THYROID HORMONE UPTAKE BY RAT HEPATOCYTES IN PRIMARY CULTURE

THYROID HORMONE UPTAKE BY RAT HEPATOCYTES IN PRIMARY CULTURE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
VRIJDAG 10 JUNI 1983 DES NAMIDDAGS
TE 2.00 UUR

DOOR

ERIC PAUL KRENNING
geboren te 's-Gravenhage

1983

grafische verzorging:
ROOD DAVIDS
ALBLASSERDAM

PROMOTOR : PROF. DR. G. HENNEMANN
CO-REFERENT : PROF. DR. W.C. HÜLSMANN

Ter nagedachtenis aan mijn vader.
Aan mijn moeder,
Ellen,
Boudewijn en
Andrea.

Financial support was provided by E. MERCK NEDERLAND, Amsterdam

CONTENTS

CHAPTER 4

REGULATION OF THE ACTIVE TRANSPORT OF 3,3',5-TRIIODOTHYRONINE (T_3) INTO PRIMARY CULTURED RAT HEPATOCYTES BY ATP

CHAPTER 5

CHARACTERISTICS OF ACTIVE TRANSPORT OF THYROID HORMONE INTO RAT HEPATOCYTES

CHAPTER 6

DECREASED TRANSPORT OF THYROXINE (T_4), 3,3',5-TRIIODOTHYRONINE (T_3) AND 3,3',5'-TRIIODOTHYRONINE (rT_3) INTO RAT HEPATOCYTES IN PRIMARY CULTURE DUE TO A DECREASE OF CELLULAR ATP CONTENT AND VARIOUS DRUGS

CHAPTER 7

PLASMA MEMBRANE TRANSPORT OF THYROID HORMONE: ITS POSSIBLE PATHOPHYSIOLOGICAL SIGNIFICANCE

ABBREVIATIONS

ATP	adenosine 5`-triphosphate
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DNP	dinitrophenol
HAS	high affinity system
K_a	equilibrium association constant
KCN	potassium cyanide
K_i	equilibrium dissociation constant of a molecule-inhibitor complex
K_m	Michaelis constant
LAS	low affinity system
PTU	propylthiouracil
rT_3	reverse T_3; 3,3`,5`-triiodothyronine
T_3	3,3`,5-triiodothyronine
T_4	thyroxine; 3,3`,5,5`-tetraiodothyronine
TBG	thyroxine-binding globulin
V_{max}	maximal velocity

CHAPTER 1

INTRODUCTION

1. Thyroid hormone synthesis and metabolism

Iodide is taken up by the thyroid follicular cell, oxydized and bound to thyroglobulin at the apical membrane facing the colloid in the follicular lumen. Iodinated colloid is subsequently engulfed by the follicular cell and hydrolysed, liberating thyroxine and triiodothyronine from their peptide linkage to thyroglobulin. Iodothyronines are then secreted into the blood stream. These and other steps in the synthesis and secretion of thyroid hormones are conditioned by the extent of thyroid stimulation by pituitary thyrotropin. The synthesis and release of thyrotropin is stimulated by the hypothalamic thyrotropin-releasing hormone, while thyroid hormone inhibits these processes (Larsen, 1982). In this way, a negative feedback mechanism is formed between the thyroid and pituitary.

The main product secreted by the human thyroid is thyroxine (mean 115 nmol/day per 70 kg body weight). Other products are triiodothyronine (mean 9 nmol/day, which accounts for 20% of the total daily production) and reverse triiodothyronine (mean 2 nmol/day; 6%) (Chopra, 1976; Chopra et al., 1978a; Visser, 1980). From these figures it will be clear that synthesis of the latter iodothyronines occurs mainly outside the thyroid gland by monodeiodination of thyroxine, the so-called peripheral production. In pathophysiologi-

cal conditions, like iodine deficiency and Graves' disease, thyroidal secretion of triiodothyronine is increased relative to that of thyroxine (Izumi and Larsen, 1977). The relative contribution of different tissues to the daily production of triiodothyronine and reverse triiodothyronine is at present unknown. However, deiodination has been observed in vitro in almost all tissues studied (see review by Visser, 1980). Based on circumstantial evidence, it is generally believed that the liver is the main site of triiodothyronine synthesis. For instance, in liver cirrhosis triiodothyronine production is decreased, while apparent reverse triiodothyronine synthesis is unaltered (Chopra, 1976). The latter finding is in favour of extra-hepatic thyroxine → reverse triiodothyronine conversion. The low serum reverse triiodothyronine levels in patients with severe, chronic renal failure may suggest that substantial amounts of reverse triiodothyronine are produced in the kidneys (Chopra et al., 1975; Weissel et al., 1977; Weissel and Stummvoll, 1981).

