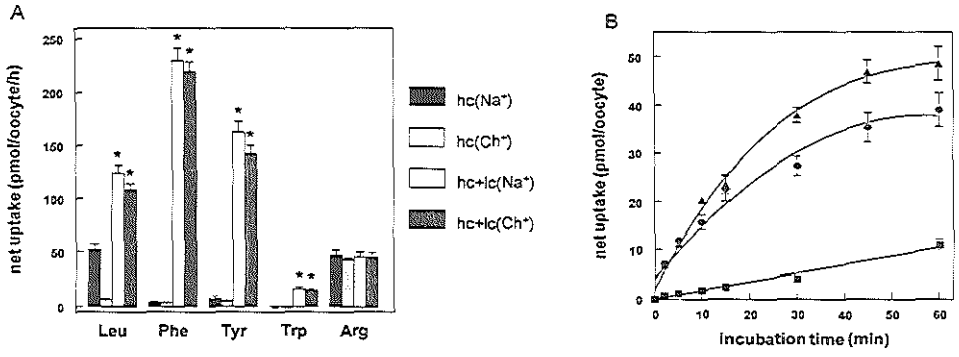

Fig.1 A. Uptake of amino acids by *Xenopus* oocytes injected with cRNA coding for *h*4F2hc alone (hc) or in combination with cRNA coding for *h*LAT1 (hc+lc). Oocytes were incubated for 60 min at 25 C with 50 µM ^{3}H-labeled Leu, Phe, Tyr, Trp or Arg in incubation medium containing Na^+ or choline (Ch^+). Data were corrected for minor uptake observed in uninjected oocytes.
B. Time course of uptake of amino acids by oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1. Oocytes were incubated for 2-60 min with 10 µM ^{3}H-labeled Leu (•), Tyr (▲) or Trp (■) in Ch^+-containing medium. Data were corrected for minor uptake observed in uninjected oocytes.
Data are presented as means ± SEM of 8-10 oocytes. *p<0.001 *vs* corresponding oocytes injected with *h*4F2hc cRNA alone.

Figure 2A shows the uptake of T_4, T_3, rT_3, or 3,3'-T_2 by uninjected oocytes and oocytes injected with cRNA coding for *h*4F2hc and *h*LAT1 after incubation for 1 h with 10 nM iodothyronine in medium with or without Na^+. As shown previously, significant uptake of iodothyronines was observed in uninjected oocytes which is a major drawback of this expression system for the cloning of thyroid hormone transporters. Iodothyronine uptake by native oocytes decreased in the order 3,3'-T_2 ≈ T_3 > T_4 > rT_3 and was somewhat lower in incubation medium containing Ch^+ instead of Na^+. Injection of oocytes with *h*4F2hc cRNA alone or with *h*LAT1 cRNA alone did not increase the uptake of any iodothyronine (data not shown). The lack of effect of injection with *h*4F2hc cRNA alone suggests that the y^+L-type transporter generated by dimerization of the exogenous heavy chain with an endogenous light chain does not mediate transport of iodothyronines. This is supported by findings that coinjection of *h*4F2hc cRNA and cRNA coding for *hy*$^+$LAT1 or *hy*$^+$LAT2 neither stimulated iodothyronine transport in oocytes (data not shown). However, injection of oocytes with both cRNA coding for *h*4F2hc and *h*LAT1 resulted in significant increases in net iodothyronine uptake, which

decreased in the order 3,3'-T_2 > $rT_3 \approx T_3 > T_4$, and in all cases was Na^+-independent. Smaller increments in iodothyronine uptake were noted after coinjection of *h*4F2hc cRNA and *m*LAT2 cRNA (data not shown).

Figure 2B shows the uptake of 0.1 µM T_3 and 3,3'-T_2 induced by injection of oocytes with both *h*4F2hc and *h*LAT1 cRNA as a function of time of incubation in Ch^+-containing medium. Transport of T_3 was linear with time for at least 60 min. Transport of 3,3'-T_2 was much faster than T_3 uptake, and linear with time for about 45 min.

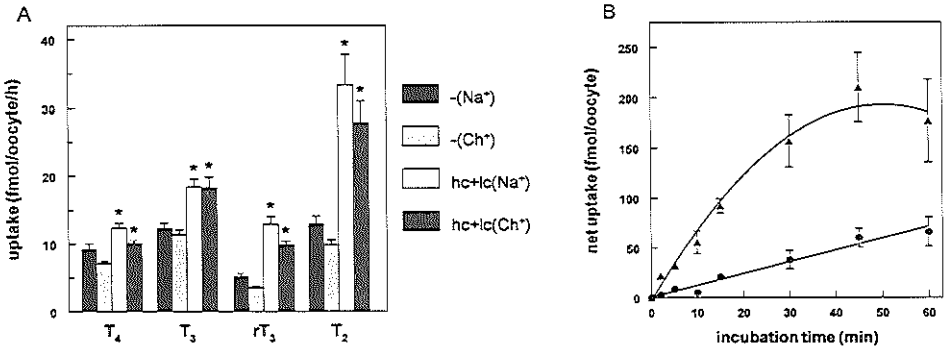

Fig. 2 A. Uptake of iodothyronines by uninjected *Xenopus* oocytes (-) or oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1 (hc+lc). Oocytes were incubated for 60 min at 25 C with 10 nM ^{125}I-labeled T_4, T_3, rT_3 or 3,3'-T_2 in incubation medium containing Na^+ or Ch^+.
B. Time course of uptake of iodothyronines by oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1. Oocytes were incubated for 2-60 min with 0.1 µM ^{125}I-labeled T_3 (•) or 3,3'-T_2 (▲) in Ch^+-containing medium. Data were corrected for uptake observed in uninjected oocytes
Data are expressed as means ± SEM of 8-10 oocytes. *p<0.01 *vs* corresponding uninjected oocytes.

The saturation kinetics of iodothyronine uptake by the heterodimeric *h*4F2hc/*h*LAT1 transporter were studied by incubation of oocytes injected with cRNA for both subunits during 1 h with 0.1-10 µM ligand in Ch^+-containing medium. Iodothyronine uptake through the oocytes' endogenous transporter(s) was determined in parallel incubations with uninjected oocytes. The results are presented in Fig. 3, showing that iodothyronine uptake was saturable in both uninjected and cRNA-injected oocytes. Michaelis-Menten analysis of the results obtained with uninjected oocytes provided apparent K_m values of 2-14 µM for the different iodothyronines. Iodothyronine transport mediated by the *h*4F2hc/*h*LAT1 transporter was determined by subtraction of the uptake rates in uninjected oocytes from those observed in oocytes injected with the cRNAs for

both subunits. Michaelis-Menten analysis of the corrected data provided apparent K_m values of 7.9 µM for T_4, 0.8 µM for T_3, 12.5 µM for rT_3 and 7.9 µM for 3,3'-T_2. V_{max} values amounted to 2.6, 1.1, 11.3 and 28 pmol/oocyte/h for T_4, T_3, rT_3 and 3,3'-T_2, respectively. The fold stimulation of iodothyronine uptake induced by injection of oocytes with cRNA for *h*4F2hc and *h*LAT1 varied with increasing ligand concentration (0.1-10 µM) from 2.1 to 2.7 for T_4, from 2.2 to 1.9 for T_3, from 4.4 to 7.6 for rT_3 and from 3.2 to 13.7 for 3,3'-T_2. The kinetics of transport of Leu and Trp by the *h*4F2hc/*h*LAT1 transporter were analyzed similarly using ligand concentrations of 1-100 µM, yielding K_m values of 46 µM for Leu and 19 µM for Trp (data not shown). These data are in good agreement with previous reports (29-32).

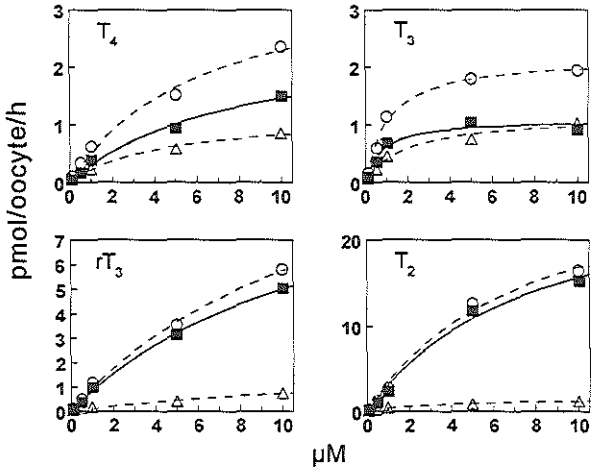

Fig. 3 Ligand concentration-dependent uptake of iodothyronines by uninjected *Xenopus* oocytes (Δ) or oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1 (○). Oocytes were incubated for 60 min with 0.1-10 µM ^{125}I-labeled T_4, T_3, rT_3, or 3,3'-T_2 in Ch^+-containing medium. Uptake induced by expression of *h*4F2hc/*h*LAT1 was calculated by subtraction of uptake in uninjected oocytes from that observed in injected oocytes (■). Curve-fitting is done using the Michaelis-Menten equation $v=V_{max}/(1+K_m/S)$. Data are presented as means of 8-10 oocytes.

Competition between iodothyronine and amino acid transport by the heterodimeric *h*4F2hc/*h*LAT1 transporter was studied by testing the effects of 10 µM unlabeled iodothyronine on the uptake of 10 µM labeled Leu, Tyr or Trp, or the effects of 100 µM unlabeled amino acid on the uptake of 0.1 µM labeled T_3. Figure 4A demonstrates that uptake of 10 µM Leu, Tyr and Trp by *h*4F2hc/*h*LAT1 was inhibited by

the different iodothyronines. The degree of competition was greatest with T_3, in agreement with its low apparent K_m value. *Vice versa*, uptake of 0.1 µM T_3 by the *h*4F2hc/*h*LAT1 transporter was almost completely inhibited by 100 µM Leu, Trp, Tyr or Phe, whereas iodothyronine uptake by the endogenous transporter(s) was not affected (Fig. 4B).

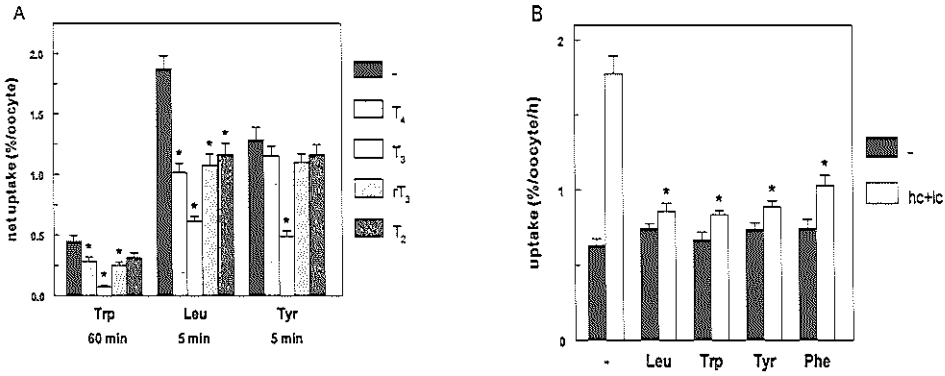

Fig. 4 A. Effects of iodothyronines on the uptake of amino acids by oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1. Oocytes were incubated for 5 (Leu, Tyr) or 60 min (Trp) at 25 C with 10 µM ^{3}H-labeled Leu, Tyr, or Trp in the absence (-) or presence of 10 µM T_4, T_3, rT_3, or 3,3'-T_2 in Ch^+-containing medium. Data were corrected for minor amino acid uptake in uninjected oocytes.
B. Effects of amino acids on the uptake of T_3 by uninjected *Xenopus* oocytes (-) or oocytes injected with cRNAs coding for *h*4F2hc and *h*LAT1 (hc+lc). Oocytes were incubated for 60 min at 25 C with 0.1 µM [^{125}I]T_3 in the absence (-) or presence of 100 µM Leu, Trp, Tyr or Phe in Ch^+-containing medium.
Data are expressed as means ± SEM of 8-10 oocytes. *p<0.01 *vs* incubation without competitor.

The specificity of iodothyronine and amino acid transport by the *h*4F2/*h*LAT1 transporter was further investigated by testing the effects of the T_3 analogs D-T_3 and Triac, the organic anions bromosulfophthalein (BSP) and taurocholate (TC), and the prototypic L-type ligand 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) on uptake of L-T_3 and L-Leu by the heterodimeric amino acid transporter (Fig. 5). Uptake was studied in the absence of Na^+ using 0.1 µM [^{125}I]T_3 or 10 µM [^{3}H]Leu as ligands and oocytes injected with cRNA for *h*4F2hc and *h*LAT1, and corrected for uptake in oocytes injected with *h*4F2hc cRNA only. In general, Leu uptake was somewhat less sensitive to the different competitors than T_3 uptake, perhaps because Leu uptake was tested at a relatively high ligand concentration. Neither [^{125}I]T_3 nor [^{3}H]Leu uptake were significantly

inhibited by 10 µM D-T_3 or Triac, in contrast to the potent inhibition by 10 µM L-T_3. Tested at 100 µM, BCH produced at least the same, marked inhibition of [^{3}H]Leu and [^{125}I]T_3 uptake as Leu itself. The prototypic organic anion transporter ligand BSP and the bile acid TC had little effect on uptake of either ligand (Fig. 5).

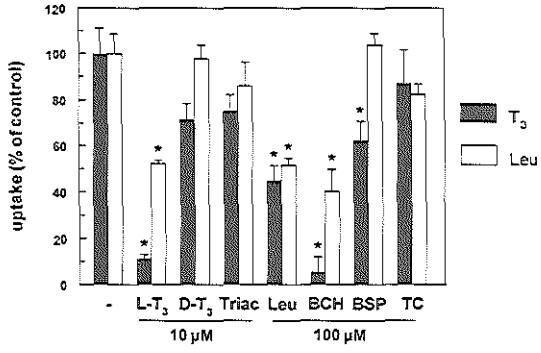

Fig. 5 Effects of various compounds on the uptake of T_3 and Leu by the *h*4F2hc/*h*LAT1 transporter. Oocytes were injected with *h*4F2hc cRNA alone or together with *h*LAT1 cRNA, and after 2 days incubated for 60 min with 0.1 µM [^{125}I]T_3 or for 5 min with 10 µM [^{3}H]Leu in Ch$^+$ medium without (-) or with 10 µM L-T_3, D-T_3, or Triac or 100 µM Leu, BCH, BSP, or TC. Uptake by *h*4F2hc cRNA-injected oocytes was subtracted from uptake by *h*4F2hc + *h*LAT1 cRNA-injected oocytes. Net uptake in the presence of competitor was expressed as percentage of control net uptake in the absence of competitor. Data are presented as means ± SEM of 8-10 oocytes. *$p<0.01$ *vs* control.

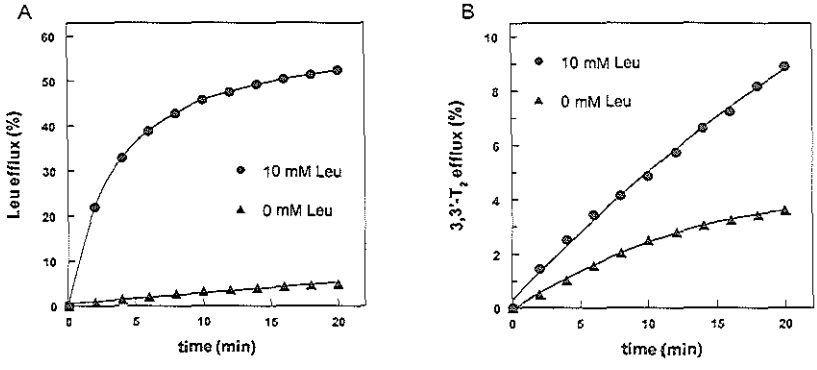

Fig. 6 Effects of extracellular unlabeled Leu on the efflux of labeled Leu (A) and 3,3'-T_2 (B) internalized by *Xenopus* oocytes injected with cRNA coding for *h*4F2hc and *h*LAT1. Oocytes were preincubated for 30 min with 10 µM [^{3}H]Leu (A) or 0.1 µM [^{125}I]T_2 (B) in Ch$^+$-containing medium. After washing, efflux of internalized radioactive ligand was determined by incubation of oocytes during successive 2-min periods at 25 C with the same medium without (▲) or with 10 mM nonradioactive Leu (•). Data are presented as means ± SEM of 8-10 oocytes.

It has been demonstrated that the *h*4F2hc/*h*LAT1 transporter mediates the exchange of intra- and extracellular amino acids (29,30). In agreement with these reports, efflux of labeled Leu taken up by *h*4F2hc/*h*LAT1-expressing oocytes was greatly stimulated by the addition of 10 mM unlabeled Leu to the efflux medium (Fig. 6A). In contrast, 10 mM extracellular Leu only slightly stimulated efflux of 3,3'-T_2 internalized by *h*4F2hc/*h*LAT1-expressing oocytes (Fig. 6B), whereas it did not affect efflux of T_3 taken up by such oocytes (data not shown).

DISCUSSION

In agreement with previous reports from our laboratory, uninjected or water-injected oocytes show significant transport of iodothyronines (44-46). Uptake of iodothyronines by native oocytes is partially Na^+-dependent and saturated at increasing ligand concentrations, with apparent K_m values of 2-14 μM, suggesting the involvement of one or more unidentified transporters. This endogenous iodothyronine transport is not inhibited by the addition of large concentrations of different amino acids, which suggest that iodothyronines are not taken up by amino acid transporters native to the oocytes. This is supported by observations that uptake of various amino acids by uninjected oocytes is negligible. However, injection of oocytes with cRNA coding for *h*4F2hc results in a marked induction of the Na^+-dependent uptake of the neutral amino acid Leu and the Na^+-independent uptake of the basic amino acid Arg. This is in agreement with previous publications from other laboratories, and is explained by the formation of a functional y^+L-type transporter by dimerization of the exogenous *h*4F2 heavy chain with an endogenous y^+LAT-type light chain (25-41). Uptake of iodothyronines is not stimulated by injection of *h*4F2hc cRNA alone, indicating that iodothyronines are not transported by y^+L-type transporters. This notion is supported by our findings that coexpression of *h*4F2hc and either *hy*$^+$LAT1 or *hy*$^+$LAT2 does not induce iodothyronine transport, although the Na^+-dependent transport of Leu as well as the Na^+-independent uptake of Arg are further markedly increased.

Injection of oocytes with *h*LAT1 cRNA alone does not induce transport of the various amino acids and iodothyronines, indicating that expression of a 4F2-like heavy

chain by native oocytes is negligible. However, if oocytes are coinjected with cRNA for both *h*4F2hc and *h*LAT1, uptake of the large, neutral amino acids Leu, Phe, Tyr and Trp, but not that of the basic amino acid Arg, is markedly stimulated above that seen after injection with *h*4F2hc cRNA alone. The *h*LAT1-induced increment in amino acid transport is completely independent of Na^+, which is conform the characteristics of the L-type amino acid transporter (23-27). Also transport of the different iodothyronines is markedly stimulated by coexpression of *h*4F2hc and *h*LAT1, and for all iodothyronines the induced transport is completely Na^+-independent. Tested at low ligand concentrations, the rate of iodothyronine uptake by the *h*4F2hc/*h*LAT1 transporter decreases in the order 3,3'-T_2 > rT_3 ≈ T_3 > T_4. This does not appear to be a simple reflection of the affinity of the different iodothyronines for the *h*4F2hc/*h*LAT1 transporter, as the apparent K_m value is much lower for T_3 than for T_4, rT_3 and 3,3'-T_2. The apparent K_m of 0.8 µM for T_3 is the lowest value reported for a ligand of the *h*4F2hc/*h*LAT1 transporter (25-27,29,30). Among the different iodothyronines, by far the highest V_{max} value is observed for 3,3'-T_2.

Since iodothyronines and large neutral amino acids are all ligands for the *h*4F2hc/ *h*LAT1 transporter, it is not surprising that they inhibit each others transport. Leu uptake is more strongly inhibited by L-T_3 than by L-T_4, L-rT_3, 3,3'-L-T_2, D-T_3 and Triac, in keeping with the low K_m value for T_3 and the stereospecificity of this L-type amino acid transporter (24-30). This is reminiscent of the competition between iodothyronine and amino acid uptake in different cell systems reported previously (13-22). Thus, uptake of T_4 and T_3 by NB41A3 mouse neuroblastoma cells is stereospecific, saturable (K_m: T_3 3 nM; T_4 6 nM), and inhibited by high concentrations of Leu and Phe but not by α-aminoisobutyric acid (AIB), a system A transporter-specific ligand (19). Similar characteristics of iodothyronine uptake were observed in the Hs683 human glioma cell line (47). Somewhat different results were reported for T_3 uptake by cultured rat astrocytes, which express both high-affinity (L1) and low-affinity (L2) system L transporters (18). Apparent K_m values for uptake of Leu and Trp by the L1 transporter amount to 8-9 µM. T_3 uptake by these cells is Na^+-independent and saturable, with an apparent K_m value of 2-3 µM. L1-mediated uptake of Leu and Trp is competitively inhibited by T_3, and T_3 uptake is competitively inhibited by Trp, with corresponding K_m and K_i values. However, T_3 uptake is not inhibited by up to 30 mM Leu (18). It is also

interesting to mention the characterization of saturable and stereospecific iodothyronine transport in GH4C1 rat pituitary tumor cells (15), showing high affinity for T_3 (K_m 0.4 μM) and T_4, low affinity for rT_3 and T_0, and strong inhibition by Leu, Phe, Tyr, Trp and the L-type transporter-specific ligand BCH. GH4C1 cells also show high-affinity transport of Leu (K_m 17 μM), which is potently inhibited by T_3 (IC_{50} 2 μM), further supporting the involvement of an L-type transporter in T_3 (and T_4) uptake (15). Also in cultured rat anterior pituitary cells, uptake of T_4 and T_3 is mediated by a common transporter and inhibited by the aromatic amino acids Phe, Tyr and Trp (13).

Blondeau and coworkers (16,17) have demonstrated that T_3 transport by rat erythrocytes is Na^+-independent, saturable (K_m 0.14 μM), and specific (L-T_3 >> D-T_3 > T_4 > rT_3 > T_0). T_3 uptake is competitively inhibited by the aromatic amino acids Trp, Phe and Tyr, but not by D-Trp or Leu. They also showed low-affinity uptake of Trp by rat erythrocytes, with an apparent K_m value of 558 μM. Trp uptake is competitively inhibited by Phe, Tyr and iodothyronine analogs, with K_i values identical to those for inhibition of T_3 transport. These results suggest the involvement of a T-type, aromatic amino acid-specific transporter in the uptake of both Trp and T_3 (16,17). Interestingly, T_3 uptake is markedly trans-stimulated by intracellular Trp, although Trp uptake is trans-inhibited by intracellular T_3 (16,17). Both T_3 and Trp uptake are inhibited by the thiol-blocking reagent *N*-ethylmaleimide. A 45 kDa protein was identified by photoaffinity labeling with [^{125}I]T_3 which may be a subunit of the T-type transporter but this was not further characterized (48).

Competition between iodothyronine and aromatic amino acid (*e.g.*, Trp) transport has also been demonstrated in other cells, *e.g.*, JAR human choriocarcinoma cells (21,22) and neonatal rat cardiomyocytes (14), but it has not been established if iodothyronine uptake in these cells is indeed mediated by amino acid transporters. Of special interest are observations of countertransport of Tyr derivatives by the system h transporter located in thyroidal lysosomal membranes (49,50). Loading of lysosomes with Tyr or 3-(mono)iodotyrosine (MIT) greatly stimulates influx of Tyr, MIT, 3,5-diiodotyrosine (DIT), Phe and Leu. Potent competition by T_4 and T_3 suggests that iodothyronines are also countertransported against Tyr derivatives. The apparent K_m value for MIT is 1.5 μM, and Tyr, DIT, T_4 and T_3 show similar high affinities. This exchange mechanism probably plays an important role in thyroid hormone biosynthesis,

since the iodotyrosines released by lysosomal hydrolysis of thyroglobulin must be transported to the cytoplasm for deiodination and the iodothyronines to the cell membrane for secretion (6).

The apparent K_m values of T_4, T_3, Leu and Trp in the 10^{-6}-10^{-5} M range for the *h*4F2hc/*h*LAT1 transporter expressed in oocytes most closely resemble those reported for their uptake by rat GH4C1 pituitary tumor cells (15), supporting the involvement of an L-type transporter. They are also in reasonable agreement with the apparent K_m values for T_4, T_3, Leu and Trp uptake by cultured rat astrocytes (18) and rat erythrocytes (16,17), but the complete lack of effect of $>10^{-2}$ M Leu on iodothyronine uptake by these cells suggests that an aromatic amino acid-specific (T type) transporter is involved. Also, the much lower K_m values reported for T_4 and T_3 uptake by the NB41A3 mouse neuroblastoma cells appear to implicate another (sub)type of amino acid transporter than 4F2hc/LAT1.

The L-type amino acid transporter mediates not only influx but also efflux of amino acids. Our results show that the release of intracellular Leu is stimulated by exchange with extracellular Leu in agreement with previous reports (29,30). Extracellular Leu only induces a small increase in the release of 3,3-T_2 from the oocytes and no release at all of internalized T_3, which may be explained by strong binding of iodothyronines to intracellular sites in oocytes.

The above-mentioned properties of the thyroidal lysosomal system h transporter, mediating the exchange of amino acids such as Leu, Tyr, iodotyrosines and iodothyronines, suggest that it may actually be an L-type amino acid transporter. This is also supported by evidence that the high uptake of radioiodine-labeled MIT and 3-iodo-α-methyltyrosine (IMT) by different tumors is mediated by an L-type transporter (51-53). This principle is utilized in nuclear medicine for the scintigraphic visualization of such tumors. If the *h*4F2hc/*h*LAT1 and/or *h*4F2hc/*h*LAT2 transporters are indeed responsible for tumor uptake of the radioactive Tyr derivatives, then the availability of cell systems overexpressing these transporters would greatly facilitate the development of improved tumor-seeking radiopharmaceuticals.

Obviously, all cells require amino acid transporters but, in contrast to the ubiquitous expression of the 4F2 heavy chain, the LAT1 and, in particular, LAT2 light chains show restricted tissue distributions: neither of them are expressed in liver (25-

33). This suggests the existence of other light chains to be identified which are involved in the uptake of aromatic amino acids in tissues that do not express LAT1 or LAT2. Presumably, one of these constitutes with 4F2hc a T-type transporter specific for aromatic amino acids, including iodothyronines (16-18). Perhaps, additional light chains exist which associate specifically with the homologous rBAT heavy chain (28), generating transporters that also accept iodothyronines. However, cellular uptake of iodothyronines is not only mediated by amino acid transporters. We and others have demonstrated recently that iodothyronines are also transported into liver by Na^+-dependent (NTCP) and Na^+-independent (OATP) organic anion transporters, although various members of the OATP family are also expressed in other tissues, in particular kidney and brain (46,54-58). Typical ligands for these organic anion transporters, BSP and TC, have no effect on iodothyronine uptake by the *h*4F2hc/*h*LAT1 transporter, in contrast to the potent inhibition by the L-type ligand BCH. However, the major, Na^+-dependent hepatic transporters for T_4 and T_3 remain to be identified. Iodothyronine uptake by uninjected oocytes is saturable (apparent K_m values 2-14 µM), suggesting that it is carrier-mediated. The lack of effect of high concentrations of different amino acids on iodothyronine uptake by native oocytes argues against the involvement of an amino acid transporter. The type of endogenous iodothyronine transporter(s) in *Xenopus* oocytes remains to be determined.

In summary, we have demonstrated that *h*4F2hc/*h*LAT1 and, albeit less effectively, also *h*4F2hc/*m*LAT2 are capable of transporting iodothyronines, in agreement with previous suggestions that thyroid hormone is taken up in different tissues via L-type amino acid transporters. Our findings are in agreement with a recent report published after completion of our study, showing iodothyronine transport by the heterodimeric transporter composed of *h*4F2hc and the IU12 light chain from *Xenopus* which is homologous to *h*LAT1 (59). One of the questions which remains to be clarified is the extent to which cellular uptake of iodothyronines through 4F2-related transporters is stimulated by countertransport of different intracellular amino acids. Of course, these transporters may also mediate cellular efflux of iodothyronines.

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Chapter 6

CHARACTERIZATION OF HEPATIC THYROID HORMONE TRANSPORTERS IN *XENOPUS LAEVIS* OOCYTES

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(*in preparation for submission*)

ABSTRACT

We have recently shown that iodothyronines, their sulfates as well as their sulfamates are transported by rat Na^+/taurocholate co-transporting polypeptide (rNTCP) and rat Na^+-independent organic anion transporting polypeptide 1 (rOATP1). This study was done to investigate the importance of these and other transporters for the uptake of thyroid hormone in *Xenopus laevis* oocytes injected with rat liver mRNA, using T_4 sulfamate (T_4NS) and T_3NS as ligands. Oocytes were injected with total or size-fractionated rat liver mRNA or cRNA coding for rNTCP, rOATP1, rOATP2, human (h) NTCP or hOATP-A. Uninjected oocytes were used as control. After 3 to 4 days, uptake was measured in oocytes incubated for 1 h at 25 C with 10 or 100 nM ^{125}I-labeled T_4, T_3, T_4NS or T_3NS or 1 μM ^{3}H-labeled taurocholate. Uptake of T_4NS and T_3NS by uninjected oocytes was much lower than uptake of T_4 and T_3. Although uptake induced by liver mRNA was similar for the different compounds, the fold increase was much greater for T_4NS and T_3NS than for T_4 and T_3. Uptake of T_4NS and T_3NS in mRNA-injected oocytes was partly Na^+-dependent and was blocked by excess bile acids, probenecid, T_3, T_4, or BSP. All organic anion transporters mediated iodothyronine transport with varying ligand specificities. Analysis of size-fractionated mRNA indicated the expression of at least 3 iodothyronine transporters in rat liver, one of which corresponded in size with rNTCP and another with rOATP1. The latter comigrated with a major, unidentified, Na^+-dependent iodothyronine transporter. Treatment with antisense oligonucleotides completely blocked induction of T_4NS and T_3NS uptake by rNTCP and rOATP1, but only partly inhibited uptake induced by total liver mRNA. These results indicate that although rNTCP and rOATP1 contribute significantly to hepatic uptake of thyroid hormone, the major iodothyronine transporter remains to be identified.

INTRODUCTION

Transport of iodothyronines across the plasma membrane is required for metabolism and action of thyroid hormone which are both intracellular events. Thyroid hormone action is initiated by the binding of the active form of the hormone T_3 to specific nuclear receptors (1,2). Conversion of the prohormone T_4 to active T_3 or inactive rT_3 as well as the further deiodination of these metabolites are effected by different deiodinases located in various tissues, among which the liver plays an important role (3,4). As iodothyronines are lipophilic compounds, it was assumed for a long time that they cross the cell membrane by simple diffusion. However, the highly polar nature of the alanine side chain forms an obstacle for membrane passage of thyroid hormone.

Studies using rat hepatocytes have shown that T_4, T_3 and rT_3 are transported across the plasma membrane by specific, mostly Na^+-dependent transporters (1,4). In recent years, we have used *Xenopus laevis* oocytes to characterize iodothyronine transporters expressed in rat liver. We observed a modest increase in T_4 and T_3 uptake by oocytes injected with rat liver mRNA, especially with size fractions of 0.8 to 2.1 kb (5). These studies were hampered by the high endogenous uptake of thyroid hormone by native *Xenopus* oocytes, which led us to test iodothyronine derivatives as potentially more selective ligands for the induced rat transporters *versus* the endogenous *Xenopus* transporters. Initially, we tested T_3 sulfate (T_3S) and T_4S which showed not only a much lower uptake by uninjected oocytes than 'free' T_3 and T_4 but also a smaller increase in uptake after injection of oocytes with rat liver mRNA. In a further attempt to improve the sensitivity of the oocytes' response to injected mRNA we studied T_4 sulfamate (T_4NS) and T_3NS, where the SO_3^- group is attached to α-amino instead of the 4'-hydroxyl group of the iodothyronines. Preliminary results showed much lower uptake of T_4NS and T_3NS than of T_4 and T_3 by uninjected oocytes, with preservation of the response to injection of rat liver mRNA.

Since T_4 and T_3 sulfates and sulfamates are anionic iodothyronine derivatives, it is not surprising that they are transported by the rat Na^+/taurocholate cotransporting polypeptide (rNTCP) and the Na^+-independent organic anion transporting polypeptide 1 (rOATP1) (6). However, we also observed transport of nonderivatized iodothyronines by rNTCP and rOATP1, and this has recently been extended to other members of the

OATP family, *i.e.*, rOATP2 (7), rOATP3 (7), rOATP4 (8), hOATP-C (9), and human and rat OATP-E (10). The present study was performed to determine the contribution of these organic anion transporters and possibly additional transporters to the transport of thyroid hormone in rat liver using *Xenopus* oocytes as expression system and T_4NS and T_3NS as alternative ligands.

MATERIALS AND METHODS

Materials

Nonradioactive iodothyronines were obtained from Henning Berlin (Berlin, Germany), taurocholate (TC) and bromosulfophthalein (BSP) from Fluka (Buchs, Switzerland), and cholate (CA) and probenecid (PBN) from Sigma Chemical Co (St. Louis, MO). [^{125}I]T_4 and [^{125}I]T_3 were purchased from Amersham Pharmacia (Uppsala, Sweden) and [^{3}H]taurocholate from NEN (Boston, MA). [^{125}I]rT_3, [^{125}I]3,3'-T_2, [^{125}I]T_4NS and [^{125}I]T_3NS were prepared as previously described (7). [^{125}I]T_4, [^{125}I]rT_3 and [^{125}I]T_4NS were purified on Sephadex LH-20 (Amersham Pharmacia) immediately before use (11).

mRNA preparation

Liver poly(A)$^+$ RNA (mRNA) was isolated from male Wistar rats as described previously (5). For size-fractionation, mRNA (100-150 µg) was loaded on a linear 6-20 % (w/v) sucrose gradient and centrifuged for 19 h at 4 C at 80,000 x g_{av} (5). mRNA and size-fractions thereof were stored in water at -80 C. Capped rNTCP, hNTCP, rOATP1, rOATP2 and hOATP-A cRNA were prepared from the cDNA clones (12-16) linearized with the appropriate restriction enzymes to create blunt or 5'- protruding ends (Roche, Mannheim, Germany), using the Ampliscribe T3 (rNTCP and rOATP2) or T7 (hNTCP, rOATP1 and hOATP-A) RNA transcription kit (Epicentre Technologies, Madison, WI). For capping, the m^{7}G[5']ppp[5']G cap analog was used (Epicentre Technologies). cRNA was stored in water at -80 C.

Oocyte isolation and mRNA injection

Oocytes were prepared as described previously (5). After 3 h collagenase B (2 mg/ml, Roche, Mannheim, Germany) treatment, the isolated oocytes were sorted on morphological criteria and defolliculated manually. Healthy-looking stage V-VI oocytes (17) were transferred to six-well tissue culture plates and incubated overnight at 18 C in modified Barth's solution, containing 20 IU/ml penicillin and 20 µg/ml streptomycin (5). The next day, oocytes were injected with 23 nl water containing 23 ng mRNA, or 2.3 ng rNTCP, hNTCP, rOATP1, rOATP2 or hOATP-A cRNA using the Nanoject system (Drummond Scientific, Broomall, PA). Uninjected oocytes were used as controls but similar results were obtained using water-injected oocytes. Injected and uninjected oocytes were maintained for 3-4 days at 18 C in modified Barth's solution.

Oligonucleotides

For antisense neutralization of rNTCP and rOATP1 as published by Hagenbuch *et al.* (18) the following oligonucleotides were purchased from Amersham Pharmacia: rNTCP-antisense1 (5' ATCGTAGATGCCTTTGCTGT 3'), rNTCP-antisense2 (5' TAACCCATCAGAAAGCCAGA 3'), rOATP-antisense1 (5' TTACACATATCCACAT 3') and rOATP1-antisense2 (5' GGCAGGCAGATAGCTT 3'). Control experiments were done with rNTCP-sense (5' GGCATTATGATATCACTAGT 3') and rOATP1-sense (5' CCAGCAGAATGTGTGA 3'). Liver mRNA (1 µg/µl) or transporter cRNA (0.01 µg/µl) were incubated with oligonucleotide (0.1 µg/µl) in 50 mM NaCl for 5 min at 60 C and for 15 min at room temperature, and placed on ice until injection.

Uptake assay

Uptake assays were performed as described previously (5). Eight to 10 oocytes were incubated for 1 h at 25 C with 10-100 nM [^{125}I]iodothyronine derivatives or 1 µM [^{3}H]taurocholate in 0.1 ml incubation buffer (100 mM NaCl or choline chloride (ChCl), 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). After 1 h, incubation buffer was removed and the oocytes were washed four times with 2.5 ml ice-cold Na^+-containing incubation buffer supplemented with 0.1% BSA. Oocytes were transferred to new tubes and counted individually.

Statistics

Data are presented as the mean ± SEM. Results presented in Fig. 1 were first analyzed with one-way ANOVA. In case of significant F-values, statistical significance was evaluated by Student's *t* test for unpaired observations.

RESULTS

Figure 1 shows the uptake of T_3 and T_4 *versus* T_3NS and T_4NS in uninjected oocytes and in oocytes injected with rat liver mRNA incubated with 10 nM ligand in Na^+ or Ch^+-containing medium. In Na^+ medium, uninjected oocytes showed higher uptake of T_3 (1.2±0.4%/h per oocyte) and T_4 (0.9±0.2%/h) than of T_3NS (0.06±0.02%/h) and T_4NS (0.08±0.02%/h). Because of the high background signal, uptake of T_3 was only slightly, but significantly higher in liver mRNA-injected oocytes ($p<0.005$), whereas uptake of T_4 by mRNA-injected oocytes was not different from uninjected oocytes. The increase in uptake induced by injection of rat liver mRNA was similar for T_3 (0.21%/h per oocyte) as for T_3NS (0.25%/h) and T_4NS (0.29%/h).

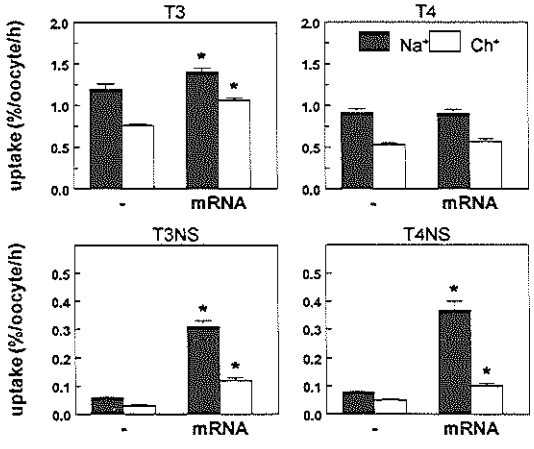

Fig. 1. Uptake of T_3, T_4, T_3NS, and T_4NS in uninjected oocytes (-) and oocytes injected with rat liver mRNA. Three to 4 days after injection of 23 ng mRNA, oocytes were incubated for 1 h at 25 C with 10 nM ligand in incubation medium containing Na^+ or Ch^+. Data are expressed as mean ± SEM of 3 (T_4, T_4NS) or 4 (T_3, T_3NS) experiments.

However, because of the lower uptake of the sulfamates by uninjected oocytes, the relative increase in T_3NS (5.2-fold) and T_4NS uptake (4.6-fold) effected by rat liver mRNA injection was much greater than for T_3 (~20%). In Ch^+ medium, uptake of the different ligands by unjected oocytes was slightly lower than in Na^+ medium, whereas uptake in rat liver mRNA-injected oocytes was more strongly reduced in the absence of Na^+. However, also in Ch^+ medium, rat liver mRNA induced a significant increase in uptake of T_3 (40%), T_3NS (4-fold) and T_4NS (2-fold).

Rat liver mRNA was size-fractionated on a 6-20 % sucrose gradient in order to enrich mRNA stimulating iodothyronine transport. Figure 2 shows a typical experiment using 100 nM T_3NS as the ligand (Upper panel *left*). Six size-fractions ranging between 0.75 and 3.25 kb were injected into oocytes. Highest Na^+-dependent uptake was induced by mRNA fractions of 1.75-2.50 kb, with a smaller Na^+-dependent signal at 1.0-1.25 kb. A distinct Na^+-independent activity was also detected in fractions of 1.75-2.75 kb which, however, was much lower than the Na^+-dependent uptake in the same fractions.

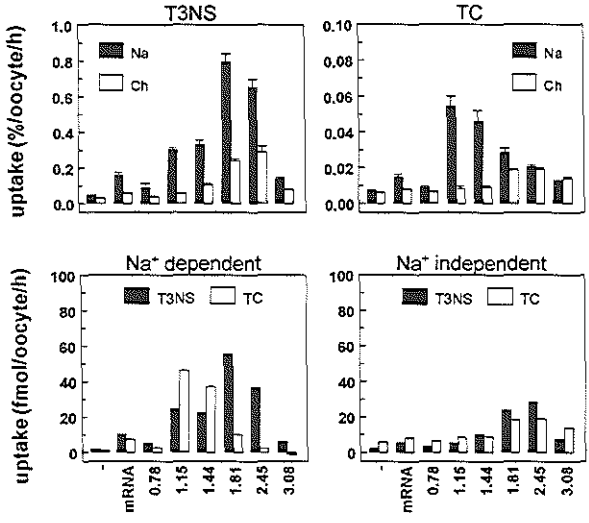

Fig. 2. Upper panels: Uptake of T_3NS or TC in uninjected oocytes (-) and oocytes injected with rat liver mRNA and fractions thereof in the presence of Na^+ or Ch^+.
Lower panels: Na^+-dependent and Na^+-independent uptake of T_3NS or TC in uninjected oocytes (-) and oocytes injected rat liver mRNA and fractions thereof.
Four days after injection of 23 ng total or size-fractionated mRNA, oocytes were incubated for 1 h at 25 C with 100 nM T_3NS or 1 µM TC. Data are expressed as mean ± SEM of 8-10 oocytes.

To monitor the activities of the organic anion transporters, rNTCP and rOATP1, the same mRNA size-fractions were also tested for uptake of 1 µM taurocholate (TC) (Fig. 2, upper panel *right*). Highest Na^+-dependent uptake was found with mRNA of 1.0-1.25 kb, with a smaller Na^+-independent activity in fractions of 1.75-3.0 kb. Thus, Na^+-dependent uptake of T_3NS and TC clearly coincided with mRNA of ~1.25 kb, whereas the Na^+-dependent peak at 1.75-2.50 kb was only detected with T_3NS as the ligand (Fig.2, lower panel *left*). The Na^+-independent uptake of T_3NS and TC coincided with the mRNA fractions of 1.75-2.75 kb (Fig. 2, lower panel *right*).

Figure 3 shows the effects of the bile acids TC and cholate (CA) and of the anionic transport inhibitor probenecid (PBN) on the uptake of T_3NS and T_4NS in uninjected oocytes and oocytes injected with rat liver mRNA with an average size of 2.0 kb. Uptake of 10 nM ligand was inhibited by 58% with 0.1 mM TC, by 70% with 0.2 mM CA and by 58% with 1 mM PBN.

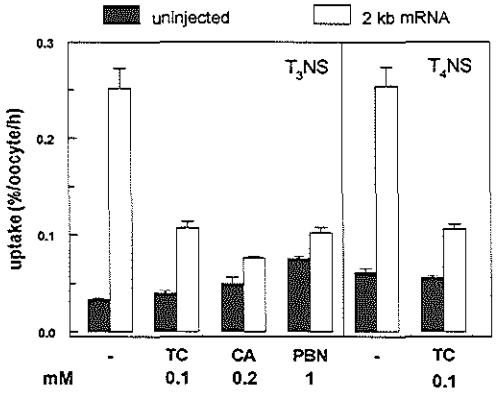

Fig. 3. Effects of taurocholate (TC), cholate (CA) and probenecid (PBN) on the uptake of T_3NS or T_4NS in uninjected oocytes and oocytes injected with ≈ 2 kb rat liver mRNA. Four days after injection of 23 ng mRNA, oocytes were incubated for 1 h at 25 C with 10 nM T_3NS or T_4NS in the absence (-) or presence of 0.1 mM TC, 0.2 mM CA, or 1 mM PBN in Na^+- containing incubation medium. Data are expressed as mean ± SEM of 8-10 oocytes.

To further analyze the specificity of the mRNA-induced T_3NS transport, oocytes injected with total rat liver mRNA or with the same size fractions as shown in Fig. 2 were tested for inhibition by T_3, T_4 and the organic anion bromosulfophthalein (BSP). Figure 4 shows that the uptake of 100 nM T_3NS in the different fractions was potently inhibited by

20 µM T_3 or T_4 or 100 µM BSP. Transport induced by mRNA of ~2 kb showed greater inhibition with T_4 than with T_3. BSP almost completely inhibited T_3NS uptake not only in oocytes injected with mRNA and size-fractions thereof but also in uninjected oocytes.

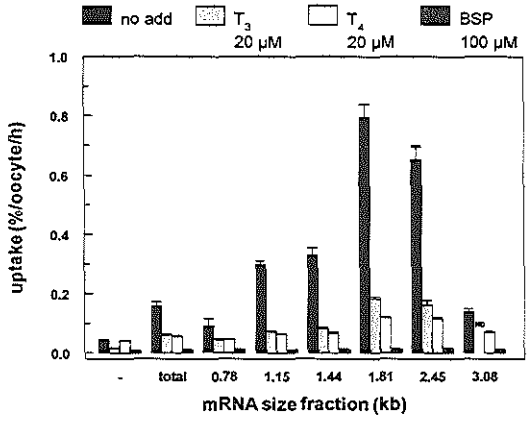

Fig. 4. Effects of T_3, T_4, and bromosulfophthalein (BSP) on the uptake of T_4NS by uninjected oocytes (-), and oocytes injected with total or size-fractionated rat liver mRNA. Size fractions are the same as shown in Fig. 2 and indicated in kb. Four days after injection of 23 ng mRNA, oocytes were incubated for 1 h at 25 C with 100 nM T_4NS in the absence (no add) or presence of 20 µM T_3, or T_4, or 100 µM BSP in Na^+- containing medium. Data are expressed as mean ± SEM of 8-10 oocytes.

Since rNTCP and rOATP1 have been shown to mediate uptake of iodothyronine derivatives (6), and T_3NS uptake induced by liver mRNA fractions partially coincided with rNTCP and rOATP1-mediated TC uptake (Fig. 2), we decided to investigate the possible contribution of rNTCP and rOATP1 to liver mRNA-induced T_3NS uptake. This was done by blocking the mRNAs coding for rNTCP and rOATP1 by incubation of liver mRNA with antisense oligonucleotides before injection into *Xenopus* oocytes. The efficacy of this method was tested using the cRNAs coding for rNTCP and rOATP1, which demonstrated decreased expression of rNTCP after incubation of the cRNA with the rNTCP antisense oligonucleotides but not with rNTCP sense or rOATP antisense oligonucleotides. Similarly, expression of rOATP1 in oocytes was decreased after incubation of its cRNA with rOATP1 antisense oligonucleotides but not with rOATP1 sense or rNTCP antisense oligonucleotides. Since maximum specific inhibitory effects were obtained with rNTCP-antisense2 and rOATP1-antisense2, the results obtained

with these oligonucleotides are presented here. Figure 5A shows that rOATP1-antisense2 almost completely blocked the expression of rOATP1 as measured with 100 nM T_4NS as the ligand, and rNTCP-antisense2 almost completely blocked expression of rNTCP. Figure 5B shows that incubation of rat liver mRNA with rNTCP-antisense2 alone did not inhibit TC uptake as rOATP1-antisense2 alone resulted in a significant reduction of TC uptake. However, incubation of rat liver mRNA with both rNTCP-antisense2 and rOATP1-antisense2 resulted in a strong reduction of TC uptake. Incubation of liver mRNA with both rNTCP-antisense2 and rOATP1-antisense2 resulted only in a partially blocked T_4NS uptake.