In the early fifties, Gross and Pitt-Rivers demonstrated the thyromimetic effect of triiodothyronine and that it is a product of thyroxine metabolism. They hypothesized that most of the thyroid hormone effects in the body are due to triiodothyronine and that thyroxine acts as its precursor (see review by Gross, 1981). Successive studies have shown that triiodothyronine has at least three receptor sites:

1. a site of action in the nucleus with a resultant effect on gene expression (Oppenheimer et al., 1976),
2. a direct effect on membrane processes, like enhancing transport of amino acids and sugars (Adamson and Ingbar, 1967; Goldfine et al., 1976;

Segal et al., 1977; Segal and Ingbar, 1979; 1982), and

3. a direct action on mitochondria to increase ATP synthesis (Sterling et al., 1980).

Moreover, it appeared that of the naturally occuring iodothyronines, triiodothyronine was the most active in all these systems.

Since thyroid hormone receptors are located predominantly intracellularly, it might be anticipated from the foregoing that triiodothyronine is primarily present inside the cell. Table 1-1 illustrates the total pool of thyroxine, triiodothyronine and reverse triiodothyronine, and the percentages of these pools confined to the extracellular, intracellular hepatic and intracellular, non-hepatic compartments (Hennemann, 1981). In spite of the fact that, in equilibrium conditions, binding to intra- and extracellularly located thyroid hormone carriers is the main determinant of the distribution of thyroid hormones (vide infra), it is striking that triiodothyronine is chiefly located inside the cell, in contrast to thyroxine. Reverse triiodothyronine is about equally distributed over both compartments.

TABLE 1-1

POOL (P) AND PERCENTAGE OF POOL IN EXTRACELLULAR ($\%P_E$), INTRACELLULAR HEPATIC ($\%P_{I.H.}$) AND INTRACELLULAR NON-HEPATIC ($\%P_{I.N.-H.}$) COMPARTMENT.

	Pnmol[a]	$\%P_E$	$\%P_{I.H.}$	$\%P_{I.N.-H.}$
T_4	1340	60	32	8
T_3	74	20	7	73
rT_3	4.8	46	-	-

[a]corrected for 1.73 m^2 body surface.

Differences in the sizes of the extracellular pools of thyroid hormones are encountered both in pathological states with abnormal production rates or metabolic clearance rates of thyroid hormones and by variations in serum binding proteins. In plasma over 99% of these hormones is protein bound. In humans, the three major carriers are thyroxine-binding globulin (TBG), prealbumin and albumin. In rats TBG is absent (Farer et al., 1962). According to Robbins and Rall (1957), however, it is the free hormone concentration in serum, which governs hormonal entry into target organs. It has been calculated that the rates of dissociation from TBG and prealbumin do not constitute a rate-limiting factor for uptake of thyroxine and triiodothyronine into the human liver (Robbins and Johnson, 1982). Thus, in this model, the intra-capillary free hormone concentration is maintained at a constant level. Similar calculations have shown that prealbumin, the predominant carrier of thyroxine in rats (Sutherland and Simpson-Morgan, 1975), can liberate thyroxine at a faster rate than is required for tissue entry during a single capillary passage. The actual sequence of events may, however, be the more complicated by the following factors:

1) transport of carrier-bound hormone into target cells, as suggested by Keller, Richardson and Yates (1969), and
2) molecular events occurring in the micro-circulation such as intra-capillary diffusion, capillary wall perfusion (Ekins et al., 1982) and return of hormone to the blood, since the entry rates exceed by far the disposal rates from the cells (Robbins and Johnson, 1982).