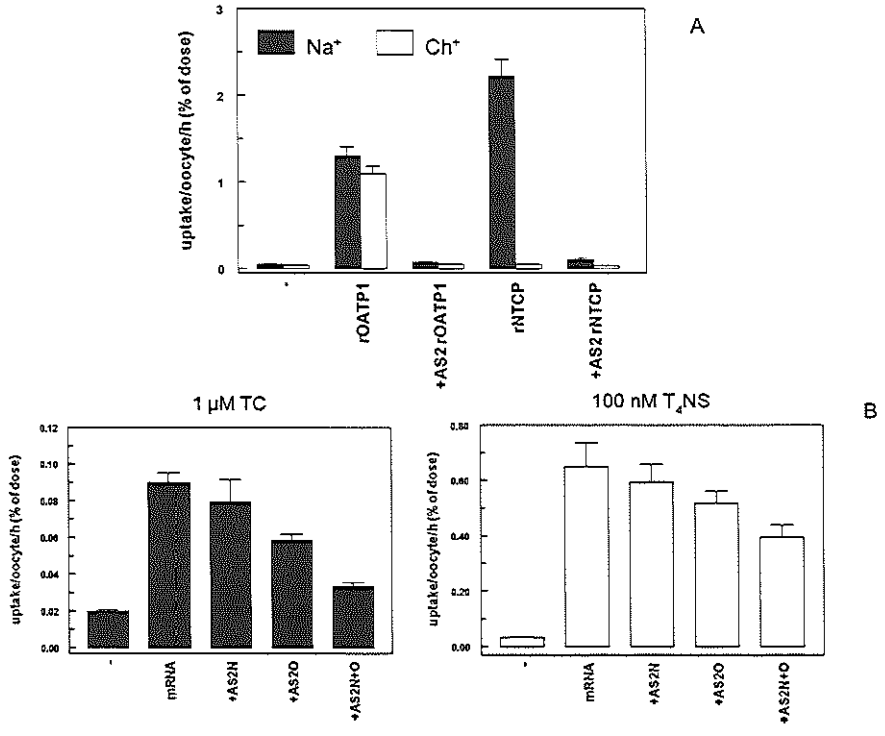

Fig. 5. Effects of pretreatment of rNTCP cRNA, rOATP1 cRNA or rat liver mRNA with rNTCP-antisense2 and/or rOATP1-antisense2 oligonucleotides on induction of uptake of TC or T_4NS.
Results of incubation of rOATP1 and rNTCP with antisense oligonucleotides for transport of 100 nM T_4NS in the presence and absence of Na^+.
B. Results of incubation of mRNA with antisense oligonucleotides for transport of 1 µM TC or 100 nM T_4NS in the presence of Na^+. Data are expressed as the mean ± SEM of 8-10 oocytes.

We have also characterized transport of iodothyronine derivatives by other members of the NTCP and OATP family. Figure 6 shows the uptake of 1 µM TC, 100 nM T_4NS or 100 nM iodothyronines by rat and human NTCP, rat OATP1 and OATP2, and human OATP-A, respectively. Since the expression levels of the different transporters are unknown, the results do not allow to assess their transport efficiencies but they allow us to compare their ligand specificities. For the NTCPs, TC and T_4NS are the preferred substrates, whereas the OATPs appeared to prefer T_4NS over TC. Besides TC and T_4NS, both rat and human NTCP transport T_4 and T_3 better than rT_3 or 3,3'-T_2. rOATP1 preferred T_4 and rT_3 like T_4NS, but showed less affinity for TC, T_3, and 3,3'-T_2. rOATP2, on the contrary, showed highest affinity for T_4, lower affinity for T_4NS and 3,3-T_2, and less for T_3 and TC. It appeared that rT_3 is not transported via rOATP2. hOATP-A showed the highest affinity for T_3, lower affinity for 3,3'-T_2, T_4, T_4NS, and rT_3, and TC is barely transported via hOATP-A. All organic anion transporters were capable of transporting the different iodothyronines.

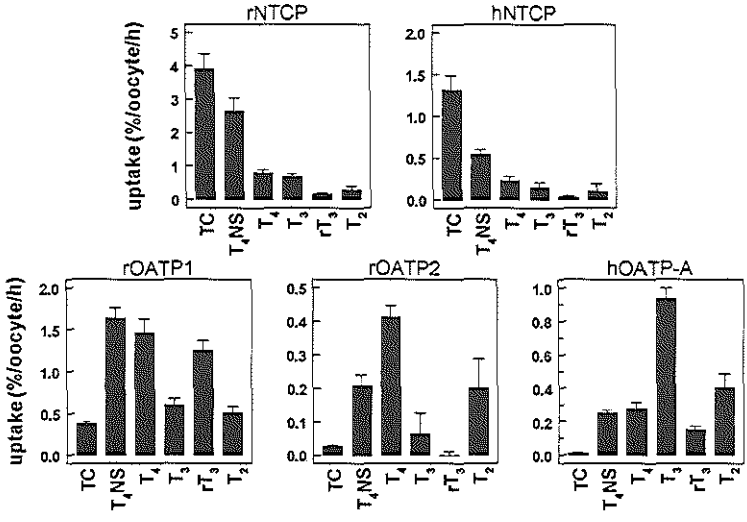

Fig. 6. Transport of TC and iodothyronines by rNTCP, hNTCP, rOATP1, rOATP2 or hOATP-A. Three days after injection of 2.3 ng cRNA for the different transporters, oocytes were incubated for 1 h at 25 C with 1 µM TC or 100 nM T_4NS, T_4, T_3, rT_3, or 3,3'-T_2. Uptake in cRNA-injected oocytes is corrected for uptake in uninjected oocytes. Data are expressed as the mean ± SEM of 8-10 oocytes.

DISCUSSION

Expression in *Xenopus* oocytes has been the most succesful method for the cloning and characterization of plasma membrane transporters for a variety of ligands. Therefore, we decided to use this system for the cloning of thyroid hormone transporters from rat liver. Previous studies using isolated rat hepatocytes in primary culture have demonstrated that uptake of iodothyronines is not a passive process but requires active transporters which are at least partially Na^+ dependent, as indicated by the inhibitory effect of ouabain (1). Initial results of studies of the expression of these transporters in *Xenopus* oocytes showed only a modest increase in T_3 uptake and no significant increase in T_4 uptake after injection of rat liver mRNA (5). To a large degree, these results were caused by the relatively high iodothyronine transport activity of uninjected oocytes. Saturation of endogenous T_4, T_3, rT_3 and 3,3'-T_2 uptake at micromolar ligand concentrations indicated the expression of low-affinity iodothyronine transporters in native oocytes. Attempts to find specific inhibitors of these endogenous transporters without affecting the transporters encoded by exogenous mRNA, and, thus, to increase the sensitivity of the *Xenopus* oocyte expression system for the cloning of iodothyronine transporters, were unsuccessful. We then decided to search for alternative ligands for hepatic iodothyronine transporters with decreased affinity for the endogenous *Xenopus* transporters. Previous studies using cultured rat hepatocytes suggested that T_3 and T_3S uptake are mediated by the same transporter(s) (19). The increased water-solubility of iodothyronine sulfates is a clear advantage for their use as alternative ligands for hepatic iodothyronine transporters expressed in oocytes. However, T_3S shows lower uptake than T_3 not only in native oocytes but also in liver mRNA-injected oocytes (5). In this study we tested iodothyronines sulfamate, derivatives generated by sulfonation of the α-NH_2 group as potentially useful ligands for iodothyronine transporters. The results indicated that T_4NS and T_3NS combine a low uptake in uninjected oocytes with an induction by rat liver mRNA that is as least as great as for T_3, suggesting that the sulfamates are attractive alternative ligands for the hepatic iodothyronine transporters.

Size-fractionation of rat liver mRNA has previously been shown to result in the enrichment of messengers inducing uptake of T_4 and T_3, with peak activities in fractions of 0.5-1.5 kb and 1.5-2.5 kb, respectively (5). Although these findings suggest partial

dissociation of mRNAs coding for T_4 and T_3 transporters, these fractionation studies are too crude to allow firm conclusions. The present study demonstrates not only that T_3NS and T_4NS uptake show much greater responses to injection of oocytes with total liver mRNA than T_4 and T_3 uptake, but also that size-fractionation results in a further enrichment of T_4NS and T_3NS transport activity. These studies provide evidence for the presence in rat liver of 3 different mRNAs coding for T_4NS and T_3NS transporters. mRNA of 1-1.5 kb induces the Na^+-dependent uptake of T_4NS and T_3NS, while mRNA of 1.5-2.5 kb stimulates both Na^+-dependent and Na^+-independent uptake of the sulfamates. The increase in T_3NS uptake *versus* uninjected oocytes amounts to 14-fold for the 1-1.5 kb mRNA induced Na^+-dependent activity, 11-fold for the 1.5-2.5 kb mRNA induced Na^+-independent activity and as much as 33-fold for the same 1.5-2.5 kb mRNA induced Na^+-dependent activity. The strong inhibition of uptake of T_3NS and T_4NS induced by the various mRNA fractions in the presence of excess T_4, T_3, the organic anions BSP and PBN, as well as the bile acids TC and CA strongly suggest that the sulfamates are ligands for organic anion transporters that mediate uptake of thyroid hormone in the liver. This is supported by findings that BSP inhibits transport of iodothyronines in human liver *in vivo* as well as in rat liver slices and rat hepatocytes *in vitro* (20,21)

The 1-1.5 kb mRNA fraction that induces uptake of T_3NS and T_4NS corresponds in size and Na^+ dependence to mRNA coding for rNTCP (1.7 kb) which has previously been shown, and confirmed in this study, to mediate uptake of different iodothyronines derivatives. Rat NTCP is a protein of 362 amino acids, containing 7 putative transmembrane domains, which is expressed exclusively in the basolateral membranes of hepatocytes. Although it is very important for hepatic uptake of bile acids, it transports a wide variety of organic anions (22). Only two other proteins homologous to NTCP have been identified, *i.e.*, ileal sodium-dependent bile acid transporter (ISBT), that is involved in the intestinal absorption of bile acids, and a protein termed P3 of unknown function (23,24). The possible role of these proteins in transport of thyroid hormone in the intestine and other tissues remains to be investigated.

The 1.5-2.5 kb mRNA species inducing the Na^+-independent uptake of T_4NS and T_3NS corresponds in size to rOATP1, which mediates the Na^+-independent transport of bile acids and a variety of other anionic (*e.g.*, conjugated steroids), neutral (*e.g.*,

unconjugated steroids), and even cationic (*e.g.*, ajmalinium derivatives) compounds (22). Rat OATP1 is a member of a large family of transporters which in rats also includes rOATP2-5 (15,7,8,25), rOATP-E (10), and the prostaglandin transporters rPGT (26) and rMOATP1 (27). In humans, the following members have been identified, hOATP-A through F (9,16,28-32), hOATP8 (33), hOATP-RP4 (34), and hPGT (35). Of these transporters, rat OATP1, OATP2, OATP3, OATP4, but not its splice variant, rat liver-specific transporter (rLST) (36), and OATP-E as well as human OATP-A, OATP-B, OATP-C (also known as human liver-specific transporter, hLST1 or OATP2), OATP-E and OATP8 have been shown to be capable of transporting T_4 and T_3. In general, OATPs show a wide tissue distribution, in particular liver, kidney and brain, although some are expressed exclusively in only one of these tissues. Usually, they consist of 650-700 amino acids and contain 12 putative transmembrane domains. The size of the mRNAs for the different rat OATPs varies between 2.0 and 3.8 kb, and amounts to 2.8 kb for rOATP1, which is in excellent agreement with the size of rat liver mRNA fraction that induces the Na^+-independent uptake of iodothyronines.

In contrast to the identification of at least 3 different mRNAs in rat liver inducing T_4NS and T_3NS uptake, only two major mRNA species appear to code for TC transport, corresponding in size and Na^+ dependence to rNTCP and rOATP1. The important role of these transporters in bile acid uptake was demonstrated by the inhibition of TC uptake after treatment of rat liver mRNA with both rNTCP and rOATP1 antisense oligonucleotides, confirming previous findings reported by Hagenbuch *et al.* (18). Uptake of T_4NS induced by rat liver mRNA was only partially inhibited by these oligonucleotides, although the uptake of T_4NS induced by rNTCP and rOATP1 cRNAs alone was completely blocked. The contribution of some OATPs can be excluded as they are not expressed in liver (*e.g.*, OAT-K1 (37) and its splice variant OAT-K2 (38)), or the size of their mRNAs does not correspond to that of the Na^+-independent liver mRNA peak fraction (*e.g.*, 3.6 kb for rOATP2). However, the contribution of other members of the OATP family, such as rOATP3-5 (all 2-2.5 kb), cannot be excluded. The tissue distribution of rOATP5 (2.5 kb), which has been cloned from kidney, is unknown. The OATP1-antisense2 oligonucleotide used in our experiments is completely complementary to the rOATP5 sequence, shows 2 mismatches with rOATP3, and deviates completely from the rOATP4 sequence. In addition, the contribution of

members of the Na^+-independent OAT family, some of which (*e.g.*, rOAT2 (39) and rOAT3 (40)) have the appropriate mRNA size and are expressed in liver, cannot be excluded either, although it is unknown if they are capable of iodothyronine transport. However, residual iodothyronine transport induced by rat liver mRNA in the presence of rNTCP and rOATP1 antisense oligonucleotides was primarily Na^+ dependent, in keeping with the size fractionation studies. Collectively, our findings strongly suggests that hepatic transport of iodothyronine sulfamates, and probably also of T_4 and T_3, is mediated largely by an as yet unidentified Na^+-dependent transporter. Future studies in our laboratory are focused on the cloning and characterization of this iodothyronine transporter.

Using the same antisense technique, Hagenbuch *et al* have found that in contrast with bile acids, Na^+-independent BSP uptake induced by rat liver mRNA was only inhibited by approximately 50% after treatment with rOATP1-antisense2 oligonucleotide (18). Similarly, Kouzuki *et al.* concluded that rOATP1 contributed only 20-30% to the Na^+-independent hepatic uptake of the sulfate conjugates of estrone and 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole (E3040) (41). These studies suggest that multiple transport mechanisms are responsible for the Na^+-independent uptake of organic anions in hepatocytes.

While T_4 and T_3 transport by various OATPs has been reported before, we carried out a more detailed study of the iodothyronine ligand specificity of rat and human NTCP, rat OATP1 and OATP2 and human OATP-A. The results indicate a large variation in ligand preference among these transporters, with both human and rat NTCP showing higher transport rates with TC and T_4NS than with the different iodothyronines as the ligands. In contrast, TC is a relatively poor ligand for the different OATPs, and underivatized iodothyronines are transported at the same or even higher rates than iodothyronione sulfamates. Interestingly, T_4 appears to be the preferred ligand for rat OATP2 and T_3 for human OATP-A. The importance of the various OATPs for uptake of iodothyronines in tissues other than liver, such as kidney and brain, also remains to be established.

In conclusion, iodothyronine sulfamates can be used as alternative ligands for the expression cloning of specific Na^+-dependent thyroid hormone transporters from liver. Our study indicates that the interference of multispecific organic anion transporters such

as NTCP and different OATPs with the characterization of specific iodothyronine transporters may be largely prevented by antisense knock-out of their mRNAs. The physiological role of the different NTCP and OATPs in mediating thyroid hormone uptake *in vivo* remains to be established.

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Chapter 7

PLASMA MEMBRANE TRANSPORT OF THYROID HORMONES AND ITS ROLE IN THYROID HORMONE METABOLISM AND BIOAVAILABILITY

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ABSTRACT

Although it was originally believed that thyroid hormones enter target cells by passive diffusion, it is now clear that cellular uptake is effected by carrier-mediated processes. Two stereospecific binding sites for each T_4 and T_3 have been detected in cell membranes and on intact cells from humans and other species. The apparent K_m values of the high-affinity, low-capacity binding sites for T_4 and T_3 are in the nanomolar range, whereas the apparent K_m values of the low-affinity, high-capacity binding sites are usually in the lower micromolar range. Cellular uptake of T_4 and T_3 by the high-affinity sites is energy, temperature, and often Na^+ dependent, and represents the translocation of thyroid hormone over the plasma membrane. Uptake by the low-affinity sites is not dependent on energy, temperature and Na^+, and represents binding of thyroid hormone to proteins associated with the plasma membrane. In rat erythrocytes and hepatocytes, T_3 plasma membrane carriers have been tentatively identified as proteins with apparent molecular masses of 52 and 55 kDa. In different cells, such as rat erythrocytes, pituitary cells, astrocytes and mouse neuroblastoma cells, uptake of T_4 and T_3 appears to be mediated largely by system L or T amino acid transporters. Efflux of T_3 from different cell types is saturable but saturable efflux of T_4 has not yet been demonstrated. Saturable uptake of T_4 and T_3 in the brain occurs both via the blood-brain barrier and the choroid plexus-CSF barrier. Thyroid hormone uptake in the intact rat and human liver is ATP dependent and rate limiting for subsequent iodothyronine metabolism. In starvation and non-thyroidal illness in man, T_4 uptake in the liver is decreased, resulting in lowered plasma T_3 production. Inhibition of liver T_4 uptake in these conditions is explained by liver ATP depletion and increased concentrations of circulating inhibitors, such as 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid, indoxyl sulfate, non-esterified fatty acids, and bilirubin. Recently, several organic anion transporters and L type amino acid transporters have been shown to facilitate plasma membrane transport of thyroid hormone. Future research should be directed to elucidate which of these and possible other transporters are of physiological significance, and how they are regulated at the molecular level.

CONTENT

I. HISTORICAL INTRODUCTION

Early reports on uptake of thyroid hormones by cells and tissues of different species appeared in the early 1950s. For about two and a half decades it was assumed that the translocation of thyroid hormones over the plasma membrane of target cells was a process of simple diffusion. This assumption was based on the fact that thyroid hormones are lipophilic and, as the plasma membrane is constituted of a lipid bilayer, there seemed apparently no need to assume any other mechanism of translocation than that of diffusion. The belief in this concept was so strong that hardly any studies testing this assumption were performed in this period of time. The studies that were performed on thyroid hormone uptake by cells and tissues were predominantly directed at investigating the influence of temperature, pH and extracellular thyroid hormone-binding proteins on the kinetics of this process. In the interpretation of the results of these studies it was often taken for granted that thyroid hormones diffuse into the cells and that the driving force of this process is the concentration of the free hormone. This so-called "free hormone hypothesis" was formulated in 1960 by Robbins and Rall (1). They stated "that the free or diffusible thyroid hormone concentration in blood and extracellular tissues would determine the rate at which thyroid hormone is distributed to its loci of action and the rates at which it is degraded and excreted". As we will see in the following sections, this assumption is only partially correct. Plasma membrane translocation is a regulated process that is rate limiting for subsequent intracellular accumulation, action and fate of the hormone. However, we will also see that, at least *in vitro*, the rate of uptake of thyroid hormones into the cell is determined not only by the efficacy of this plasma membrane translocation process but also by variations in the free hormone concentration in physiological and pathophysiological conditions. *In vivo* the situation is more complicated in that circulating inhibitors of thyroid hormone tissue uptake may be operative as well.

It is remarkable that, to the best of our knowledge, the first publication on thyroid hormone transport points to an energy-dependent uptake process (2). In this report, transport of T_3 into ascites carcinoma cells was inhibited by KCN, a metabolic blocker that suppresses ATP formation, indicating that energy is involved in the

uptake mechanism. The authors of this study concluded that, "this amino acid does not escape the cellular concentration process to which all other amino acids so far studied are subjected". This report apparently escaped attention and was 'rediscovered' by Sorimachi and Robbins in 1978 (3).

In a review in 1957 (4), Robbins and Rall proposed that thyroid hormone action is a function of the free hormone in the blood. However, in view of the extremely low concentration of unbound T_4 in blood, they suggested that tissues are extraordinarily sensitive to thyroid hormone, or that T_4 has to be concentrated in target cells. This latter suggestion leaves open the possibility of an active transport process. On the basis of their studies using tissue slices at different incubation temperatures and metabolic activities, Freinkel *et al.* (5) concluded that the establishment of concentration differentials for T_4 between tissue slices and suspending media constitutes an equilibrium binding phenomenon rather than an active transport. Hogness *et al.* (6) suggested that the higher concentration of T_4 and T_3 in rat diaphragm as compared with that in the incubation media was evidence for a true chemical binding. They did not consider the possibility of energy-dependent transport against a concentration gradient. Two groups of investigators, Beraud *et al.* (7) and Ingbar and Freinkel (8), were of the opinion that extra- and intracellular thyroid hormone binding-proteins govern transmembrane transfer of free diffusible hormone. In their studies of the uptake of T_4 and T_3 by rat diaphragm, Lein and Dowben (9) assumed that the kinetics of uptake they observed were based on diffusion into the tissue and subsequent binding of hormone to intracellular proteins. In his review on distribution and metabolism of thyroid hormone, Tata (10) suggested that the plasma membrane did not play an active role in the movement of free hormone from the vascular to the tissue compartments. Hillier (11) published a series of studies related to uptake and release of T_4 and T_3 in different organs. To our knowledge, he was the first to assess saturability of these processes. Studying the perfused rat heart, saturation of these processes could not be detected using free hormone concentrations ranging from 13 pM to 1.3 μM. As we will see below (sections II, III), the highest concentration used is sufficient to saturate the high-affinity component of the uptake process detected in rat hepatocytes and many other cell types, although discrepancies have been described. One of the reasons why any

saturation of the uptake mechanism might have escaped detection is that the conditions under which the studies were performed were not optimal to maintain intracellular ATP concentrations. This means that any energy-dependent, carrier-mediated process might have become undetectable. This possibility is in line with another observation from the same study (11), that thyroid hormone uptake was independent of changes in incubation temperature. In a follow-up study (12), Hillier concluded that extracellular thyroid hormone binding-proteins are an important factor determining the total amount of hormone taken up by the rat heart. Studying uptake and release of T_4 and T_3 in rat liver under similar 'ATP-poor' conditions and using hormone concentrations up to 0.13 μM, he arrived at similar conclusions, in that uptake and release were temperature independent and that uptake was importantly influenced by extracellular hormone-binding sites (13). The assumption that thyroid hormones easily penetrate plasma membranes was strengthened by Hillier's next studies (14) using liposomes prepared from egg-yolk lecithin. He reported that these membranes were readily permeable to T_4 and that the binding of both T_4 and T_3 to liposomes and to rat heart tissue is similarly dependent on pH.

In summary, until 1970 it was generally believed that thyroid hormones enter target cells by simple diffusion. This assumption was based on the fact that thyroid hormones are lipophilic and could therefore easily traverse the lipid-rich bilayer of the cell membrane. Transport of thyroid hormones into cells was envisaged to be mainly regulated by binding forces of extra- and intracellular thyroid hormone-binding proteins, directing the free moiety of thyroid hormone passively through the plasma membrane.

II. BINDING OF THYROID HORMONES TO ISOLATED CELL MEMBRANES

A. Binding kinetics

The earliest studies analyzing specificity of binding of thyroid hormones to plasma membranes of target cells were reported in 1975 by Tata (15) and in 1976 by Singh *et al.* (16). Although detecting saturability of binding of thyroid hormones to

different cellular constituents, including plasma membranes, Tata questioned the biological relevance of these binding sites (15). Singh and his group studied inhibition of binding of T_3 and T_4 to intact hemoglobin-free erythrocyte membranes by thyroid hormone analogs (16). Specificity of binding was demonstrated for both T_4 and T_3 by structure-dependent inhibition by the analogs. The major finding of this study was that the avidity of erythrocyte membranes was greater for T_3 analogs than for T_4 analogs but was similar for L-T_3 and L-T_4.

Several reports concerned binding of thyroid hormones to plasma membranes of rat hepatocytes (17-20). Pliam and Goldfine (17) reported on two binding sites for L-T_3, one with high affinity and low capacity and one with low affinity and high capacity. Mean apparent K_d values were 3.2 nM and 220 nM, respectively (Table 1). Similar values were found by others (18), who also reported on high and low-affinity binding sites for L-T_4, with mean apparent K_d values of 0.57 nM and 23.8 nM, respectively, distinct from the T_3 binding sites (Table 1). Specific T_4 binding was inhibited by thiol-blocking agents and by proteases. L-T_4 was bound with high specificity regarding iodine substituents and alanine side chain modifications (20). Studies of L-rT_3 binding to rat hepatocyte membranes also revealed two binding sites, the high-affinity site being different from that of L-T_4 (21).

A number of studies have also reported on the binding of thyroid hormones to human and rat erythrocyte membranes (22-29). Both in human and rat erythrocyte membranes, two saturable binding sites for L-T_3 were identified; a high-affinity, low-capacity and a low-affinity, high-capacity binding site. Apparent K_d values for the high-affinity binding site in human erythrocytes varied between 0.2 nM and 140 nM and for the low-affinity binding site between 5 nM and 26 µM (22,24,25,27). Specific binding was dependent on the presence of reduced protein-SH groups and showed high specificity for L-T_3, with L-T_4 being far less avidly bound (24). For rat erythrocyte membranes apparent K_d values for T_3 varied between 9 pM and 4.5 nM for the high-affinity site and between 0.4 nM and 50 µM for the low-affinity site (23,26-29) (Table 1). Also here, specific binding was dependent on the reduced state of protein–SH groups, and the high-affinity binding site appeared to be related to the amino acid transport system T (27,28).

Table 1. Specific binding of L-T_3 and L-T_4 to isolated plasma membranes of different tissues from different species (mean values)

	T_3		T_4		
Tissue	Kd_1[a]	Kd_2[b]	Kd_1[a]	Kd_2[b]	Ref.
Rat hepatocytes	3.2 nM	220 nM			17
Rat hepatocytes	15 nM	270 nM	0.57 nM	23.8 nM	18
Rat hepatocytes	15.8 nM	237 nM	4.54 nM	127.0 nM	18
Rat kidney			10 nM		19
Human erythrocytes	140 nM	26 µM			22
Human erythrocytes	0.2 nM	5 nM			24
Human erythrocytes	34 nM	ND[c]			25
Human erythrocytes	0.2 nM	18 µM			27
Rat erythrocytes	19 pM	20 nM			23
Rat erythrocytes	9 pM	0.4 nM			26
Rat erythrocytes	20 pM	ND[c]			27
Rat erythrocytes	21 nM	50 µM			28
Rat erythrocytes	4.5 nM	ND[c]			29
Rat testis	266.0 nM	ND[c]	27.77 nM	285.7 nM	18
Rat spleen	ND[c]	ND[c]	ND[c]	ND[c]	18
Human placenta	2.0 nM	18.5 µM			30
Mouse neuroblasts	8.4 nM	7.3 µM			31

[a] High-affinity binding site; [b] Low-affinity binding site; [c] Not detected

Binding was (stereo)specific, in that D-T_3 and L-T_4 were less potent in competing for these sites than L-T_3, whereas rT_3 and triiodothyroacetic acid (TRIAC) were inactive (23). The considerable variation in apparent K_d values reported in these studies is probably due to differences in test conditions and techniques, but may also be caused by involvement of multiple transporters (see section IX).

Binding of thyroid hormones to plasma membranes of other cell types and species was also reported. High-affinity binding sites for T_3 and T_4 in plasma

membranes of rat kidney and testis were characterized by apparent K_d values in the low nanomolar range, whereas those of the low-affinity binding sites were in the high nanomolar range (Table 1). Specific binding sites for L-T_3 and L-T_4 could not be detected in rat spleen (18). In plasma membranes of human placenta, two specific L-T_3 binding sites were found with apparent K_d values of 2.0 nM and 18.5 µM (30). D-T_3, L-rT_3, L-T_4 and D-T_4 were less effective in displacing L-T_3 from both binding sites. In plasma membranes of a mouse neuroblastoma cell line, L-T_3 binding sites showed apparent K_d values of 8.4 nM and 7.3 µM, with lower affinity of both sites for D-T_3 (31).

B. Analysis of binding protein(s)

A series of publications by Cheng and coworkers (30,32-35) concerned the identification of T_3 and/or T_4-binding membrane proteins in different cell types by affinity-labeling techniques. In their experiments using human placenta (30), GH3 cells (32,33), mouse Swiss 3T3 fibroblasts (33), and human A431 epitheloid carcinoma cells (33), the proteins were envisaged to be associated with the plasma membrane and to have a molecular mass between 55 (32,33) and 65 kDa (30). Peptide mapping of the proteins labeled with *N*-bromoacetyl-[^{125}I]T_3 (BrAc[^{125}I]T_3) or BrAc[^{125}I]T_4 showed very similar patterns (33), indicating that the same protein was probably involved. Later immunocytochemical studies, using four different monoclonal antibodies against the 55 kDa thyroid hormone-binding protein, showed that this protein was loosely associated with the endoplasmic reticulum and nuclear envelope, although some association with the plasma membrane could not be excluded (34). In a later study by Kato *et al.* (35), this protein was shown to be identical to protein disulfide isomerase (PDI). This finding was confirmed by Horiouchi *et al.* (36), who detected both T_3-binding and PDI activity in a 55 kDa protein isolated from a plasma membrane-enriched beef liver fraction. Although some PDI may indeed be associated with the plasma membranes, most of this enzyme is located in the lumen of the endoplasmic reticulum (37). In contrast to the high reactivity of PDI towards BrAcT_3 and BrAcT_4, it shows only low affinity for underivatized T_3 and T_4 (38). Since, moreover, PDI is not an integral membrane

protein (37,38), it seems unlikely to be involved directly in plasma membrane transport of thyroid hormone.

Photoaffinity-labeling of erythrocyte membranes with L-T_3 has identified a protein with an apparent molecular weight of 55 kDa (39). T_3 binding to this protein was critically dependent on the presence of phospholipids. Tryptophan but not leucine or D-T_3 competed with the L-T_3 binding site, indicating stereospecificity and a possible relationship with the amino acid transport system T (39). Using a monoclonal antibody that specifically inhibited uptake of T_3 in rat hepatocytes, a putative carrier protein was detected with an apparent molecular mass of 52 kDa (40). Affinity-labeling of mouse neuroblastoma plasma membranes with BrAc[^{125}I]T_3 has detected a 27 kDa protein (31). Since the size of this protein is identical to that of the type I iodothyronine deiodinase, which is also readily labeled with BrAcT_3 (38), it is unlikely to be related to a thyroid hormone transporter.

In summary, the first studies showing specific binding of thyroid hormones to isolated cell membranes appeared in the mid-1970s. Most extensively studied were cell membranes from human and rat erythrocytes and rat hepatocytes. For each T_3 and T_4, two stereospecific binding sites were detected in these membranes; one with apparent K_d values in the lower nanomolar range, and the other in the (sub)micromolar range. Specific binding for both hormones was dependent on the reduced state of protein-SH groups. T_3-binding proteins have been identified in rat erythrocyte and hepatocyte membranes with apparent molecular masses of 55 and 52 kDa.

III. TRANSPORT OF THYROID HORMONES INTO ISOLATED CELLS

The first evidence, to our knowledge, that transport of thyroid hormones into intact cells is not a passive, but an energy-dependent, process was reported by Christensen *et al.* in 1954 (Ref 2; see also section I) but unfortunately temporarily escaped attention. It was not until 1976 that Rao *et al.* (41) and our laboratory (42,43) in 1978 independently published the saturable and energy-dependent transport of T_3 and T_4 into rat hepatocytes. Since then a whole series of reports from different

laboratories have confirmed carrier-mediated, mostly energy- and Na^+-dependent transport of iodothyronines into a variety of cells from different species.

A. Transport into hepatocytes

In Table 2 the kinetics of thyroid hormone uptake by hepatocytes are summarized. In most studies two saturable processes have been discerned: a high-affinity, low-capacity and a low-affinity, high-capacity process (41-55). In the majority of the studies, the apparent K_m values of the high-affinity systems for T_4, T_3 or rT_3 uptake are in the nanomolar range (42-55). This process is thought to represent the translocation process across the plasma membrane as it is energy and temperature dependent (41-55). Studies testing the possible Na^+ dependence of the high-affinity uptake of iodothyronines have produced controversial results in rats (44-47,50), confirmatory results in human hepatocytes (52), and negative results in trout hepatocytes (54,55). The energy-, temperature-, and Na^+-independent, low-affinity uptake process may represent binding of thyroid hormone to cell surface-associated proteins (45). T_4 and T_3 mutually inhibit their high-affinity uptake processes in rat hepatocytes, but kinetic analysis of these inhibitions indicates that T_3 and T_4 cross the plasma membrane by different pathways (47,55). This finding was confirmed by others who found differences in the dependence of the T_3 and T_4 transport systems on the cell phase of the rat hepatocyte and on sodium butyrate stimulation (56). Preliminary results in rat hepatocytes suggest that rT_3 shares the same transport system with T_4 (48), but kinetic studies of plasma iodothyronine clearance in humans suggest different plasma to liver transfer mechanisms for rT_3 and T_4 (57), in line with different binding sites for rT_3 and T_4 in (rat) liver plasma membrane (21). In addition to the metabolic condition of hepatocytes in culture, in particular with regard to ATP concentration, the free T_4 concentration in the medium is also a determinant for the amount of hormone that is taken up by the cell and subsequently metabolized (58). Stereospecificity of T_3 and T_4 uptake has been demonstrated in rat and trout liver cells (51,54,55).

Table 2. Kinetics of thyroid hormone transport into hepatocytes *in vitro* (mean values).

Species	K_m T_4	K_m T_3	K_m rT_3	Temperature dependent	Energy (ATP) dependent	Na^+ dependent	Stereo-specific	Ref.
Rat								
1[a]		52 nM		yes	yes			41
2[b]		144 nM		yes	yes			
Rat								
1[a]	1.2 nM[c]	21 nM[c]		yes	yes	yes		42,45-47,50
2[b]	1.0 µM	1.8 µM		no	no			
Rat								
1[a]		86 pM		yes	yes	no		44
2[b]		726 pM		yes	yes	no		
Rat			≈6 nM[d]		yes			48
Rat hepatoma		680 nM			yes		yes	51
Human		NR[e]		yes	yes	yes		52
Human								
1[a]		3.6 nM			yes			53
2[b]		503 nM						
Trout	0.52 µM[a]	74 nM[a]			yes	no	yes	54,55

[a] High-affinity uptake system; [b] Low-affinity uptake system; [c] T_4 and T_3 have different transport systems
[d] rT_3 transport system possibly shared with T_4; [e] Not reported

B. Transport into other cell types

Many studies have confirmed carrier-mediated, often energy- and Na^+-dependent transport of thyroid hormones in various cell types from different species, *i.e.*, human (22,59-62), rat (63-65), and trout (66,67) erythrocytes; normal (68,69) and clonal (70) rat pituitary cells, brain cells such as human glioma cells (71), rat glial cells (72), astrocytes (73), cerebrocortical neurons (74), and brain synaptosomes (75); mouse neuroblastoma cells (76), rat skeletal (77) and cardiac (78) myocytes; human (79,80) and mouse (81) fibroblasts; human epithelial carcinoma cells (81); Chinese hamster ovary cells (81); human trophoblasts (82); human choriocarcinoma cells (83-86); rat adipocytes (87); human peripheral leukocytes (88,89); and mouse thymocytes (90,91) (Table 3).

1. T_3 transport. Similar to hepatocytes, apparent K_m values for the high-affinity uptake of T_3 in other cell types are mostly in the nanomolar range. Some authors (22,73), including our laboratory (80), have also detected a low-affinity T_3-binding site, like that present on hepatocytes, apparently depending on the use of protein (albumin)-containing incubation media and probably reflecting the association of protein-bound T_3 with/around the cells (45). When studied, the energy dependence of T_3 transport was invariably demonstrated in the different cell types. In contrast, the Na^+ dependence of this process differed between cell types. Thus, transport of T_3 in erythrocytes of human, rat and trout origin (22, 59-67), in rat astrocytes (72,73), and human choriocarcinoma cells (82-86) was not dependent on the Na^+ gradient over the plasma membrane, whereas this was the case in rat pituitary cells (68-70), rat brain synaptosomes (73), rat neonatal cardiac myocytes (78), human fibroblasts (80), and mouse thymocytes (90,91). In some cell types the influence of pH on transport was studied and found to be of importance, in the sense that T_3 uptake decreased when pH increased in mouse thymocytes (91), while the reverse was true in rat brain astrocytes (75). When studied, T_3 transport was invariably (stereo)specific, *i.e.*, in human and rat erythrocytes, human and rat nerve and brain cells, rat skeletal myoblasts, human choriocarcinoma cells and mouse thymocytes (Table 3). In general, different L-iodothyronine analogs and the D-isomers of T_3 and T_4 were less potent in inhibiting T_3 and T_4 uptake than L-T_3 and L-T_4.

2. T_4 transport. T_4 transport into intact cells has been less well studied than T_3 transport (Table 3). The most probable explanation for this, at least in liver cells, is the greater requirement of an optimal energy charge of the cells under study for transport of T_4 than for uptake of T_3. This is explained by the much steeper slope of the relationship between cellular ATP concentration and the rate of T_4 (and rT_3) transport in hepatocytes than that of the relationship between ATP and T_3 transport (Fig. 1) (46). Even a small decrease in cellular ATP concentration results in a major reduction in T_4 (and rT_3) transport but only slightly affects T_3 uptake. This may also be the reason why some authors could not observe specific, energy-dependent transport of T_4 in liver cells (44,92). Others (93) did find saturable but energy-

Table 3. Kinetics of thyroid hormone uptake in different cell types *in vitro* (mean values).

Cells	K_m T_4	K_m T_3	Temperature dependent	Energy (ATP) dependent	Na^+ dependent	Stereo-specific	Ref.
Human erythrocytes							
1[a]		16 nM			yes		22
2[b]		3.3 µM			no		
Human erythrocytes		128 nM		no	no		59
Human erythrocytes		248 nM					60
Human erythrocytes	Diffusion?	67 nM		no	no	yes	61
Human erythrocytes		59.9 nM					62
Rat erythrocytes	No uptake	53 nM[c]	yes		no	yes	63,64
Rat erythrocytes		160 nM					65
Trout erythrocytes	0.1-1.1 nM	70-119 nM	yes	no	no		66,67
Rat pituitary	NR[d]	400 nM[e]		yes	yes	yes	68-70
Human glioma cells	0.46 nM	2.17 nM	yes	yes		yes	71
Rat astrocytes	1.02 µM[g]	0.52 µM[g]		no	no	yes	72,75
Rat brain synaptosomes							
1[a]	Diffusion ?	50 pM	yes	yes	yes		73
2[b]		3.1 nM					
Mouse neuroblastoma	6.07 nM	2.38 nM		yes		yes	74
Rat brain neurons	≈300 nM[f]	≈400 nM[f]				yes	76
Rat skeletal myoblasts		17 nM	yes	yes		yes	77
Rat neonatal cardiac myocytes	Diffusion?	NR[d]		yes	yes		78
Human fibroblasts	Diffusion?	108 nM					79
Human fibroblasts							
1[a]	1.9 nM	29 nM		yes	yes		80
2[b]	141 nM	650 nM					
Mouse fibroblasts		NR[d]		yes			81
Humane epithelial carcinoma		NR[d]		yes			81
Hamster ovary		NR[d]		yes			81
Human trophoblasts		755 nM					82
Human choriocarcinoma	59.4 nM[h]	378-586 nM[h]	yes	yes	no	yes	83-86
Rat adipocyte	0.30 nM	0.29 nM					87
Human leucocytes		NR[d]					88,89
Mouse thymocytes	Diffusion?	0.8 nM		yes	yes	yes	90,91

[a] High-affinity uptake system; [b] Low-affinity uptake system; [c] *trans*-inhibition of T_3 in- and efflux by T_3

[d] Not reported; [e] T_4 and T_3 share same transport system; [f] T_4 and T_3 have different transport systems

[g] Na^+-H^+ exchanger dependent; [h] K_m rT_3 3.04 µM

independent uptake not only of T_4 but also of T_3 in rat hepatocytes under far from optimal cellular ATP conditions. In other cell types, such as erythrocytes, rat neonatal cardiac myocytes, rat brain cells, pituitary cells, and fibroblasts, some laboratories observed that, in contrast to T_3, T_4 was apparently taken up by diffusion only or not at all, whereas other laboratories did find (stereo)specific, mostly energy-dependent T_4 uptake in the same cell types (Table 3). It is not known whether these discrepancies are related to the different energy requirements of the T_4 and T_3 transport processes as mentioned above or due to other factors such as the use of different techniques.

C. Interactions of various compounds with thyroid hormone transport

1. Amino acids. Interrelationships between amino acid and thyroid hormone transport have been studied in different cell types from different species. It should be noted that the effects of amino acids on thyroid hormone transport cited below were usually obtained at physiological serum concentrations of free amino acids in the micromolar range.

a. Erythrocytes. In rat erythrocytes, the aromatic amino acids tryptophan (Trp), phenylalanine (Phe), and tyrosine (Tyr) competitively inhibited T_3 transport, while transport of Trp was similarly inhibited by T_3, D-T_3, T_4 and thyronine (T_0) (94). *N*-ethylmaleimide (NEM) irreversibly inhibited Trp and T_3 transport, and both ligands protected each others transport from inactivation by this compound. These data indicated common or closely linked transport systems for T_3 and for aromatic amino acids, *i.e.*, the system T amino acid transporter, at least in erythrocytes (94). Similar results were obtained for binding of T_3 and Trp to rat erythrocyte membranes (28). Further studies suggested a common carrier for T_3 and Trp which also facilitates countertransport such that the uphill transport of T_3 is driven by hetero-exchange with intracellular aromatic amino acids (95). Evidence for uptake of T_3 by the system T amino acid transporter or a closely linked transporter was also obtained using human and trout erythrocytes (62,67).

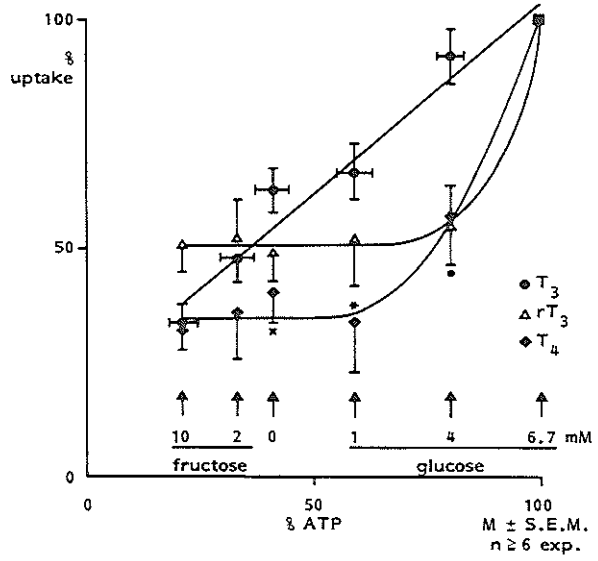

Fig. 1 Uptake of T_3 (•), rT_3 (Δ) and T_4 (◆) *vs.* ATP concentration in rat hepatocytes preincubated with different concentration of glucose or fructose. [Reproduced with permission from E.P. Krenning *et al.*: *FEBS Lett* 140:229-233, 1982 (48).]

No such relationship was found between T_4 and system T amino acid transport in trout erythrocytes (67).

b. Other cell types. In rat hepatocyte sinusoidal membrane vesicles, Trp transport occurs via a NEM-resistant (system T) and a NEM-sensitive (system L) pathway, and T_3 and T_4 mainly inhibit Trp transport via system T (96). The inhibitory activity of T_3 and T_4 is dependent on the thyroid status of the donor rat, *i.e.*, decreasing in the order hyperthyroid > euthyroid > hypothyroid. T_3 and T_4 share the same stereospecific uptake carrier in the rat pituitary (68,69), and the potent inhibition of T_3 and T_4 uptake by leucine (Leu) suggests the involvement of amino acid transport system L (70). This system was also found to participate in T_3 and T_4 transport in mouse neuroblastoma cells (74) and in T_3 transport in rat astrocytes (97). In Ehrlich ascites cells, the neutral amino acids Phe, α-aminoisobutyric acid and cycloleucine did not compete with transport of T_4, indicating that the system A, L and

ASC amino acid pathways were not involved (98). In rat hepatocytes, participation of the amino acid transport system A in uptake of T_3 and T_4 was ruled out (51,99). A weak interaction was found between uptake of system L and T amino acids and uptake of T_3 in human JAR choriocarcinoma cells (100).

2. Drugs and other chemicals. As shown in Table 4, a variety of compounds has been demonstrated to inhibit thyroid hormone uptake in different cells. Despite their widely different properties, the inhibitory activity of most of these substances is suggested to be based on competition because of structural similarity with thyroid hormone (19,48,51,53,101,103-107,111,112). The antiarrhythmic drug amiodarone is also known to inhibit binding of T_3 to its nuclear receptors on the basis of structural similarity (114). The concentration of amiodarone shown to inhibit uptake of thyroid hormone in rat hepatocytes was ≈1 μM, which is similar to therapeutical serum levels in humans (114). However, since in serum, amiodarone is primarily bound to albumin that circulates at a concentration of ≈4% but was used in the hepatocyte incubations at a concentration of 1%, the free amiodarone concentrations obtained *in vitro* may be higher than in treated humans. Nevertheless, *in vivo* kinetic data in patients treated with amiodarone also show decreased net tissue uptake of thyroid hormone (115). This decrease can be explained by inhibition of thyroid hormone transport into tissues and/or by inhibition of thyroid hormone binding to intracellular proteins. Cholecystographic agents usually reach serum concentrations between 100 and 700 μM in humans (116) and were tested *in vitro* (at lower albumin levels) at concentrations between 10 and 100 μM (48). These agents not only inhibit thyroid hormone transport into rat hepatocytes, supposedly on the basis of molecular structural similarity (19,48), but also displace T_4 from the human liver *in vivo* (117). The non-bile acid cholephils, sulfobromophthalein, bilirubin, and indocyanine green, also inhibit thyroid hormone transport and binding in rat hepatocytes on the basis of structural similarity (19,51). Diphenylhydantoin, the non-steroidal antiinflammatory phenylanthranilic acids, flufenamic acid, meclofenamic acid, and mefenamic acid, and the structurally related compounds 2,3-dimethyldiphenylamine and diclofenac, all competitively inhibit rat hepatocyte and pituitary uptake of thyroid hormone (51,107,111,113). Analysis of the structure-activity relationship for inhibition of T_3

uptake in rat hepatocytes by the phenylanthranilic acids demonstrated that inhibitory potency was highly dependent on the hydrophobicity of the inhibitor (107). Phloretin, a glucose transporter inhibitor that is structurally related to thyroid hormones, competitively inhibited T_3 uptake into human HepG2 hepatocarcinoma cells (53).

Table 4. Chemical inhibitors of thyroid hormone uptake into cells *in vitro*.