Recent studies (Obregon et al., 1979; Larsen, 1982; van Doorn et al., 1982) have pointed to differences in the contribution of circulating triiodothyronine and triiodothyronine derived from local conversion of thyroxine to the intracellular triiodothyronine levels. Especially in liver and kidneys the major part of nuclear bound triiodothyronine is derived from the extracellular pool. Thus, also because of the high capacity to produce triiodothyronine, the liver and probably to a lesser extent the kidneys, have to be regarded as organs which are important in the production of triiodothyronine to fulfil general demands. It is clear that thyroxine, triiodothyronine and reverse triiodothyronine (vide supra) have to cross the plasma membrane of hepatocytes for further metabolism. The aim of this thesis is to study the mechanism of the translocation of iodothyronines (vide infra). The experiments were carried out with primary cultures of rat hepatocytes. Since in these studies this cell type has been used, both some quantitative and qualitative aspects of the mammalian liver and the use of isolated cells in liver studies are briefly discussed in the next paragraphs.

2. Some quantitative and qualitative aspects of the mammalian liver

Over the last 10 to 15 years, morphologic observations and studies with preparations of purified liver cell subpopulations have provided an extensive basis in defining the morphology, ultrastructural characteristics and function of single cell types. Furthermore, these studies have allowed estimates of

the contribution of parenchymal cells (hepatocytes) and the cells which line the blood sinusoids (for a major part Kupffer and endothelial cells) to the total liver mass. About $110x10^6$ hepatocytes, $42x10^6$ endothelial cells and $12x10^6$ Kupffer cells are present per gram liver in a 3-months-old rat (Seglen, 1973; Knook and Sleyster, 1980).

Hepatocytes occupy 78%, (peri-)sinusoidal cells 6% and extracellular space 16% of the total liver volume. The latter is divided in 5% space of Disse, 11% sinusoidal lumina and 0.4% bile canaliculi (Blouin, 1977).

Broadly outlined, the following functions are attributed to hepatocytes and sinusoidal cells:

hepatocytes,
- bile formation
- control of carbohydrate and fat metabolism
- conjugation, inactivation and detoxification of many compounds
- synthesis of many compounds, such as, with regard to this thesis, the active form of thyroid hormone, triiodothyronine

sinusoidal cells,
- uptake and degradation of circulating lipo-proteins
- selective clearance of circulating glyco-proteins, lipopolysaccharides (e.g. endotoxin) and mucopolysaccharides (e.g. heparin)
- clearance of foreign materials, senescent erytrocytes and micro-organisms

According to their localisation, three distinct cell membrane regions in hepatocytes are distinguished:

1) the sinusoidal membrane, which accounts for 40-50% of the total hepatocyte surface and covers the part that abuts on the space of Disse. Numerous microvilli project from the hepatocytes into the space of Disse, providing an enormous surface, over which hepatocytes are in direct contact with plasma from the sinusoids, since the lining between sinusoids and the space of Disse does not constitute a continous membrane (Reeve and Chen, 1970),
2) the intercellular membrane (30-40% of the total surface), which forms the line of contact between the cell membranes of adjacent hepatocytes,
3) the canalicular membranes (about 10% of the total surface, Sips, 1981), which are bound together tightly by a junctional complex to form the lining of a bile canaliculus.

Enzymes are used as markers of specific types of membrane. For instance, Na^+-K^+-ATPase is a marker of the sinusoidal and intercellular membrane (Erlinger, 1982). The surface area of the plasma membrane contributed by each cell type to the total surface area of plasma membranes of the liver, amounts for hepatocytes, endothelial and Kupffer cells to 73%, 15% and 4%, respectively (Blouin, 1977; Praaning-Van Dalen et al., 1981). The plasma membrane of fat-storing cells accounts for the main part of the remainder.

3. Use of isolated cells in liver studies

Before the publication of the article of Berry and Friend in 1969 entitled "High yield preparation of isolated rat liver parenchymal cells" and the establishment of these cells in primary monolayer cultures (Bissel et al., 1973), most in vitro studies on liver

functions have been made on perfused liver and liver slices. However, both techniques have several disadvantages, which can be summarized as follows (Ichihar et al., 1980):

- limited number of simultaneous experiments can be made with liver perfusions,
- many cells in liver slices are damaged,
- both techniques can be used for only a few hours,
- no information of a single cell population will be obtained.