Inhibitor	Cell type	Ligand	Supposed mechanism of inhibition	Ref.
Ouabain, monensin	Rat hepatocytes, rat skeletal muscle, pituitary	T_3	Abolition Na^+ gradient	48,49,68
KCN, dinitrophenol, bacitracin, oligomycin	Rat hepatocytes, mouse thymocytes	T_3, T_4	ATP depletion	48,90
Vinblastin, colchicin, cytochalasin	Rat hepatocytes, mouse thymocytes	T_3, T_4	ATP depletion + perturbation cytoskeleton	48,90
D- and L-propranonol	Rat hepatocytes, mouse thymocytes	T_3, T_4	ATP depletion + membrane stabilization	48,90
Amiodarone	Rat hepatocytes	T_3, T_4	Competitive	48,114
Cholecystographic agents	Rat hepatocytes	T_3, T_4	Competitive	20,51
Nifedipine, verapamil, diltiazem	Rat hepatocytes, rat myoblasts, human hepatocytes	T_3	Interacteraction with calmodulin (like-protein)	101,112
Bromosulphthalein, indocyanine green	Rat hepatocytes, rat brain astrocytes	T_3, T_4	Competitive	19,51
Bilirubin and conjugates	Rat hepatocytes	T_3	?	106,109
Diphenylhydantoin, phenylanthranilic acid and phenylacetic acid derivatives	Rat hepatocytes, rat pituitary	T_3	Competitive	51,107, 111,113
Phloretin	Human hepatocytes	T_3	Competitive	53
3,5-Dibromo-3'-pyridazinone-L-thyronine (L-94901)	Rat myoblasts, rat hepatocytes, rat neuroblasts	T_3	Un- or noncompetitive	102
Benzodiazepines	Human hepatocytes, human neuroblast, rat pituitary	T_3	Direct or indirect interaction with T_3 carrier	103-105
CMPF, indoxyl sulfate	Rat hepatocytes	T_4	Unknown	108
NEFA	Rat hepatocytes	T_4	Unknown	109,110

Many of the here discussed inhibitors of thyroid hormone uptake also interact competitively with thyroid hormone-binding sites on serum proteins and nuclear T_3 receptors (51,107,113,118). Amiodarone, cholecystographic agents, and bilirubin have been shown to interact with deiodinases (114,119). The benzodiazepine drugs do not interact with nuclear T_3-binding sites, but inhibit T_3 uptake in different cell types from human and rat origin (Table 4) by competing for the T_3 carrier without being transported themselves (104). The structure-activity relationships were studied for inhibition of T_3 uptake in HepG2 cells by benzodiazepine and thyromimetic compounds. The results of these studies, along with computer-assisted molecular modeling techniques, predicted a "tilted crossbow" conformation of the inhibitor for interaction with the iodothyronine transporter (105).

The three different types of organic calcium channel blockers, nifedipine, verapamil, and diltiazem, inhibit T_3 uptake in different cell types (Refs. 101 and 112; Table 4). It is considered unlikely that the inhibitory effect is due to dependence of the uptake process on extracellular Ca^{2+}, on Ca^{2+} fluxes via voltage-dependent or receptor-operated calcium channels, or on the interaction of Ca^{2+} with protein kinase C (PKC). A plausible mechanism for the inactivation of the uptake process is by interaction of the calcium blockers with calmodulin in the plasma membrane. Calmodulin is found in high concentrations in plasma membranes; it binds T_3 and may play a role as such in the translocation process of thyroid hormone (101). 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), indoxyl sulfate, and non-esterified fatty acids (NEFAs) are substances that circulate in increased amounts in patients with nonthyroidal illness (NTI) and inhibit thyroid hormone uptake in liver cells (Refs. 108-110; see section VII B).

Little information is available about stimulatory factors of thyroid hormone uptake *in vitro*. The histamine H1 receptor antagonist, telemastine, and phenobarbital enhance the specific, energy-dependent uptake of T_4 in rat hepatocytes but not in hepatocytes from guinea pig or beagle dog (120). The exact mechanism of this induction in rat hepatocytes is unknown but appears to be a primary effect on the plasma membrane transport system. Telemastine did not influence T_3 uptake in rat hepatocytes, underscoring the functional difference in the uptake systems of T_3 and T_4 in the liver (121).

In summary, transport of T_4 and T_3 has been studied extensively in human, rat, and trout hepatocytes. Both for T_4 and T_3, high-affinity, low-capacity and low-affinity, high-capacity uptake processes have been identified. The high-affinity processes have apparent K_m values in the nanomolar range and represent the translocation of the hormones over the plasma membrane. This transport is temperature, energy and Na^+ dependent, and rate limiting for subsequent hormone metabolism. T_4 and T_3 mutually inhibit their high-affinity uptake processes but they are transported by different carriers. The low-affinity processes represent binding to cell surface-associated proteins and are not involved in transport. High-affinity, energy-dependent T_3 transport systems similar to those in hepatocytes have also been identified in many other cell types, although their Na^+ dependence varies. T_4 transport has been less well studied in other cell types and results are variable, possibly because of its greater requirement for an optimal energy charge of the cells.

T_3 uptake in different cells (rat erythrocytes, pituitary cells, astrocytes and mouse neuroblastoma cells) is inhibited by Trp, Phe, Tyr and/or Leu, suggesting the involvement of system L or T amino acid transporters. A large variety of chemicals (Table 4) inhibit cellular uptake of thyroid hormones on the basis of structural similarity or by decreasing the cellular energy charge. Alternatively, inhibition is mediated by a decrease in the Na^+ gradient over the plasma membrane, or by other as yet unknown mechanisms. The inhibitory activities of amino acids and other compounds are in the concentration range observed in humans and may interfere with *in vivo* tissue uptake of thyroid hormone.

IV. CELLULAR EFFLUX OF THYROID HORMONES

Efflux of thyroid hormones has been studied in a number of cell types from different species, *i.e.*, hepatocytes (122-124), erythrocytes (60,61,64,125,126), placenta cells (84,127,128), pituitary cells (129), FRTL-5 thyroid cells (130), NIH-3T3 cells (130), thymocytes (90), lymphocytes (131), and Ehrlich ascites cells (98).

We reported on absence of energy dependence of T_3 and T_4 efflux from cultured rat hepatocytes (122). Cellular efflux consisted of two components,

representing release of hormone bound to the outer cell surface and of intracellularly located hormone. We also observed a lack of saturability of T_3 efflux after loading of rat hepatocytes using free T_3 concentrations up to 54 nM (122). However, further results suggested saturation of T_3 efflux after loading of the cells using a free T_3 concentration of 1.5 µM. Others also observed saturability of T_3 efflux, by both T_3 and T_4, from a poorly differentiated rat hepatoma cell line (HTC) (123). The same authors also demonstrated that verapamil inhibited thyroid hormone efflux from these cells as well as from isolated rat hepatocytes, cardiomyocytes, and fibroblasts (123). Furthermore, they observed increased verapamil-inhibitable T_3 efflux from HTC cells adapted for resistance to a permeable bile ester (HTC-R cells). The authors suggested that the carrier protein involved in export of thyroid hormone is related to the family of the multidrug resistance-related ABC transporters as these membrane proteins are overexpressed in HTC-R cells (123). The same group also found verapamil inhibition of T_3 efflux from FRTL-5 thyroid cells and NIH-3T3 cells (130). Others assessed T_4 and T_3 efflux from multidrug-resistant pituitary tumor cells but did not find kinetics to be different from control pituitary tumor cells (129). Neither was any effect detected by verapamil on thyroid hormone efflux in both cell types. Possible saturability of thyroid hormone efflux was not tested by these authors (129).

Efflux of T_3 from rat erythrocytes was found to be a saturable process that is stimulated by aromatic amino acid countertransport, much as T_3 uptake is stimulated by counter efflux of aromatic amino acids (61,64). Efflux of T_4 from these cells occurred apparently by diffusion as is the case with T_4 and rT_3 efflux from human JAR choriocarcinoma cells, while also in these latter cells efflux of T_3 is saturable (84,128). No inhibitory effect on thyroid hormone efflux by neutral system A, L and ASC amino acids was observed in Ehrlich ascites cells (98). In many of the *in vitro* studies discussed in this article, it has been shown that thyroid hormone-binding proteins, including T_4-binding globulin (TBG), transthyretin (TTR), albumin, and lipoproteins have a permissive effect on efflux of thyroid hormones, probably by facilitating diffusion of thyroid hormone through the water layer around the cell (122,124,126).

In summary, efflux of T_3 from rat hepatocytes, cardiomyocytes, and fibroblasts has shown to be a saturable but energy-independent process. The efflux carriers in

these cells may be related to the multidrug resistance-related ABC transporter family. In rat erythrocytes, T_3 efflux is also saturable and is stimulated by aromatic amino acid counter transport. Saturability of T_4 efflux was not observed in these cells nor of T_4 and rT_3 efflux from human JAR choriocarcinoma cells, in contrast to the saturable efflux of T_3. Little is known about the role of efflux mechanisms in the regulation of intracellular hormone concentrations.

V. TRANSPORT OF THYROID HORMONE INTO ISOLATED ORGANS

Transport of thyroid hormones into perfused organs isolated from animals has been extensively studied. The advantage of studying an isolated organ is that its function can be evaluated without interference from other influences in the intact organism. Compared with experiments using isolated cells, the study of intact organs better represents the function of the tissues *in vivo*, although conditions are still appreciably different from the (patho)physiological situation. The results of thyroid hormone uptake studies using perfused, isolated organs from different species will be discussed in this section.

A. Transport into the liver

Transport of thyroid hormones into the intact liver has been studied mostly using organs isolated from rats. In 1979, Jennings *et al.* (132) reported on the effect of starvation on T_3 production from T_4 taken up by the perfused rat liver. They found that the reduced T_3 production was not caused by impaired deiodination of T_4 to T_3 in the liver but by reduced transport of T_4 into the liver, underlining the regulatory role of transport of thyroid hormone in subsequent hormone metabolism (132). One of the explanations that these authors mentioned was that T_4 uptake was inhibited by decreased activity of a 'specific' transport system. We extended these studies to T_3 and also found inhibition of T_3 uptake in the intracellular compartment of livers from fasted *vs.* normally fed rats perfused with medium lacking glucose, insulin and cortisol (133). This inhibition was reverted to normal by a 30-min preperfusion of

fasted livers with medium containing a combination of glucose, insulin, and/or cortisol but not by the individual additions. On the basis of these results, we explained the diminished T_3 uptake by a decrease in cellular ATP induced by fasting, which was restored by preperfusion with energy-rich medium (133). Further studies using fructose in the perfusate to (transiently) lower cellular ATP stores in the rat liver showed a parallel decrease in T_4 uptake in the intracellular compartment of the liver, thus underscoring the regulatory role of the energy charge of the cell in the transport process (Fig. 2, Ref. 134). Similar to the results in cultured rat hepatocytes, we found that, in addition to the energy state of the liver, the free hormone concentration in the perfusion medium determined the amount of hormone taken up by the intracellular compartment of the liver (135). Studies using livers from amiodarone-treated animals indicated that transport of T_4, but not of T_3, was inhibited (136), in agreement with hepatocyte studies (47,55) showing that T_4 and T_3 are transported differently across the liver plasma membrane. Efflux of T_3 from the isolated perfused trout liver was stimulated by addition of T_4, epinephrine, or TSH to the perfusion medium, and efflux of T_4 was stimulated by addition of T_4 to the medium. The stimulating effect of extracellular thyroid hormone on efflux of T_4 and T_3 may be caused by inhibition of reuptake, stimulation of an exchange mechanism, and/or displacement of hormone from intracellular binding sites (137,138). However, the stimulation of T_3 efflux by epinephrine and TSH remains unexplained.

B. Transport into other organs

As the choroid plexus is known to synthesize TTR (139,140), the specific role that this tissue plays in transport of thyroid hormone to brain cells was evaluated. Isolated choroid plexus of the rat was found to accumulate T_4 and T_3 from surrounding medium by a nonsaturable process (141). The authors proposed a positive role of choroid plexus-derived TTR in the transport of thyroid hormones from the blood to the cerebrospinal fluid (CSF) and subsequently to brain cells. Others found partly saturable uptake of T_4 in the choroid plexus of the rabbit (142). Measurement of T_3 uptake at the blood face of isolated sheep choroid plexus showed both saturable and nonsaturable transport (143). T_3 uptake lacked stereospecificity

and was Na^+ independent, but was inhibited by T_4 and by large neutral amino acids. Uptake of T_3 at the CSF side of sheep choroid plexus was also partially saturable and independent of the Na^+ gradient over the plasma membrane (143).

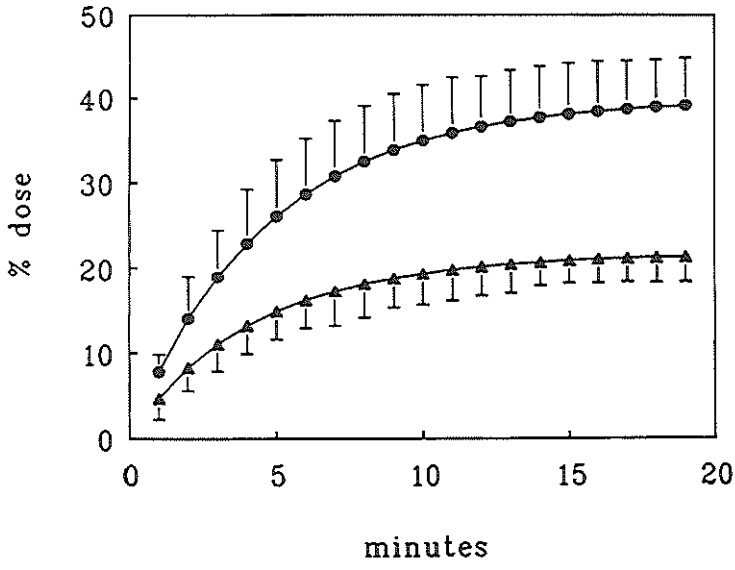

Fig. 2 T_4 liver uptake (in % dose) in rat livers during glucose (●) and glucose/fructose (▲) perfusion. [Reproduced with permission from M. de Jong *et al.: Am J Physiol* 266:E768-E775, 1994 (134).]

Incubation of whole soleus muscle isolated from rats showed stereospecific, energy- and Na^+-dependent uptake of T_3, but T_4 uptake was considered to be a diffusion process (49,144). Addition of insulin to the incubation medium stimulated T_3 uptake but did not affect T_4 uptake (145). T_3 uptake in the perfused rat heart showed a saturable process with an apparent K_m value of 80 μM (146). This value is about 1 order of magnitude higher than the apparent K_m values obtained in *in vitro* studies using isolated cardiomyocytes (Tables 2 and 3). This difference may be explained by the fact that T_3 uptake in the perfused rat heart was determined after a single capillary passage that proceeds within seconds and differs fundamentally from techniques in which initial uptake rates in cells are measured over a period of minutes. The question is if the former method represents uptake of the ligand by the

cardiomyocytes, since this assumes that the hormone has already passed the endothelium after such a short time lapse. Another explanation, of course, is that the experiments using cultured cells provide data that are more remote from the *in vivo* situation than data obtained from isolated organ studies. In contrast to the rat liver (132), fasting did not decrease uptake of T_4 by the isolated perfused rat kidney, but T_4 uptake was decreased in kidneys of diabetic rats (147,148).

In summary, uptake of T_4 and T_3 is decreased in isolated livers from fasted *vs.* fed rats perfused with the same 'energy-poor' medium. Changing the perfusate to an energy-rich medium restores uptake in 30 min, suggesting restoration of cellular ATP. Perfusion of fed livers with fructose results in a lowering of cellular ATP and a parallel decrease in thyroid hormone uptake. Analysis of transport in livers from amiodarone-treated rats showed that also in the intact liver T_3 and T_4 are taken up by different mechanisms. Apart from the cellular energy charge, the free and not the protein-bound fraction of thyroid hormone determines the amount of hormone taken up by the cellular compartment of the liver. Uptake of T_4 and T_3 in isolated rat or sheep choroid plexus was found to be nonsaturable by some investigators but partly saturable by others. Saturable transport of T_3, but not of T_4, was observed in the isolated rat soleus muscle. Saturable T_3 transport was also found in the perfused rat heart.

VI. *IN VIVO* PLASMA MEMBRANE TRANSPORT OF THYROID HORMONES IN ANIMALS

To assess plasma membrane transport of thyroid hormones to different organs *in vivo*, animals were injected with tracer amounts of labeled hormones after which entry of hormones into the isolated organs was analyzed.

A. Brain

Several questions related to transport of thyroid hormone to the brain have been addressed. One aspect is whether entry of thyroid hormone into brain proceeds

via a passive process or via a carrier-mediated mechanism. When dogs were injected intravenously with tracer T_4, allowing entry in the brain via the blood-brain barrier (BBB) and the CSF, brain uptake was saturable under conditions of T_4 loading, indicating that transport occurred via a carrier-mediated process (149). In mice, transport of T_3 into the brain was saturable but, under the conditions of the experiment, no saturation of T_4 transport was observed. Efflux of both T_3 and T_4 from the brain appeared to proceed by a carrier-mediated mechanism (150).

Another point of interest is to what extent transport through the BBB and the choroid plexus-CSF barrier (CP-CSFB) contributes to overall brain uptake of thyroid hormone. To investigate this, rats were injected either intravenously or intrathecally with radioactive thyroid hormones. When administered *intravenously*, hormones have access to the brain via both the BBB and the CP-CSFB. However, hormone injected *intrathecally* represents entry into brain cells via the CP-CSFB. After injection of radioactive hormones via these two routes and subsequent autoradiography of the brain, distribution of thyroid hormone over brain areas could be documented as well as the contribution of the BBB and the CP-CSFB to brain accessability (151-153). These studies demonstrated that T_3 and T_4 enter the brain mainly via the BBB for distribution throughout the brain, but that localization in the ependymal cells and in the circumventricular organs occurs via the CP-CSFB. In contrast, rT_3 is excluded by the BBB but has limited access to the brain via the CP-CSFB (Fig. 3).

Also, by *in vivo* injection of tracer hormones, the question of whether TTR has a special role in transport of thyroid hormone to the brain via the CP-CSFB was addressed. Results of studies in rats and sheep, showing accumulation of thyroid hormone in the choroid plexus, led to the proposal of a model for T_4 transport from the bloodstream into the CSF, involving uptake of T_4 by the choroid plexus, binding of the hormone to newly synthesized TTR, and secretion of the complex into the CSF (140,154-156). Recent studies in the TTR-null mouse mutant showed that total lack of TTR seems to have no consequences for normal development and fertility (157,158). In these mice, serum levels of free T_4, free T_3, and TSH were normal as were the type I and II deiodinase activities (being very sensitive to the thyroid status of the tissue) in liver and brain, respectively (157).

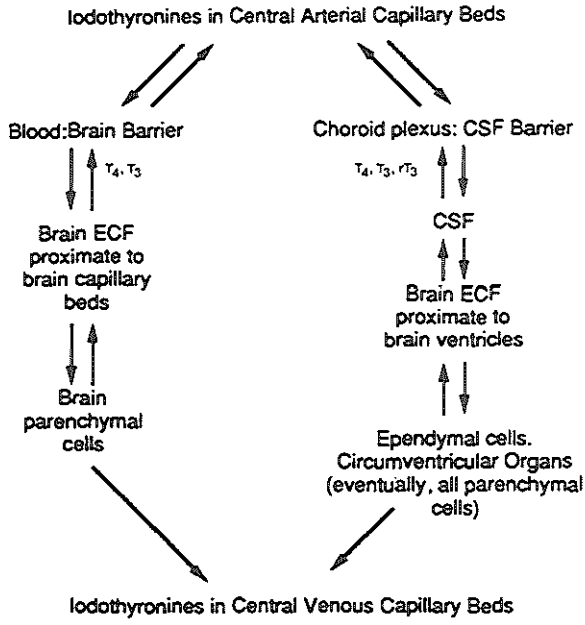

Fig. 3 Routes of iodothyronine transport between blood and brain. According to autoradiographic results, rT_3 crosses the CP-CSFB but not the BBB, whereas T_3 and T_4 cross both BBB and CP-CSFB. [Reproduced with permission from L.Y. Cheng *et al.: J Neurophysiol* 72:380-391, 1994 (153).]

Analysis of tracer hormone kinetics showed that T_4 tissue content of liver and kidney was little affected, but was decreased in the brain. T_3 content of these tissues was normal. The low T_4 content of brain was explained on the basis of absence of TTR-T_4 complexes, apparently without repercussion for normal local T_3 production from T_4. These studies show that TTR is not essential for sufficient transport of thyroid hormones into brain and other organs. It seems that as long as the free hormone concentration is kept constant, probably by virtue of the presence of other thyroid hormone-binding proteins in blood and other body fluids, no apparent harm is done to tissue metabolism. In this respect, it is noteworthy that a similar situation exists in humans with complete TBG deficiency, who also show no apparent biological abnormality (159). However, it is remarkable that genetic abnormalities associated with complete TTR deficiency have so far not been documented in humans or animals.

B. Other organs

The liver is another organ that has been studied in animals for plasma membrane transport of thyroid hormone. Pardridge *et al.* published a series of *in vivo* studies in the rat (for review see Ref. 160). From their studies the authors concluded that thyroid hormone delivery to the liver "occurs via the free intermediate mechanism, *i.e.*, protein-bound hormone debinding is an obligatory intermediate step in the transport process". Although they found that transport of T_4 into rat brain via the BBB is a saturable process, they could not find saturability of plasma membrane transport in rat liver, and suggested that this occurred via passive diffusion. The authors used for their studies a single capillary pass technique for analysis of initial kinetics of transport (160). The model used by Pardridge *et al.* and their interpretation of the data were strongly contested (161,162). The main criticism concerned the rate-limiting role in the transport process that was attributed to the dissociation of hormone from serum binding proteins. No such role could be envisaged, both on theoretical and experimental basis, by these opponents. Others documented hepatic uptake in mice, injected *in vivo* with radioactive T_3, using autoradiography (163). Excess unlabeled T_3 resulted in 90% inhibition of liver uptake of labeled T_3. Time sequence autoradiographic analysis showed that the plasma membrane is initially labeled before internalization of T_3 occurs (163). These results clearly document *in vivo* specific binding of T_3 to the liver plasma membrane as an initial step to internalization of the hormone. *In vivo* injection of rats with radiolabeled T_4 and subsequent measurement of uptake in heart and lung tissue, isolated at different time intervals, showed that T_4 transport in these organs was also saturable, in accordance with a carrier-mediated transport mechanism (164).

In summary, brain entry of T_4 in dogs appears to proceed via a carrier-mediated mechanism. This was also found for brain uptake of T_3, but not of T_4, in the mouse. It was further shown in the rat that T_3 and T_4 mainly enter the brain via the blood-brain barrier for distribution throughout the brain, and via the CP-CSF barrier for restricted distribution in circumventricular areas. Although it has been envisaged for a long time that TTR expressed in the choroid plexus plays an essential role in the transport of thyroid hormones into the brain, total lack of the protein in TTR knock-out

mice has no effect on concentrations of plasma free thyroid hormones and TSH or on tissue thyroid hormone status. *In vivo* studies have shown saturable T_3 uptake into rat liver and saturable T_4 uptake into mouse lung and heart.

VII. PLASMA MEMBRANE TRANSPORT IN HUMANS

A. Introduction

In healthy individuals, about 80% of plasma T_3 is produced outside the thyroid gland, the remaining 20% being secreted directly by the thyroid (165). In the extra-thyroidal pathway, T_3 is produced by outer ring deiodination of T_4, and in this process the type I deiodinase in the liver (and kidneys) plays an important role (165,166). Another organ that may be involved in this pathway in humans is skeletal muscle, expressing the type II deiodinase that also catalyzes the conversion of T_4 to T_3 (167). To reach the intracellular T_3-producing enzymes, T_4 has to cross the plasma membrane of these tissues. It has been established in rats that the extents to which nuclear receptor-bound T_3 is derived from plasma T_3 and from local T_3 production from T_4 varies among the tissues. Thus, for instance, nuclear T_3 in cerebral cortex is derived for ≈80% from local conversion of T_4, in pituitary for ≈50%, in skeletal muscle for ≈40%, and in liver for only ≈5% (168,169). In other words, for exertion of biological activity by nuclear T_3, both T_4 and T_3 have to cross the plasma membrane of target cells. It follows that the activity of these transport processes may have an important influence on the regulation of the biological activity of thyroid hormone. Although the exact contribution of the different sources of nuclear T_3 in human tissues is unknown, it will also depend to varying degrees on plasma membrane transport of T_3 and its precursor T_4.

Many reports have dealt with the measurement of thyroid hormone distribution and metabolism in humans. However, few of these are concerned with analysis of unidirectional transport of thyroid hormones into tissues. To study regulation of biological processes, it is in general necessary to analyze these under circumstances of perturbation of the physiological steady state. This is certainly also true for the

study of the regulation of thyroid hormone transport into tissues. Both in starvation and in so-called nonthyroidal illness (see section VII.C), plasma T_3 production is decreased. As the diminution in plasma T_3 production may be substantial and thyroidal secretion of T_3 contributes only little to total plasma T_3, the main cause of this diminution in T_3 production must consequently be located in the extrathyroidal pathway. Both starvation and nonthyroidal illness, have been used as models to study regulation of thyroid hormone penetration into target tissues. Two possibilities have been suggested to be responsible for the lowered T_3 production in these situations, *i.e.*, a decrease in outer ring deiodinase activity in plasma T_3-producing tissues and/or a decrease of T_4 transport into these tissues as substrate for T_3 production. There is evidence in animals, but not in humans (170), that outer ring deiodination is indeed lowered in starvation and in nonthyroidal illness, but this aspect will not be further discussed here. For further orientation the reader is referred to Ref. 171. In this section we will discuss plasma membrane transport of thyroid hormones in human tissues both in starvation and in nonthyroidal illness.

B. In starvation

In caloric deprivation, like in nonthyroidal illness (see section VII.C), abnormalities in serum thyroid function parameters are invariably present. The most constant and thus characteristic abnormality is a low serum T_3 concentration; hence the term "low T_3 syndrome" for this entity. Serum T_4 and TSH are usually normal, whereas serum rT_3 is usually elevated (for a review see Ref. 172). To our knowledge the first published study that was primarily designed to evaluate unidirectional transport of thyroid hormones into tissues before and during caloric deprivation in man was published in 1986 by our laboratory (170). In this study T_4 and T_3 kinetics were studied using a three-pool model of thyroid hormone distribution and metabolism in 10 obese but otherwise healthy subjects before dieting and while on a 240 kcal diet. During caloric restriction, unidirectional transport of T_4 and T_3 into the rapidly equilibrating tissues (liver) was decreased by 50% and 25%, respectively, when corrected for changes in free hormone concentration. The decrease in plasma T_3 production amounted to 42%, about equaling the reduction in T_4 transport into the

liver. T_4 to T_3 conversion rate decreased by an insignificant 8%. Therefore, the lowered T_3 production during caloric deprivation is largely, if not fully, explained by a decrease of T_4 entry into T_3-producing tissues. The fasting-induced decrease in liver T_4 transport may be explained, at least in part, by a decrease in the energy charge of liver cells. This explanation is based on at least two points. First, it has been shown that starvation leads to ATP depletion of the liver as assessed by ^{31}P-magnetic resonance spectroscopy (173). Second, tissue T_4 transport was much more affected by caloric deprivation than transport of T_3, similar to findings of T_4 and T_3 transport in cultured rat hepatocytes deficient in ATP (Ref. 48 and Fig. 1). To further substantiate the effect of the intracellular ATP concentration on hepatic T_4 uptake *in vivo* in humans, liver T_4 uptake was measured in four healthy human volunteers, using T_4 tracer plasma kinetics, before and after an intravenous bolus injection of fructose, which is known to transiently decrease liver ATP levels. Obviously, hepatic ATP could not be measured, but fructose was found to induce an increase in serum lactic acid and uric acid concentrations, refecting a decrease in liver ATP. After fructose administration there was a temporary decrease in liver T_4 uptake that normalized after fructose was metabolized and hepatic ATP concentrations were restored, as reflected by the normalization of serum lactic acid and uric acid levels (134). In contrast to the transient effect of fructose, transport of T_4 into the liver remained suppressed when the same subjects were studied on a calorie-restricted diet (Fig. 4). As will be discussed in section VII.C, NEFAs that circulate in increased concentrations during caloric restriction have an additional inhibitory effect on T_4 uptake by the liver.

We also studied renal handling of T_4 and T_3 in humans during fasting (174). The results suggested inhibition of T_4 and T_3 uptake at the basolateral membrane of the tubular cells in the kidney. As to the cause of this inhibition, several factors were proposed, including a decreased energy state of the cells, the existing acidosis and/or inhibition of transport by the increased serum NEFA concentration.

C. In nonthyroidal illness

NTI may be defined as any acute or chronic illness, not related to the thyroid gland, that is accompanied by an abnormal pattern of thyroid function parameters. Other terms that are synonymously being used are the "low T_3 syndrome" because serum T_3 is invariably low in NTI, and the "euthyroid sick syndrome" because patients are usually clinically euthyroid despite the low serum T_3 and sometimes also low T_4 levels. With an increase in severity of disease there is a progressive decrease in serum T_3 and, in most diseases, an increase in serum rT_3 that eventually plateaus. Serum T_4 is usually normal but may be slightly increased in mild disease and lowered in critical illness (Fig. 5). Serum TSH is usually normal but may be depressed in severe illness (175,176). Many studies of thyroid hormone distribution and turnover kinetics in patients with NTI have been reported (for reviews see Ref. 171,175-177). In general, they show that T_4 production rates are normal, except in severe illness when it is decreased, but that T_4 transport into tissues is decreased.

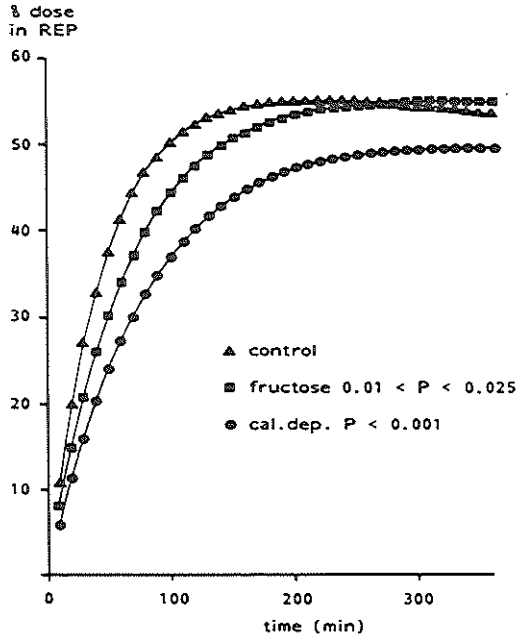

Fig. 4 Computed kinetics of T_4 uptake into the rapid equilibrating pool (REP, representing largely liver) in four obese volunteers, before (▲) and during (●) caloric deprivation and after intravenous fructose (■). [Redrawn with data from Refs. 134 and 170.]

Plasma rT_3 production, virtually all originating in type III deiodinase-containing tissues, such as brain (177), is normal in NTI, while the plasma rT_3 clearance, almost exclusively by the liver (178), is decreased. Plasma T_3 production rates are invariably decreased in proportion to the severity of disease, while plasma T_3 clearance is generally little affected (175). Few studies, mostly by Kaptein *et al.* (179-183), reported on the analysis of unidirectional T_4 transport into tissues during NTI to determine its possible contribution to low plasma T_3 production. Thus, in a group of 11 patients with acute critical illness, T_4 transport into tissues was inhibited by ≈50% and plasma T_3 production decreased by ≈70%. From this analysis it is not known to what extent inhibition of T_4 transport occurs in T_3-producing tissues, predominantly the liver (see section VII.A). In another study in 15 patients with NTI due to various causes (180), these authors found an inhibition of T_4 transport into the rapidly equilibrating pool (representing liver and kidneys) by ≈30% and into the slowly equilibrating pool (representing the remaining tissues) by ≈65%. Plasma T_3 production rates were not reported in this study. In patients with chronic renal failure, tissue transport of T_4 was inhibited by ≈50%, but no data were presented for T_3 production (181). In contrast to most patients with NTI, who show normal plasma rT_3 production but decreased plasma rT_3 clearance (see section VII.A) and thus elevated rT_3 plasma concentrations, this and other studies (for review see Refs. 182,183) demonstrate that patients with CRF have normal plasma rT_3 levels, clearance rates and production rates. The fact that plasma T_4 clearance is much more affected than that of T_3 is in agreement with similar findings in fasting humans (see section VII.B), and suggests that hepatic ATP depletion may also be important here, which does not seem illogical since NTI patients are mostly, if not always, in a negative energy balance.

We also considered the possibility of circulating inhibitors of thyroid hormone uptake in NTI. In the presence of serum from patients with severe NTI, T_4 uptake by rat hepatocytes was ≈50% lower than in the presence of serum from healthy controls, without any direct effect on the deiodination process (184).

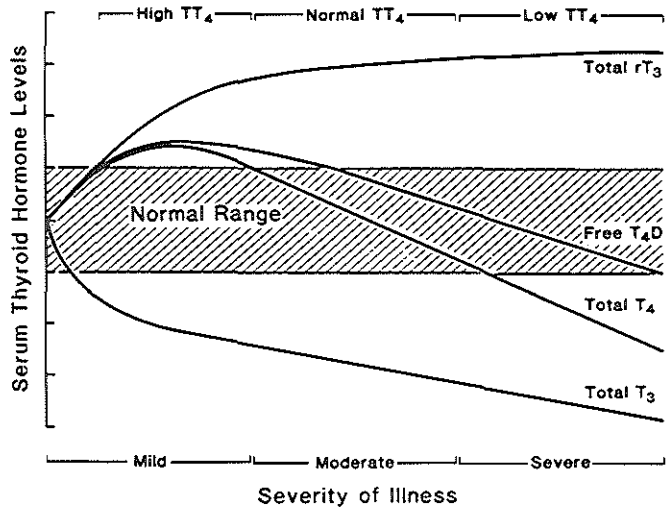

Fig. 5 Schematic representation of the changes in serum thyroid hormone levels in patients with nonthyroidal disorders relative to the severity of the illness. [Reproduced with permission from E.M. Kaptein. In: Thyroid hormone metabolism. New York: Marcel Dekker,1986 (175).]

Further characterization of the factors responsible for this inhibition identified several compounds circulating at increased serum concentrations in patients with NTI, including CMPF and indoxyl sulfate in patients with renal failure (108), and bilirubin and NEFAs in nonuremic critically ill patients (109). It also appeared that in mild NTI and during caloric restriction in obese subjects, serum NEFAs are increased to levels that inhibit hepatocyte uptake of T_4 (110). Remarkably, T_4 uptake in the rat pituitary is not inhibited by concentrations of CMPF, indoxyl sulfate, and bilirubin that inhibit T_4 uptake in hepatocytes (185,186). In addition, T_3 and T_4 uptake was normal in rat pituitary cells with low ATP concentration due to culture in an energy-poor medium. These phenomena indicate different effects of pathophysiological factors on the common pituitary transporter for both T_4 and T_3 (Table 3, Refs. 68-70) compared with the specific T_4 transporter in the liver. We hypothesized that this differential transport handling may serve to maintain low T_3 production in starvation and NTI, by allowing T_3, T_4 and the bioactive metabolites TRIAC and 3,5-diiodothyronine (187,188), which circulate at increased levels in NTI (189,190), and possibly also 3,3',5,5'-tetrathyroacetic acid (191,192), to enter the pituitary to prevent any compensatory

increase in TSH (193). As a low T_3 level is associated with conservation of energy and possibly also protein, it is considered by some as a defense mechanism in situations of stress. This point, however, is controversial as conflicting results have been obtained in studies of this protein-sparing effect. For further orientation about this subject, the reader is referred to Ref. 171.

In summary, most plasma T_3 is produced by conversion of T_4 in peripheral tissues, in particular the liver. Nuclear receptor-bound T_3 in different tissues is derived to varying extents from plasma T_3 or from local deiodinaton of T_4. Thus, the exertion of the biological activity of thyroid hormone requires the transport of T_4 and T_3 across the plasma membrane. Analyses of thyroid hormone kinetics in humans during caloric restriction revealed a 50% inhibition of hepatic T_4 transport, roughly equal to the 40% decrease in plasma T_3 production, whereas the T_4 to T_3 conversion in the liver was not affected. These findings suggest a rate-limiting role of hepatic T_4 transport for plasma T_3 production. The inhibition of T_4 transport was ascribed to hepatic ATP depletion by fasting. Liver ATP depletion by fructose infusion in humans indeed leads to a concomitant decrease of hepatic T_4 transport. In nonthyroidal illness, apart from a decrease of liver ATP, increased plasma concentrations of compounds such as CMPF, indoxyl sulfate, bilirubin, and NEFAs may inhibit T_4 transport into the human liver, thereby contributing to the low plasma T_3 production in this condition. NEFA concentrations are also elevated in starvation and may thus contribute to decreased hepatic T_4 uptake and T_3 production during caloric deprivation.

VIII. REQUIREMENTS FOR A REGULATORY ROLE OF PLASMA MEMBRANE TRANSPORT IN THE BIOAVAILABILITY OF THYROID HORMONE

Although it has been amply discussed in the previous sections that in most, if not all, cells thyroid hormones cross the plasma membrane by a carrier-mediated (often energy-dependent) mechanism, its significance for the regulation of the bioavailability of thyroid hormone has not yet been addressed. This will be done in the following sections.

Certain requirements have to be fulfilled before it can be concluded that the process of transport across the plasma membrane of target cells is potentially regulatory for the bioavailability of thyroid hormone and thus may have a role in the regulation of thyroid hormone bioactivity. These requirements are depicted in Table 5 and are discussed below.

A. Specificity of plasma membrane transport

Specificity of transport indicates that only structurally related substances are being transported or compete with the transport system. These systems are saturable and usually have limited capacity. Specificity of thyroid hormone transport into target cells has been substantiated for many cell types from many species as discussed in the different sections above. In some, but not all, cell types two systems have been detected for uptake of iodothyronines (Tables 1-3). If two systems were identified, the high-K_m site was attributed to binding of thyroid hormone to protein trapped in the water layer around the cell or associated with the cell surface (45). There is little doubt that in most cell types stereospecific transport of thyroid hormone across the plasma membrane occurs. The reported K_m values of transport varied but were mostly in the nanomolar range (Tables 2 and 3). The use of different conditions and techniques as well as the tissue-specific distribution of different transporters (see section IX) may account for this variation. A point of apparent discrepancy is the fact that some laboratories could not identify a specific T_4 transport system whereas others could. This fact is probably related to the phenomenon that T_4 transport into cells, at least into the hepatocyte, is much more sensitive to suboptimal cellular ATP concentrations than T_3 transport (Ref. 48, section III.B.2 and Fig 1). When studies of T_4 transport are not focused on this aspect (92,93), T_4 transport may become undetectable.

Table 5. Characteristics of plasma membrane transport of thyroid hormone required for its potential function in the regulation of thyroid hormone bioavailability

1. Specificity of plasma membrane transport
2. Absence of significant diffusion
3. Plasma membrane transport is subject to regulation
4. Transport is rate limiting for subsequent metabolism

B. Absence of significant diffusion

If a significant proportion of thyroid hormone transport across the plasma membrane would take place by diffusion, it is obvious that this would diminish the role of the plasma membrane in the regulation of hormone uptake. There is substantial evidence, on both theoretical and experimental grounds, that little or no diffusion occurs in the transport process. Thus, although overall iodothyronines are lipophilic compounds, the highly polar zwitter-ionic nature of the alanine side chain prevents passage of the molecule through the hydrophobic inner core of the lipid bilayer of the plasma membrane. Experimental evidence has also been provided that diffusion hardly takes place if at all. Thus, using an electron spin resonance stop-flow technique, it was shown that a spin-label derivative of T_3 does not flip-flop at any appreciable rate in phospholipid bilayers and that, after partitioning into the membrane, it remains in the outer half of the bilayer (194). In other words, if no specific transport sites were present in the membrane of target cells, thyroid hormones would not be able to cross the plasma membrane. Using a monoclonal antibody raised against a rat hepatocyte surface epitope involved in thyroid hormone transport, a concentration-dependent inhibition of the transport of T_3 and T_4 was observed, with 100% inhibition at a low (1:100) antiserum dilution (40). The same monoclonal antibody also strongly inhibited uptake of T_4, T_3, and rT_3 in cultured human hepatocytes (52). In rat anterior pituitary cells and also in *Xenopus laevis* oocytes, minimal, if any, uptake of T_3 sulfate (T_3S) was detected, in contrast to specific uptake of T_4 and T_3 in these cell types (195,196). However, injection of rat liver mRNA induced uptake of T_3S in these oocytes (Ref. 197 and Fig. 6). These

observations indicate that diffusion plays no role in transmembrane transport of sulfated iodothyronines.

C. Plasma membrane transport is subject to regulation

As the serum concentrations of free T_3 and free T_4 are in the picomolar range, whereas their apparent K_m values for the plasma membrane transporters are in the nanomolar range, no regulation of transport occurs by the process of saturation. However, as pointed out above (see sections III, V, and VII) thyroid hormone transport into cells, except maybe for erythrocytes, is dependent on the energy state of the cell and often on the Na^+ gradient over the plasma membrane. Thus, cellular ATP and the Na^+ gradient may be important factors in the regulation of the activity of thyroid hormone transporters (Tables 2 and 3), while thyroid hormone uptake will also depend on the number of transporters located in the cell membrane. The latter is determined not only by the balance between the rates of synthesis and degradation of these proteins but also by mechanisms regulating their translocation between intracellular organelles and the plasma membrane.

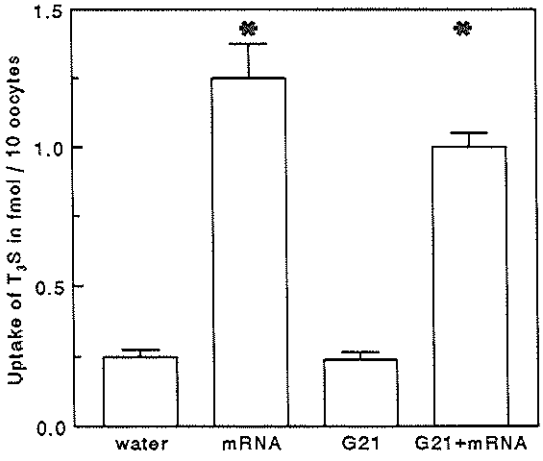

Fig. 6 Initial uptake of T_3S in *X. laevis* oocytes injected with water (control), fractionated rat liver mRNA, cRNA for rat liver type 1 deiodinase (G21), or both (G21 + mRNA). Values are means ± SEM; *, $P < 0.001$ *vs.* water. [Modified reproduction with permission from R. Docter *et al.*: *Endocrinology* 138:1841-1846, 1997 (197). © The Endocrine Society.]

Circulating inhibitors such as CMPF, indoxyl sulfate, bilirubin, NEFAs, and amino acids (Refs. 108-110 and section III.C.1) are also involved in the regulation of thyroid hormone uptake, especially in starvation and nonthyroidal illness. However, in tissues in which thyroid hormone is taken up by amino acid transporters that mediate exchange between extra- and intracellular ligands, hormone uptake is subject not only to *cis*-inhibition by extracellular amino acids but also to *trans*-stimulation by intracellular amino acids.

The possible effects of thyroid state on the rate of thyroid hormone uptake has been studied in rat liver. When livers of hypothyroid rats were perfused, uptake of T_3 was not different from normal, but T_3 metabolism was decreased. In livers of hyperthyroid rats, uptake of T_3 was decreased and T_3 metabolism was increased. These data suggest an adaptation mechanism at the cellular level to maintain tissue T_3 levels when T_3 supply is abnormal (198). When expression of mRNA of thyroid hormone transporters in rat liver was studied, using *Xenopus laevis* oocytes as expression system, no thyroid state-dependent differences were seen in the expression of these transporters, not excluding, however, any regulation of transporter activity at the translational or post-translational level (199).

Thus, although questions remain, a number of factors, both intracellular and circulating, have been identified that determine the amount of thyroid hormone taken up by target cells.

D. Transport is rate limiting for subsequent metabolism

Plasma membrane transport is rate limiting for cellular thyroid hormone metabolism if any change in transport results in proportional alterations in subsequent metabolism. This implies that influx of thyroid hormones is independent of intracellular metabolic capacity. When rat hepatocytes in primary culture were incubated with T_4, T_3, or rT_3 in the presence of an iodothyronine transport-blocking monoclonal antibody or ouabain to lower the Na^+ gradient over the plasma membrane, a decreased clearance from the medium of these iodothyronines was found that paralleled a decreased iodide production (Table 6). As it was shown that the added compounds had no effect on intracellular deiodinase activity, it was

concluded that the decreased iodide production was caused by the inhibition of iodothyronine uptake (50). In addition it was reported from different laboratories that compounds that inhibit T_3 uptake at the plasma membrane level, and do not influence nuclear binding of T_3 *per se*, effected a decrease in nuclear occupancy that paralleled the inhibition of uptake, indicating that cellular uptake controls T_3 access to its receptors (77,122,200).

Table 6. Remaining iodothyronine and iodide released in medium after incubation of rat hepatocytes in monolayer culture with T_4, T_3, or rT_3 in the absence (control) or presence of uptake inhibitors ER-22 (monoclonal antibody) and ouabain

	Percentage (mean ± SEM)	
	Iodothyronine	Iodide
T_4		
Control	82.9±0.8	12.7±0.4
ER-22	90.7±1.2[a]	6.9±0.6[a]
Ouabain	92.2±1.2[a]	7.0±0.6[a]
T_3		
Control	32.0±1.4	51.5±0.6
ER-22	64.8±1.4[a]	24.6±0.6[a]
Ouabain	66.2±1.4[a]	21.5±0.6[a]
rT_3		
Control	45.8±0.9	54.1±0.5
ER-22	62.8±1.1[a]	36.9±0.7[a]
Ouabain	56.8±1.6[a]	41.0±0.9[a]

[a] Significantly different from control, $P<0.001$. [Reproduced with permission from G. Hennemann *et al.*: *Endocrinology* 119:1870-1872, 1986 (50). © The Endocrine Society.]

These findings were obtained using rat pituitary tumor cells, hepatocytes, and skeletal myoblasts. Furthermore, uptake of T_3S induced in *X. laevis* oocytes by injection of fractionated rat liver mRNA was not affected by coinjection with cRNA coding for type I deiodinase. Thus, an increase in the capacity of oocytes to

metabolize T_3S did not affect T_3S uptake (Fig. 6 and Ref. 197). Obviously, the rate of T_3S metabolism was stimulated by both induction of T_3S transport and induction of deiodinase activity. A remarkable finding was reported by our laboratory in support of the clinical relevance of inhibited hepatic T_4 transport as a cause for a decrease in T_3 production (58,201). When rat hepatocytes in primary culture were incubated with T_4 in the presence of serum from patients with NTI, a strong correlation ($r = 0.69$) was observed between residual transport of T_4 into the hepatocytes and the serum T_3 concentration in these subjects (Fig. 7). In other words, the more inhibition of T_4 transport exerted by the serum, the lower the serum T_3 concentration of that particular patient.

There is evidence that *in vivo* inhibition of T_4 transport into the liver is also rate limiting for total plasma T_3 production in humans. In a female in her 60s, an increased serum free T_4 concentration was present in combination with a low plasma T_3 concentration in the absence of NTI or any abnormality of serum thyroid hormone-binding proteins (202). Iodothyronine kinetic studies revealed that T_4 uptake (and content) in the rapidly equilibrating compartment, comprising mainly the liver (and kidneys), was inhibited, but uptake in the slowly equilibrating compartment, consisting of the other tissues, was normal (Fig. 8). T_3 uptake was normal in both compartments. Plasma T_3 production was subnormal, but the ratio of T_3 production over hepatic T_4 uptake or T_4 content was normal. It was concluded from these data that the lowered plasma T_3 production was caused by inhibition of T_4 uptake into the liver, leading to a decrease in substrate available for conversion to T_3, whereas the liver capacity to produce and secrete T_3 was unimpaired (202). We have identified this abnormal serum thyroid hormone profile also in another subject (203). In this latter subject, serum TBG was elevated and normalized upon administration of physiological amounts of T_3. As TBG may be elevated in hypothyroidism, this suggests that the lowered T_3 production caused hypothyroidism at the level of the liver. These human studies suggest that inhibition of T_4 transport into the liver, leading to lowered T_3 production, has biological consequences.

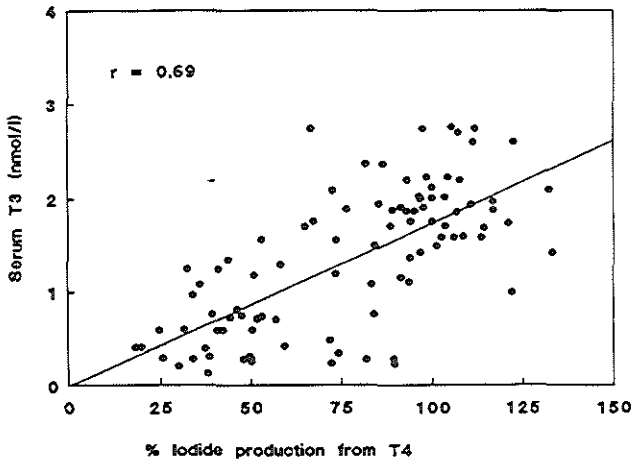

Fig. 7 Relationship between iodide production from T_4 (corrected for differences in free hormone concentration) in the presence of 10% NTI serum, expressed as percentage of iodide production in the presence of 10% serum of healthy controls and serum T_3. [Reproduced with permission from R.A. Vos *et al.: J Clin Endocrinol Metab* 80:2364-2370, 1995 (58). © The Endocrine Society.]