Since 1969, many reports appeared using freshly isolated cells in suspension for functional studies. In the beginning, cells were isolated by perfusion of livers with collagenase (0.05%) and hyaluronidase (0.10%). It appeared, however, that addition of hyaluronidase to the perfusion medium significantly reduced the glycogen content of the isolated cells without affecting the cell yields (Wagle, 1975). At present, most investigators use a perfusion medium with collagenase as the only proteolytic enzyme. It appeared that by exposing the liver to collagenase various functions of the freshly isolated hepatocytes were greatly affected, resulting in a reduced rate of protein synthesis, loss of active transport of amino acids, low inducibility of enzymes, little or no ability to respond to hormones, and catabolic state of protein turnover (Kato et al., 1979). Furthermore, the ATP content of these cells (about 40 nmol per 2 x 10^6 cells) (Dickson and Pogson, 1977) is relatively low as compared to hepatocytes cultured in monolayer for 4 h (64 nmol per 2 x 10^6 cells) (chapter 6, this thesis). Even in the presence of Eagle's essential medium with 10% fetal calf serum, after several hours of incubation, cells in suspen-

sion showed a decrease in ATP content. This observation accords with the experience that these cells are viable for only a few hours.

Isolated hepatocytes readily attach to and spread on the surface of polystyrene culture dishes in the presence of serum. Attachment is virtually completed in 20-30 min, whereas spreading beyond the initial circumference of the cell, i.e. flattening of the cell, is evident only after 2.5-3 h. Cell contacts are formed at the margins of spreading cells and, provided that cells are seeded at a high density, a continous monolayer can be formed in 3-4 h (Seglen and Fossa, 1978). This interval is sufficient for incorporation of proteins into the plasma membrane (Ray et al., 1968). Liver plasma membranes of cycloheximide treated rats continued to incorporate labeled protein for at least 3 h, in spite of the reduced (by 97%) liver protein synthesis. It appears that the proteins taken up by the membrane, are formed several hours previously.

The use of primary cultures of hepatocytes to study cell function offers several advantages over freshly isolated cells such as:

- cell viability for several days in stead of only a few hours,
- recovery of impaired function caused by damage to the plasma membrane during the isolation procedure,
- the preservation of metabolic processes like consumption of amino acids for protein synthesis and other processes, such as gluconeogenesis. A corollary of these metabolic events might be the above mentioned increase in ATP content,
- easy separation of viable and non-viable cells (viz. non-viable cells lose attachment and are discarded via the medium), and

- easy separation of cells and medium.

A major disadvantage of the use of monolayers in culture dishes is the poor mixing and, therefore, the suboptimal contact between the cells and the bulk of the medium. As a consequence, when investigating movement phenomena of substances over the plasma membrane, influences of the unstirred water layer around the cell may be encountered (Lerner, 1978). With respect to thyroid hormone uptake studies, this problem can be solved by using optimal concentrations of binding protein in the medium, as is described in chapter 3.

4. Scope of the thesis

In previous years, it was generally accepted that thyroid hormone enters the cellular compartment by simple diffusion. One major argument in favour of this hypothesis was the lipophilicity of the hormone, favouring the passage through the lipid bilayer of the membrane. The rates of transfer through the plasma membrane would then be conditioned by the simple competitive binding equilibrium of the hormone between intracellular and extracellular binding proteins (Hasen et al., 1968). According to the "free hormone hypothesis", viz. the hypothesis that the free hormone concentration in serum governs hormone dilivery to the cell and ultimately regulates hormonal action, it is the non-protein bound moiety, which is translocated into the tissues (Robbins and Rall, 1957). In other words, the free thyroxine concentration is more closely correlated with the thyroxine turnover rate than is the total concentration (vide

supra, thyroid hormone synthesis and metabolism). This hypothesis has been challenged, however, as for example a normal thyroxine turnover is found in healthy euthyroid subjects with thyroxine-binding globulin deficiency and lowered free hormone concentrations (Hennemann et al., 1971). In addition, in this study no correlation was found between serum free thyroxine concentration and thyroxine turnover in a group of healthy euthyroid individuals. Other examples are: normal thyroxine turnover in non-thyroidal illness despite increased free thyroxine serum levels (Inada and Sterling, 1967; Bellabarba et al., 1968) and similarly normal thyroxine turnover has been found in the post-operative state in which decreased thyroxine-binding pre-albumin with elevated free thyroxine concentrations can be encountered (Bernstein et al., 1967). It was postulated that other factors, possibly directly related to cellular handling of thyroxine, may be operative. Amoung other things, these discrepancies warranted further investigations at the peripheral level. Starting thyroid hormone uptake studies meant an extension of the work in our laboratory in the field of peripheral thyroid hormone metabolism, viz. investigations regarding nuclear receptors (Docter et al., 1976), cytosol binding proteins (Visser et al., 1976) and deiodination (Visser, 1980; Fekkes, 1982). Since the liver has an essential part in thyroid hormone metabolism, it was decided to use isolated rat hepatocytes for the uptake studies.