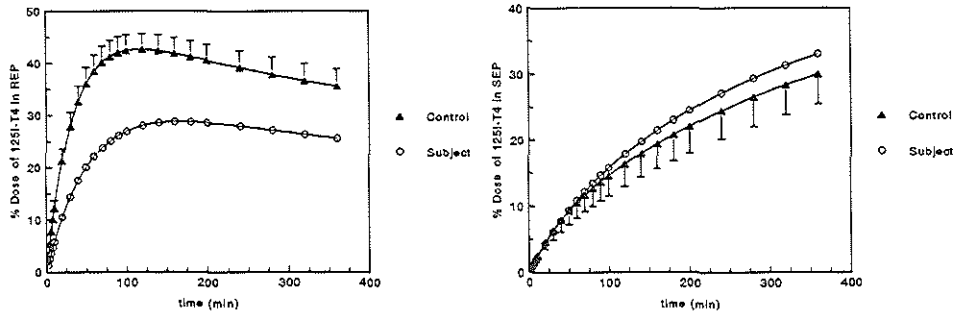

Fig. 8 T_4 uptake into (a) the rapidly equilibrating pool (REP) and (b) the slowly equilibrating pool (SEP) of a subject with reduced peripheral T_3 production (○) and control subjects (▲) during the first 400 min of T_4 tracer kinetics. Values are the mean ± SEM. [Reproduced with permission from G. Hennemann *et al.: J Clin Endocrinol Metab* 77:1431-1435, 1993 (202). © The Endocrine Society.]

In summary, to play an important role in the regulation of tissue thyroid hormone bioavailability, the mechanism of transport of thyroid hormone over the plasma membrane must fulfill certain requirements (Table 5). Thus, plasma membrane transport should be specific, subject to regulation, and rate limiting for subsequent thyroid hormone metabolism. This implies that there is only limited or no diffusion into target cells such that influx of hormone is largely effected by specific transporters. Collectively, the studies discussed in this section have demonstrated that this is indeed the case in liver and many other tissues. Hepatic uptake of thyroid hormone is regulated by the energy charge of the cells, and also by compounds that circulate at increased levels in humans during starvation (NEFAs) and nonthyroidal illness (NEFAs, CMPF, indoxyl sulfate, and bilirubin). The reduced T_4 transport into the liver is a major cause for the decreased plasma T_3 production in these conditions.

IX. IDENTIFICATION OF THYROID HORMONE TRANSPORTERS

A. Organic anion transporters

Recently, we have explored the possibility to clone iodothyronine transporters from rat liver using *X. laevis* oocytes as an expression system (197,204-207). A modest increase in T_4 and T_3 uptake was induced by injection of oocytes with rat liver mRNA, in particular the 0.8-2.1 kb size fraction, above the background iodothyronine uptake by native oocytes (197). Much lower background uptake was observed with the sulfonated iodothyronine derivatives, T_3 sulfate (T_3S), T_4 sulfate (T_4S), T_3 sulfamate (T_3NS) and T_4 sulfamate (T_4NS), resulting in much larger relative inductions by injection with rat liver mRNA (197,204). Uptake of these water-soluble derivatives was competitively inhibited by T_4 and T_3, suggesting that they are alternative ligands for the iodothyronine transporters (197,204). Since the sulfonated compounds are organic anions, we tested the hypothesis that hepatic uptake of iodothyronine derivatives is mediated, at least in part, by organic anion transporters, in particular Na^+/taurocholate cotransporting polypeptide (NTCP) and the (Na^+-independent) organic anion transporting polypeptides (OATPs) (208,209).

Human and rat NTCP are 349- to 362-amino acid proteins containing 7 putative transmembrane domains and 2 glycosylation sites with an apparent molecular mass of ≈50 kDa (208-211). This transporter is now also known as solute carrier family 10, member 1 (SLC10A1). NTCP is only expressed in hepatocytes, where it is localized selectively to the basolateral cell membrane (208,209). It is the major transporter of conjugated bile acids in liver, but it also mediates uptake of unconjugated bile acids and a number of non-bile acid amphipathic compounds, including estrogen conjugates such as estrone 3-sulfate (208,209). A homologous bile acid transporter is expressed in ileum and kidney, where it is localized to the apical cell membrane (212-215). The OATPs constitute a large family of homologous Na^+-independent transporters, which are now also comprised in the solute carrier family 10 (SLC10). Seven members of this family have been identified in rats, *i.e.*, *r*OATP1-5 (216-221), *r*OAT-K1 (222), and splice variant *r*OAT-K2 (222,223), and the prostaglandin transporter *r*PGT (224); eight members in humans, *i.e.*, *h*OATP-A to -F (225-230), *h*OATP8 (231), and *h*PGT (232); and two members in mice, *i.e.*, *m*OATP1 (233,234) and *m*PGT (235). *r*OATP1 was the first identified member of this transporter family, representing a 670-amino acid protein with 12 transmembrane domains and 2 glycosylation sites with an apparent molecular mass of 80 kDa (208,209). The other OATP transporters have similar structures. The tissue distribution of the OATPs varies among the different members, *e.g.*, *r*OATP1 and *r*OATP2 are expressed in liver, kidney and brain, *r*OATP4 and *h*OATP-C (*alias h*LST-1, liver-specific transporter) are expressed exclusively in liver, and *r*OAT-K1 and -K2 are expressed selectively in kidney. Like NTCP, the OATPs expressed in liver are localized to the basolateral cell membrane. It is interesting to note that in brain both *r*OATP1 and *r*OATP2 show prominent localization in the choroid plexus, which may be an important gate of thyroid hormone to the brain (209). The OATPs are multispecific transporters, mediating the uptake of a wide variety of amphipathic ligands, not only anionic (*e.g.*, conjugated and unconjugated bile acids, conjugated steroids, bromosulfophthalein), but also neutral (*e.g.*, steroids, cardiac glycosides), and even cationic (*e.g.*, ajmalinium) compounds (208,209). For different OATPs, it has been demonstrated that they faciltate the exchange of intra- and extracellular anions (236,237). Intracellular reduced glutathione (GSH) is an important intracellular ligand, the efflux of which down its large electrochemical gradient provides the driving force

for uptake of extracellular ligands (236). Figure 9 shows the phylogenetic tree of the OATP transporter family.

We observed marked stimulation of the uptake of native iodothyronines as well as their sulfamate and sulfate derivatives after injection of oocytes with cRNA for *r*NTCP, *h*NTCP, *r*OATP1, *r*OATP2, or *h*OATP-A (206,207). The Na^+ dependence of the NTCPs and the Na^+ independence of the OATPs were confirmed with all these ligands. Significant transport of T_4 and T_3 has also been reported by others for *r*OATP2 (218), *r*OATP3 (218), and *r*OATP4 (219), but not its splice variant *r*LST-1 (220), for *h*OATP-C, *alias h*LST-1 (227,228), and human and rat *h*OATP-E (238). The degree of stimulation of iodothyronine uptake varied among the different OATP family members, *e.g.*, *r*OATP1 showed highest iodothyronine transport with T_4 and rT_3, and *h*OATP-A with T_3 as ligand (207). Apparent K_m values were determined for T_4 and T_3 transport by *r*OATP2, *r*OATP3, and *h*OATP-C, and found to be in the micromolar range (218,227). Together, these data suggest that tissue uptake of thyroid hormone may be mediated in part by different Na^+-dependent and Na^+-independent organic anion transporters, although the NTCPs and OATPs do not represent the high-affinity iodothyronine transporters detected in different tissues. Studies of the induction of iodothyronine transport by injection of *Xenopus* oocytes with liver mRNA size fractions have indicated the existence of a major Na^+-dependent transporter in addition to *r*NTCP and *r*OATP1 (204,207).

B. Amino acid transporters

A large number of amino acid transporters has been characterized in recent years, including the 4F2-related heterodimeric transporters (239,240). The 4F2 or CD98 cell surface antigen is expressed in many tissues, especially on activated lymphocytes and tumor cells, and has recently been identified as a family of amino acid transporters (239,240). These transporters are now comprised in the solute carrier family 7 (SLC7). These heterodimeric transporters consist of a common 4F2 heavy chain (4F2hc) linked through a disulfide bond to one member of a family of homologous light chains, seven of which have now been cloned (239-262). 4F2hc is a glycosylated protein with a single transmembrane domain, whereas the light chains are not glycosylated and have 12

transmembrane domains (239,240). However, most investigators agree that one of the light chains ($b^{0,+}AT$) dimerizes preferentially with rBAT (for 'related to basic amino acid transport'), another heavy chain homologous to 4F2hc (256-259). Cystine is an important ligand for the rBAT/$b^{0,+}AT$ transporter (Table 7), and mutations in the rBAT heavy chain have been identified in patients with type I cystinuria (263), while mutations in the $b^{0,+}AT$ light chain have been found in patients with non-type I cystinuria (256-258). The characteristics of the different heterodimeric amino acid transporters are summarized in Table 7. The several 4F2 and rBAT-related heterodimeric transporters facilitate exchange of extra- and intracellular amino acids (239,240).

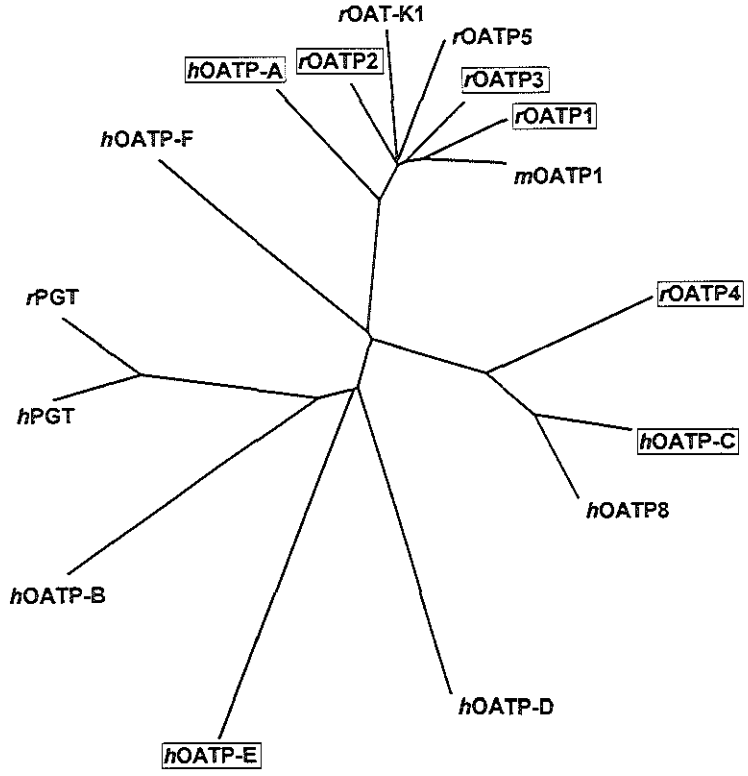

Fig. 9. Phylogenetic tree of the family of human, rat and mouse OATP organic anion transporters, based on the alignment of the amino acid sequences using the ClustalW program (http://www.ebi.ac.uk), and constructed using the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). *Boxed* transporters have been shown to transport iodothyronines.

We have studied possible transport of iodothyronines (T_4, T_3, rT_3, and 3,3'-T_2) by four heterodimeric amino acid transporters, consisting of *h*4F2hc and either *h*LAT1, *m*LAT2, *hy*$^+$LAT1 or *hy*$^+$LAT2 in *Xenopus* oocytes (264). The LAT1 and LAT2-containing heterodimers represent isoforms of the system L amino acid transporters, which mediate the Na^+-independent uptake of neutral amino acids. The 4F2hc/LAT1 transporter shows preference for large neutral (branched chain and aromatic) amino acids such as Leu, Tyr, Trp, and Phe, whereas 4F2hc/LAT2 also transports small neutral amino acids such as Gly, Ala, Ser, and Thr (241-250). The heterodimers containing the y$^+$LAT1 or y$^+$LAT2 light chains mediate the Na^+-dependent transport of neutral amino acids such as Leu as well as the Na^+-independent transport of basic amino acids such as Arg, which is characteristic of the system y$^+$L amino acid transporters (251-255).

Iodothyronine uptake in *Xenopus* oocytes was not affected by coexpression of 4F2hc and either y$^+$LAT1 or y$^+$LAT2, although the Na^+-dependent transport of Leu, Phe and Tyr, and the Na^+-independent uptake of Arg were markedly increased (264). This indicates that thyroid hormone transport is not mediated by 4F2-related, system y$^+$L amino acid transporters. However, coinjection of oocytes with cRNA for both 4F2hc and LAT1, but not for each subunit alone, resulted in marked increases in (Na^+-independent) uptake of the system L ligands Leu, Phe, Tyr and Trp, and of the different iodothyronines. At subsaturating ligand concentrations, the rate of iodothyronine uptake by the h4F2hc/hLAT1 transporter decreased in the order 3,3'-T_2>T_3~rT_3>T_4. Apparent Km values were found to be in the micromolar range, being lowest for T_3 (1.5 µM), which is the lowest value reported for a ligand of the *h*4F2hc/*h*LAT1 transporter (241-245). Apparent K_m (>10 µM) were highest for rT_3, but V_{max} values were highest for 3,3'-T_2 (264). Significant but smaller increases in uptake of the different iodothyronines was observed in oocytes coexpressing 4F2hc and LAT2 (264). In addition, Ritchie *et al.* (265) have reported on the stimulation of T_3 transport in oocytes injected with cRNA for 4F2hc and for the IU12 *Xenopus* LAT1 homolog. These results, therefore, strongly confirm previous findings suggesting that thyroid hormone uptake in different cell systems is mediated by L type amino acid transporters (see section III.C). However, the T type amino acid transporter thought to be involved in the uptake of thyroid hormone in erythrocytes (94) has yet to be characterized.

Table 7. Characteristics of heterodimeric amino acid transporters

Light chain	Heavy chain	Amino acids transported	Localization	Refs.
LAT1	4F2hc	Large neutral (Na^+-independent) *e.g.*, Leu, Phe, Tyr iodothyronines	*e.g.*, brain, spleen, testis placenta, stomach, skeletal muscle	241-245
LAT2	4F2hc	Broad, neutral (Na^+-independent) iodothyronines	*e.g.*, kidney, intestine, placenta, brain, liver skeletal muscle	246-250
y^+LAT1	4F2hc	Basic (Na^+-independent), *e.g.*, Arg, Lys, and neutral (Na^+-dependent), *e.g.*, Leu	*e.g.*, kidney, intestine	251-254
y^+LAT2	4F2hc	Basic (Na^+-independent), *e.g.*, Arg, Lys, and neutral (Na^+-dependent), *e.g.*, Leu	*e.g.*, brain, intestine heart, kidney, testis	252,254,255
$b^{0,+}$AT	rBAT	Broad, basic and neutral (Na^+-independent), *e.g.*, Lys, Arg, cystine, Leu	*e.g.*, kidney, intestine	256-259
xCT	4F2hc	Cystine, Asp, Glu	macrophage, brain	260
Asc-1	4F2hc	Small neutral amino acids *e.g.*, Gly, Ala, Ser, Thr, Cys	*e.g.*, brain, placenta, kidney, skeletal muscle, heart	261,262

In contrast to the ubiquitous expression of the 4F2hc, the LAT1 and, in particular, LAT2 light chains show restricted tissue distributions (239-250). This suggests the existence of additional light chains involved in the uptake of aromatic amino acids and iodothyronines in tissues that do not express LAT1 or LAT2, one of which may be the subunit for the system T transporter. It has not been tested whether iodothyronines are transported by the rBAT/$b^{0,+}$AT heterodimeric transporter. Perhaps, other light chains combine with rBAT and mediate transport of iodothyronines. Iodothyronines may also be ligands for completely different classes of neutral (aromatic) amino acid transporters, such as the recently cloned Na^+-dependent $B^{0,+}$ transporter (266).

In summary, recent studies have identified plasma membrane transporters that are capable of mediating cellular uptake of thyroid hormone. These include 1)

the rat and human Na^+-dependent organic anion transporter (NTCP), which is expressed exclusively in the basolateral liver cell membrane, 2) different members of the rat and human Na^+-independent organic anion transporter (OATP) families, which show different tissue distributions, and 3) the L type heterodimeric amino acid transporters, comprised of the human 4F2 heavy chain and the LAT1 or LAT2 light chains, which are expressed in different, largely extrahepatic tissues. The physiological relevance of these transporters for tissue thyroid hormone uptake, however, remains to be established.

X. SUMMARY AND CONCLUSIONS

There is little doubt that thyroid hormones and their analogs are transported into target cells via plasma membrane carriers. Although variations exist in reported K_m values, explained in part by differences in laboratory techniques and conditions, but also by different tissue distribution of the various transporters, it seems that the mechanism of saturation does not play a role in the regulation of thyroid hormone access to cells. Most laboratories report apparent K_m values in the nanomolar range (Tables 1-3) that are 3 orders of magnitude higher than serum free hormone concentrations. However, other factors have been identified that are involved in regulating thyroid hormone cellular uptake. Cellular factors include the energy charge, in particular cellular ATP concentrations, the number of carriers per cell, and the Na^+ gradient over the plasma membrane. Extracellular factors comprise the free hormone concentration, and possibly competition by circulating amino acids. Several groups of amino acids were shown to inhibit thyroid hormone transport at physiological serum concentrations. Also substances circulating in increased concentrations in NTI and starvation, such as CMPF, indoxyl sulfate, bilirubin, and NEFAs, and several drugs may influence thyroid hormone tissue uptake.

Strong evidence exists that plasma membrane transport of thyroid hormone is rate limiting for subsequent thyroid hormone metabolism. As in man about 80% of plasma T_3 is produced outside the thyroid gland from T_4 in plasma T_3 producing tissues, regulation of uptake of T_4 in these tissues is potentially determinant for

overall plasma T_3 production and thus exertion of thyroid hormone activity at the tissue level. This process probably plays a major role in the lowered T_3 production in NTI and starvation in man, in contrast to the situation in the rat, where a diminished T_4 production plays an important if not major role in the cause of the low T_3 syndrome (267,268). The contributions of plasma derived T_3 and of local T_3 production from T_4 differs between tissues. Thus, not only regulation of T_4 uptake but also of T_3 uptake at the level of the plasma membrane is important for overall regulation of thyroid hormone bioactivity. Plasma membrane carriers for thyroid hormone may be different in different organs. For instance, in the liver there are probably different carriers for T_3, T_4, and rT_3, whereas in the pituitary only one transport mechanism has been identified for both T_3 and T_4. Transport mechanisms may also differ in various tissues and species with regard to Na^+ dependence and maybe other, as yet unidentified, factors. Few publications deal with cellular efflux of thyroid hormone. When tested, T_3 efflux is found to be a saturable process, albeit at supra-physiological hormone levels. Efflux of thyroid hormone, even if carrier mediated, seems to be independent of the energy charge of the cell. This suggests that carrier mediated efflux of thyroid hormone does not play a major role in the regulation of the cellular free hormone concentration.

A very recent development is the identification of different thyroid hormone transporters belonging to different families. This field is developing rapidly; nonetheless information in the following areas is insufficient: 1) how and to what extent these transporters compete for thyroid hormone transport; 2) how they are distributed over the different tissues; and 3) in what way other ligands for these transporters interact with thyroid hormone transport into tissues. Insufficient information is also available about the rank order of physiological importance of the different transporters. Once more, knowledge has been accumulated about this aspect, but studies must be done on the regulation at the molecular level of the activity of physiologically important thyroid hormone transporters and the mechanisms by which they regulate bioavailability of thyroid hormone.

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Chapter 8

GENERAL DISCUSSION

DISCUSSION

In this thesis, studies on the characterization of thyroid hormone transporters are described. Previous studies in our laboratory using rat hepatocytes have demonstrated that uptake of iodothyronines proceeds via plasma membrane transporters. This is likely to be an active mechanism mediated by one or more transporters because iodothyronine uptake is dependent on temperature, the intracellular ATP concentration, and the Na^+ gradient over the cell membrane (see Chapter 7 for a review). Sofar, the molecular structure of the responsible proteins is unknown. Therefore, a major challenge is to identify the putative thyroid hormone transporters (THTs) and to characterize the transport process in molecular biological terms. While we have focussed our studies on the characterization of thyroid hormone transport in the liver, there is evidence that the expression of THTs is regulated in a tissue-specific manner (see Chapter 7 for a review). If this is caused by, for example, expression of different THT genes or by splice variants and / or tissue specific expression of the same THT gene, is not known. Such studies can only be done once the THTs have been identified.

Cloning approaches of transporters

Several approaches have been described in the past decades to isolate putative transport proteins. These include:

1. *Expression cloning*

   In the introduction, functional expression cloning in *Xenopus laevis* oocytes has already been described as the most successful method for the cloning and characterization of plasma membrane transporters. It is also possible to use a bacteria (*e.g.*, *Escherichia coli*), yeast (*e.g.*, *Saccharomyces cerevisiae*), or mammalian cells for this purpose. However, *E. coli*, for example, is a prokaryotic cell system that can not recognize the signal peptide that has to guide the eukaryotic transmembrane proteins into the cell membrane. The disadvantage of yeast cells is that they have such an impermeable cell wall that it is difficult to

introduce foreign DNA into the cells and to study transport through the cell membrane. Because of their low transfection efficiency mammalian cells are not commonly used for cloning purposes. Only once a new transporter has been cloned, these cells can be transfected with the cDNA of interest to test functional expression in a more *in vivo* related system than *X. laevis* oocytes.

2. *Linkage analysis*

This approach is based on the availability of human pedigrees in which a disease, due to defects in transport mechanisms, is segregating according to Mendelian laws. The linkage method to isolate such disease proteins is based on co-segregation of DNA markers (with a known chromosomal location) with the disease gene. When this co-segregation is observed, the chromosomal area can be investigated for presence of "candidate genes", mutations in which explain the disease. Examples of such diseases where this approach was successfully applied, include cystic fibrosis (CF) (1) and Pendred syndrome (PDS) (2). The CF gene codes for a transmembrane conductance regulator channel (CFTR) containing 12 putative membrane-spanning regions which presumable form a transmembrane pore for transport of chloride (Cl^-). Mutations in this CFTR protein lead to reduced Cl^- permeability, which impairs fluid and electrolyte secretion and results in luminal dehydration (3). The PDS gene codes for pendrin, a protein expressed mainly in the thyroid containing 12 putative transmembrane-spanning domains and which was shown to transport Cl^- and I^- (4,5). The Pendred syndrome is an autosomal recessive disease and mutations in the PDS gene lead to congenital sensorineural deafness and thyroid goitre (6). The molecular basis of the thyroidal defect and the link with abnormal cochlea function is unknown.

However, the availability of families in which a "transport disease" is segregating, is crucial for this approach to be successful. For thyroid hormone transport currently any such (large) pedigree with multiple patients whose disease can be attributed unambiguously to defects in thyroid hormone transport is unknown. Only two individual patients have been identified in our hospital that could have a THT defect (7). Other researchers have suggested that a patient of them, clinically euthyroid despite an elevated serum T_4 concentrations, has a

defect in the type I iodothyronine deiodinase (D1) However, further investigations revealed no mutations in the D1 gene and they concluded that this patient could also have an impaired transport of T_4 and rT_3 into liver and kidney cells (8,9).

3. *Homology screening*

Once approach 1 or 2 have led to the identification of a transport protein, knowledge of the gene sequence and amino acid sequence can be used to find additional homologous genes and proteins, also in other species. Such homologous proteins can then be tested in expression systems such as *X. laevis* oocytes for their functionality. This approach, homology screening, has been widely used, for example, to isolate the human organic anion transporting polypeptide (OATP) (10), the human Na^+ /taurocholate cotransporting polypeptide (NTCP) (11), and the human Na^+/I^- symporter (NIS) (12), etc. Transporters can also be identified on the basis of homology between ligands. For example, thyroid hormone is similar in chemical structure to the amino acid tyrosine, while the thyroid hormone sulfamate derivatives are similar in chemical behavior to organic anions. Thus, these features can be used to look for thyroid hormone transport activity based on homology with the tyrosine amino acid transporter and/or with the organic anion transporters, both of which have already been cloned.

The *X. laevis* expression system for cloning THTs

a) Optimization of signal:noise ratio

As expression in *X. laevis* oocytes has been the most successful method for the cloning and characterization of plasma membrane transporters, we have first tested the suitability of *X. laevis* oocytes for the cloning and characterization of thyroid hormone transporters from rat liver. Injection of rat liver mRNA into *X. laevis* oocytes has resulted in only a modest increase in T_3 uptake. Due to the relatively high iodothyronine transport activity of uninjected oocytes, transport of T_4 was not induced significantly after injection of rat liver mRNA. To exclude binding of thyroid hormone to the cell membrane of the oocyte, we measured metabolism of thyroid

hormone by the oocytes. However, iodothyronines are not metabolized by native oocytes, except for rT_3. In contrast to the substrate preference of rat and human cytoplasmic sulfotransferases, oocytes rapidly sulfate rT_3 instead of 3,3'-T_2. Thyroid hormone sulfation has also been studied in oocytes of the axolotl *Ambystoma mexicanum*, a neotenous amphibian (13). Preliminary results suggest that the sulfotransferases present in those oocytes also show a substrate preference for rT_3. As oocytes do not express iodothyronine deiodinases (14), we coinjected cRNA coding for rat type I iodothyronine deiodinase (D1) together with rat liver mRNA. T_3 is a poor substrate for D1, but T_3 sulfate (T_3S) is rapidly deiodinated by D1, initially in the inner ring and subsequently in the outer ring to release radioactive iodide. The finding that the deiodination of T_3S was highest in oocytes injected with cRNA coding for D1 together with rat liver mRNA indicates that T_3S has been transported to the interior of the cell in order to be metabolized by D1. This means that thyroid hormone transporters are present in rat liver and that functional expression of these membrane transporters is possible in *X. laevis* oocytes.

Endogenous uptake of the iodothyronines at micromolar ligand concentrations indicates the expression of low-affinity thyroid hormone transporters in native *X. laevis* oocytes. To increase the sensitivity of the oocyte expression system for the cloning of thyroid hormone transporters, we tried to find specific inhibitors of these endogenous transporters without affecting the transporters encoded by rat liver mRNA. As these attempts were unsuccessful, we used iodothyronine sulfate derivatives, generated by sulfonation of the 4'-OH group and sulfamate derivatives, generated by sulfonation of the α-NH_2 group as alternative ligands. The increased water-solubility of the sulfates and sulfamates is a clear advantage for their use as alternative substrates. However, T_3S showed a lower uptake than T_3 not only in native oocytes but also in liver mRNA-injected oocytes. The results obtained by using the sulfamates, indicate that both T_4 -sulfamate (T_4NS) and T_3NS combine a low uptake in uninjected oocytes with an induction by rat liver mRNA that is at least as great as for T_3.

b) Testing of transporters with potential substrate homology

1. Organic anion transporters

Iodothyronine sulfates and sulfamates are sulfonated derivatives of thyroid hormone changing the hydrophobic thyroid hormones towards more water-soluble organic anions. In the literature, different organic anion transporter families for transport of bile acids and other organic anions have been reported (15). Human and rat NTCP contain 7 putative transmembrane domains and are localized to the basolateral cell membrane in the liver. NTCPs are expressed exclusively in liver and are the major Na^+-dependent transporters for conjugated bile acids. Two other proteins homologous to NTCP have been identified, *i.e.*, the ileal sodium-dependent bile acid transporter (ISBT), that is involved in the intestinal absorption of bile acids, and a protein termed P3 of unknown function (16,17). The possible role of these two proteins in transport of thyroid hormone remains to be investigated.

OATPs contain 12 putative transmembrane domains and are not only expressed in liver but also in kidney and brain. rOATP1 is localized to the basolateral liver cell membrane. This protein codes for a multispecific transporter mediating Na^+-independent uptake of a wide variety of ligands. Our results have demonstrated marked stimulation of uptake not only of sulfates and sulfamates but also of native iodothyronines after injection of oocytes with cRNA coding for rNTCP and rOATP1. We have also found uptake of these substrates with oocytes injected with the human homolog of rNTCP, another rat OATP type 2 (rOATP2) and a human variant of OATP (hOATP-A). Also other laboratories have recently published transport of native iodothyronines by members of the OATP family, *i.e.*, rOATP2 and rOATP3 (18), rOATP4 (19), hOATP2 or hLST-1 (both renamed to hOATP-C) (20,21), human and rat OATP-E (22), and hOATP-A, hOATP-B, and hOATP8 (23). However, the Na^+ independence of the OATP family makes this transporter a less likely candidate to be involved in active thyroid hormone transport.

2. Amino acid transporters

As thyroid hormones are iodinated amino acid derivatives built of two tyrosine molecules and indications in literature were found for competition between amino acids (for example, leucine) and thyroid hormone (24-28), we tested a heterodimeric human system L amino acid transporter for uptake of thyroid hormone. This heterodimeric transporter consists of a 4F2 heavy chain (4F2hc) combined with the light chain of system L amino acid transport type 1 (LAT1). 4F2hc is a glycosylated protein with a single transmembrane domain, whereas LAT1 has 12 putative transmembrane domains. This dimeric protein transports large neutral amino acids such as leucine (Leu), tyrosine (Tyr), tryptophan (Trp), and phenylalanine (Phe) in a Na^+-independent manner. Injection of oocytes with cRNA coding for both subunits induced Na^+-independent transport of all iodothyronines tested. Iodothyronines inhibit transport of the large neutral amino acids induced by the 4F2hc/LAT1 transporter and *vice versa*. In contrast to the ubiquitous expression of the 4F2 heavy chain, the LAT1 light chain is not expressed in liver. So, it can be concluded that this heterodimeric amino acid transporter is not responsible for the uptake found in liver mRNA injected oocytes. Furthermore, the induced uptake in oocytes injected with both subunits is completely Na^+-independent again making this transporter a less likely candidate to explain active thyroid hormone transport.

Besides uptake via system L amino acid transporters, system T amino acid transporters can also transport iodothyronines. In rat erythrocytes, researchers have found that the aromatic amino acids Trp, Phe, and Tyr competitively inhibited T_3 transport (29). Similar results were obtained for binding of T_3 and Trp to rat erythrocyte membranes (30). Evidence for uptake of T_3 by the system T amino acid transporter or a closely linked transporter was also obtained using human and trout erythrocytes (31,32). Recently, a system T amino acid transporter (TAT1) has been cloned and characterize to exhibit Na^+-independent transport of aromatic amino acids such as Trp, Tyr, and Phe (33). However, TAT1 does not transport iodothyronines.

c) Cloning strategy

Size-fractionation of rat liver mRNA has shown to result in the enrichment of messengers inducing transport of T_3 and also T_4, with maximal transport activity in fractions of 0.5-1.5 kb and 1.5-2.5 kb, respectively. These findings suggest partial dissociation of mRNAs coding for the T_4 and T_3 transporters, but the fractionation technique is too crude to allow this conclusion. Injection of oocytes with size-fractionated mRNA results in a further enrichment of T_4NS and T_3NS transport activity.

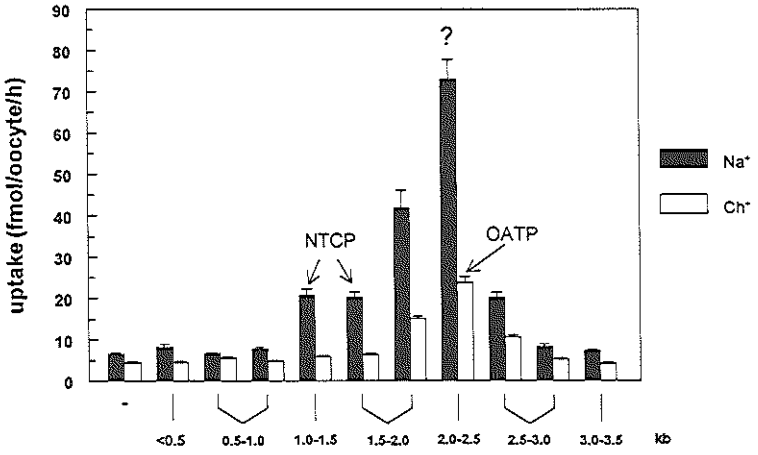

Fig. 1. Uptake of 100 nM T_3NS in uninjected oocytes (-) and oocytes injected with different size-fractions rat liver mRNA. Sizes ranges from less than 0.5 kb to 3.5 kb.

Evidence is provided for the existence of at least 3 different mRNAs coding for sulfamate transporters present in rat liver (Fig. 1). One transporter may represent NTCP, because of the size of the mRNA and the Na^+ dependent uptake of thyroid hormone sulfamate and taurocholate, respectively. A second transporter may represent OATPs, also because of the size of the mRNA and the Na^+ independent uptake characteristics for thyroid hormone sulfamate and taurocholate, respectively. However, the third transporter with mRNA size of 1.5-2.5 kb exhibits Na^+-dependent transport of thyroid hormone sulfamate, but TC is not transported. The contribution of both organic anion transporters to uptake of taurocholate was demonstrated by the almost complete loss of induction of taurocholate uptake after treatment of rat liver

mRNA with the NTCP and OATP antisense oligonucleotides. However, uptake of thyroid hormone sulfamates induced by rat liver was only partially blocked by these antisense oligonucleotides. These findings strongly confirm that hepatic transport of iodothyronine sulfamates, and probably also of T_4 and T_3 as uptake of iodothyronine sulfamates is competitively inhibited by 10 µM T_4 and T_3, is mediated largely by an as yet unidentified Na^+-dependent transporter. The functional cloning using the described and optimized *X. laevis* expression system with the iodothyronine sulfamates as ligands for the THTs is the major challenge of future experiments.

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SUMMARY

SUMMARY

The thyroid is the largest endocrine organ and produces predominantly the prohormone thyroxine (T_4) (Chapter 1). The biologically active thyroid hormone 3,3',5 triiodothyronine (T_3) is mainly formed by deiodination of T_4 in the liver while action of T_3 is initiated by binding to the specific nuclear thyroid hormone receptors. As both the deiodinase enzymes and the thyroid hormone receptors are localized intracellularly, thyroid hormones (THs) have to cross the plasma membrane. There is now accumulating evidence that this transport is mediated by one or more specialized proteins, which I here call thyroid hormone transporters (THTs). Sofar, such THTs have not been identified. Therefore, the aim of the research described in this thesis was to characterize these THTs to elucidate the role of transport in regulating the bioactivity of THs. I have tested several known proteins for their ability to transport THs, referred to as candidate THTs, and also designed and optimized a system to clone unknown mRNA species with THT characteristics.

The initial experiments were designed to express rat liver mRNA in *Xenopus laevis* oocytes, the most important testsytem used (Chapter 2). After injection of total mRNA and size-fractions thereof, rat liver mRNA was demonstrated to contain THTs for T_3 as well as for T_4. Size-fractionation also revealed that maximal stimulation of T_3 uptake could be obtained with mRNA of 0.5-1.5 kb and of T_4 uptake with mRNA of 1.5-2.5 kb. This suggests that T_4 and T_3 transporters are translated from different mRNAs. Injection of cRNA coding for the rat type I iodothyronine deiodinase (D1), which is an intracellularly expressed enzyme, showed that the substrate T_3 sulfate (T_3S) is indeed actively transported to the cell interior as radioactive iodide release from T_3S, catalyzed by D1, could be measured.

In Chapter 3, we characterized the endogenous THT activity of the test system used to identify mammalian THTs. During the expression studies with rat liver mRNA, it was found that native *X. laevis* oocytes themselves also actively transport THs. This indicates the presence of one or more endogenous THTs in *X. laevis* oocytes. Furthermore, the oocytes also metabolize 3,3',5' triiodothyronine (rT_3) by sulfation. This sulfation of rT_3 is rather unique as rat and human sulfotransferases have a substrate preference for 3,3'-T_2, while rT_3 is a relatively poor substrate.

In Chapters 4 to 6, experiments are described involving candidate THTs, *i.e.*, proteins with potential TH transport capacity. However, in order to minimize the endogenous uptake of THs by native oocytes, we have used TH sulfamates and sulfates as alternative substrates for putative THTs (Chapter 4). As especially TH sulfamates are organic anions, two already cloned rat liver organic anion transporters were tested for their ability to transport THs. Uptake studies with oocytes injected with cRNA coding for the rat Na^+/taurocholate cotransporting polypeptide (rNTCP) or the rat organic anion transporting polypeptide (rOATP1) demonstrates that uptake of THs and their metabolites is indeed possible via rNTCP and rOATP1. In Chapter 6 we tested the human homolog of the rat NTCP and two additional members of the large OATP family. This showed that all these transporters do have THT activity, but with varying ligand specificities.

THs are iodinated amino acid derivatives built of two tyrosine molecules and it is well-established that amino acids, including tyrosine, can compete with TH uptake. I therefore also tested a group of amino acid transporters capable of transporting large neutral amino acids (such as tyrosine) as candidate THTs (Chapter 5). The structure of these amino acid transporters is different from the NTCP or OATP1 in that they are heterodimers consisting of a heavy (*i.e.*, 4F2) and a light chain (*i.e.*, LAT1, LAT2, y^+LAT1, y^+LAT2). Injection of oocytes with cRNA coding for both the heavy and each one of the four different light chains tested, indeed induced Na^+-independent transport of THs. Interestingly, this TH-specific uptake was observed predominantly with the combination 4F2 and LAT1.

In Chapter 6 we focussed on the identification of the unknown THT activities, as observed in mRNA from rat liver after expression in oocytes. Analysis of size-fractionated mRNA indicated the expression of at least three different THT entities. One of these transporters may represent NTCP activity (in the mRNA size-fraction of 1-1.5 kb) and another may represent OATP1 activity (in the mRNA size-fraction of 1.5-2.5 kb). However, since all the OATP members act Na-independently it is unlikely that one or more of them represent the true THT activity. In addition, although NTCP mediated transport is Na-dependent, the mRNA size is most likely too small to represent the true THT activity. Thus, the major Na^+-dependent THT activity, as found in the mRNA size-fraction of 1.5-2.5 kb, remains to be identified.

The current knowledge about TH transport and its role in TH metabolism and bioavailability is reviewed in Chapter 7. A general discussion on the findings described in this thesis are presented in Chapter 8 together with suggestions for further research, mainly based on the results described in Chapter 6. Contrary to the results obtained with rat liver mRNA, the candidate THTs tested in this thesis have been found to be Na^+-independent, to have a low K_m (µmolar range), to be incompatible with respect to mRNA size and/or tissue-specific expression. This strongly suggests the existence of one or more true THT(s) which explain the majority of TH transport in the liver. Thus, the functional cloning of this true THT using the *X. laevis* expression system, is the major challenge for future experiments.

SAMENVATTING

SAMENVATTING

De schildklier is het grootste endocriene orgaan en produceert voornamelijk het prohormoon thyroxine (T_4). Het biologisch actieve schildklierhormoon 3,3',5 trijodothyronine (T_3) wordt grotendeels gevormd door dejodering van T_4 in de lever. De uiteindelijke werking van T_3 wordt geïnitieerd door binding aan de specifieke schildklierhormoon kernreceptoren. Omdat zowel de enzymen voor de dejodering als ook de schildklierhormoonreceptoren binnen in de cel aanwezig zijn, moet schildklierhormoon de celmembraan passeren. Er zijn thans veel aanwijzingen dat dit transport gebeurt door middel van een of meerder eiwitten die ik hier schildklierhormoontransporters heb genoemd (SHTs). Totnutoe zijn deze SHTs echter nog niet geïdentificeerd. Het doel van het in dit proefschrift beschreven onderzoek was dan ook deze SHTs te karakteriseren om zo de rol van transport in de regulatie van de bioactiviteit van schildklierhormoon op te kunnen helderen. Ik heb hiertoe verschillende bekende eiwitten (ook wel kandidaat SHTs genoemd) getest op hun capaciteit om schildklierhormonen te kunnen transporteren. Tevens heb ik een systeem verder ontwikkeld en geoptimaliseerd om nog onbekende mRNAs te klonen die schildklierhormoon kunnen transporteren.

Verschillende experimenten werden gedaan om rattenlever mRNA tot expressie te brengen in *Xenopus laevis* oocyten, het belangrijkste testsyteem dat is gebruikt (Hoofdstuk 2). Na injectie van totaal mRNA en van mRNA-fracties van verschillende grootte werd gevonden dat rattenlever mRNA SHTs voor zowel T_3 als voor T_4 bevat. Grootte-fractionering heeft ook nog bewezen dat maximale stimulatie van T_3 opname werd gevonden met mRNA van 0.5-1.5 kb en van T_4 opname met mRNA van 1.5-2.5 kb. Dit duidt erop dat de T_4- en T_3-transporters afkomstig zijn van verschillende mRNAs. Injectie van het cRNA van de rat type I jodothyronine dejodase (D1), een enzym dat intracellulair tot expressie komt, liet zien dat het substraat voor dit enzym T_3 sulfaat (T_3S) inderdaad naar het binnenste van de cel wordt getransporteerd, aangezien jodide productie werd gemeten hetgeen vrijkomt als gevolg van D1 activiteit.

De karakterisering van de endogene activiteit van het oocyten testsyteem dat gebruikt wordt om zoogdier SHTs te identificeren, wordt beschreven in Hoofdstuk 3.

Tijdens de mRNA expressie-experimenten werd gevonden dat de *X. laevis* oocyten zelf ook actief schildklierhormoon transporteren. Verder werd ook gevonden dat de oocyten in staat zijn 3,3',5' trijodothyronine (rT_3) om te zetten door sulfatering. Deze sulfatering van rT_3 is opvallend omdat rat en humaan sulfotransferases een substraat voorkeur hebben voor 3,3'-T_2, waarbij rT_3 een relatief slecht substraat is.

In de Hoofdstukken 4-6 worden de experimenten beschreven met kandidaat SHTs, dat wil zeggen eiwitten met een potentiële SHT-capaciteit. We hebben echter schildklierhormoon-sulfamaten en -sulfaten gebruikt als alternatieve substraten voor de kandidaat SHTs, om de endogene opname van schildklierhormoon door de natieve oocyten te minimaliseren (Hoofdstuk 4). Echter, omdat vooral sulfamaten organische anionen zijn, werden twee reeds eerder gekloneerde rattenlever organische anion transporters getest op de capaciteit om schildklierhormoon te transporteren. Opname studies met oocyten geïnjecteerd met het ratten Na^+/taurocholaat cotransporterend polypeptide (rNTCP) of het ratten organische anion transporterend polypeptide (rOATP1) tonen aan dat rNTCP and rOATP1 inderdaad in staat zijn tot opname van schildklierhormoon en zijn metabolieten. In Hoofdstuk 6 zijn het humane homoloog van het rat NTCP getest alsmede twee additionele leden van de grote familie van OATPs Deze proeven toonden aan dat al deze transporters inderdaad SHT activiteit laten zien, hetzij met een van de ligand afhankelijke specificiteit.

Schildklierhormoon is een gejodeerd aminozuur-afgeleide dat is opgebouwd uit twee tyrosine moleculen. Verder zijn er inmiddels veel aanwijzingen dat aminozuren, inclusief tyrosine, kunnen competeren met de opname van schildklierhormoon. Ik heb om die reden dan ook een groep van aminozuur transporters die in staat zijn grote neutrale aminozuren te transporteren, getest als kandidaat SHTs (Hoofdstuk 5). De structuur van deze eiwitten verschilt met die van NTCP en OATP1, omdat ze als heterodimeren bestaan uit een zware (4F2) en een lichte keten (LAT1, LAT2, y^+LAT1, y^+LAT2). Injectie van oocyten met cRNA coderend voor de zware keten en een van de lichte ketens, veroorzaakte inderdaad (Na^+ - onafhankelijk) transport van schildklierhormoon. Interessant was dat deze schildklierhormoon-specifieke opname met name werd waargenomen voor de combinatie van 4F2 en LAT1.

In Hoofdstuk 6 werden de SHT acitviteiten die aanwezig zijn in rattenlever mRNA nader onderzocht. Analyses van de grootte-fracties van het mRNA suggereren het bestaan van tenminste drie verschillende SHT entiteiten. Een hiervan kan NTCP zijn (in de mRNA grootte-fractie van 1-1.5 kb) en een ander kan OATP1 zijn (in de mRNA grootte-fractie van 1.5-2.5 kb). Echter, aangezien alle OATPs Na-onafhankelijk zijn is het onwaarschijnlijk dat zij de echte SHT activiteit verklaren. Voor NTCP geldt dat, hoewel dit eiwit Na-afhankelijk is, de mRNA grootte te klein is om de echte SHT actviteit te verklaren. Het eiwit(complex) dat verantwoordelijk is voor de belangrijkste Na^+-afhankelijke SHT activiteit in de mRNA groottefractie van 1.5-2.5 kb moet daarom nog geïdentificeerd worden.

Hoofdstuk 7 bevat een overzicht van wat thans bekend is omtrent schildklierhormoontransport en zijn rol in metabolisme en de biologische beschikbaarheid van schildklierhormoon. Hoofdstuk 8 geeft een algemene discussie aangaande de bevindingen die zijn beschreven in dit proefschrift. In dit hoofdstuk zijn tevens suggesties gedaan voor verder onderzoek, grotendeels gebaseerd op de resultaten beschreven in Hoofdstuk 6. In tegenstelling tot de resultaten behaald met het rattenlever mRNA zijn de kandidaat-SHTs die getest zijn in dit proefschrift Na-onafhankelijk, hebben een lage K_m (in het µmolair gebied), en hebben een andere mRNA grootte en/of een andere weefsel-specifieke expressie. Er zijn dus sterke aanwijzingen dat er een (of meerder) echte SHTs bestaan die de meerderheid van het schildklierhormoontransport in de lever voor hun rekening nemen. De functionele klonering van deze SHT, gebruik makend van het *X. laevis* expressie systeem, is daarom de grote uitdaging voor toekomstige experimenten.

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CURRICULUM VITAE AUCTORES

De auteur van dit proefschrift werd op 30 mei 1970 geboren te Heerlen. In het najaar van 1974 verhuisde het gezin naar Hattem (Gld.) alwaar het lager onderwijs werd gevolgd. Vanaf 1982 werd het middelbaar onderwijs doorlopen aan het Thomas à Kempis College te Zwolle alwaar het VWO diploma (Gymnasium B) in 1988 werd behaald. Aansluitend werd dat jaar begonnen met de studie Scheikunde aan de Rijks Universiteit van Groningen en op 25 augustus 1994 werd het doctoraal examen Scheikunde, specialisatie Biochemie, behaald. De bijbehorende afstudeerstages werden uitgevoerd op het laboratorium van Biochemie te Groningen onder leiding van Prof.dr. D.B. Janssen en Drs. J.R. van der Ploeg met als onderwerp "kloneren en karakteriseren van het epoxide hydrolase gen van *Pseudomonas* AD1" en op het Unilever Research Laboratorium Vlaardingen, sectie "Gene Technology and Fermentation" onder leiding van Dr. L.G.J. Frenken met als onderwerp "klonering en expressie van kameel antilichaam-fragmenten in *Aspergillus awamori*".

Vanaf november 1994 tot augustus 1999 werd promotieonderzoek gedaan als Assistent in Opleiding (AIO) op de afdeling Inwendige Geneeskunde III aan de Erasmus Universiteit Rotterdam onder leiding van Prof.dr. G. Hennemann, Dr.ir. R. Docter, en later ook Prof.dr.ir. T.J. Visser. De resultaten van dit onderzoek staan in dit proefschrift beschreven. Vanaf september 1999 volgde een aanstelling in tijdelijk dienstverband als wetenschappelijk medewerker op de afdeling Inwendige Geneeskunde, alwaar het onderzoek aan SHTs wordt voortgezet.

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