The main subject of this thesis is to answer the question if thyroid hormone enters the intracellular compartment by diffusion only or that other types of translocation are present as well, such as adsorptive or fluid endocytosis (Munniksma et al., 1980). A

distinction between these types of endocytosis is that, in contrast to fluid endocytosis, adsorptive endocytosis shows saturation kinetics.

FEBS LETTERS 91(1978) 113-116

CHAPTER 2

ACTIVE TRANSPORT OF TRIIODOTHYRONINE (T_3) INTO ISOLATED RAT LIVER CELLS

E.P. Krenning, R. Docter, H.F. Bernard, T.J. Visser and G. Hennemann

1. Introduction

Since the receptors for thyroid hormones are located within the cell (Bernal and Refetoff, 1977), these hormones have to be transported from the extracellular compartment through the plasma membrane into the cell. Only scarce and conflicting data concerning this transport mechanism are available. Both diffusion (Freinkel et al., 1957; Lein and Dowben, 1961; Tata, 1964) and carrier-mediated processes (Stitzer and Jacquez, 1975; Rao et al., 1976; Pliam and Goldfine, 1977) have been described or suggested. In view of the fact that amino acids (Oxender, 1972) are transported through the plasma membrane by active or mediated processes, it is not likely that thyroid hormones enter the cell by diffusion only. In an attempt to gain more insight into the mechanism of cellular uptake of T_3 the present study was performed. As a model we have chosen non-proliferative cultures of parenchymal cells from adult rat liver. The use of primary cultures offers a distinct advantage over freshly prepared liver cell suspensions. Cells are given time to recover from the damage associated with the isolation, such as temporary changes in the in-

ability to concentrate amino acids (Schreiber and Schreiber, 1973) and the increased catabolic state during the first 2-4 h after isolation (Seglen, 1977). In this report, we describe the basic characteristics of T_3 uptake in this system. A preliminary account of this work has been published (Docter et al.,1977).

2. Materials and methods

2.1. Materials

3,3',5-Triiodo-L-thyronine (L-T_3), L-Thyroxine (L-T_4), piperazine-N,N'-bis[2-ethane sulfonic acid] (Pipes), N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (Hepes), N,N-bis[2-hydroxy ethyl]-2-amino ethane sulfonic acid (Bes), dinitrophenol (DNP), potassium cyanide (KCN), oligomycin, collagenase (type I), bovine serum albumin (type V) (BSA), insulin and D-glucose were purchased from Sigma, St. Louis, MO; fetal bovine serum (FBS), penicillin/streptomycin (P/S) and Ham's F10 from Flow Laboratories, Irvine.

All glass-ware was siliconized (Siliclad^R, Clay Adams, New York, NY). Plastic culture dishes were purchased from Costar, Cambridge, MA.

The purity of [^{125}I]T_3 tracer (Radiochemical Centre, Amersham), spec. act. >1200 μCi/μg, was verified by cellulose thin-layer chromatography (Zappi, 1967) (acetone : 0.5 M acetic acid, 30 : 70).

2.2. Methods

Parenchymal cells were isolated from livers of male Wistar rats (150-300 g) according to the method of Berry and Friend (1969) with minor modifications. These consist of a preperfusion of the liver for 10 min with Ca^{2+}-free Hank's solution followed by perfusion with collagenase (0.05%) and Ca^{2+} (2 mM) in Hank's solution. About $2x10^6$ cells in 4 ml culture medium (Ham's F10, 10.6 mM Pipes, 11.2 mM Bes, 8.9 mM Hepes, 12 mU/ml insulin, 15% FBS, 2 mM $CaCl_2$ and 10 U/ml P/S, pH 7.4) were inoculated into 60 mm dishes. After 4 h culture at 37° C the medium was replaced by 4 ml incubation medium (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 6.7 mM glucose and 1% BSA, pH 7.4). Incubations were performed in triplicate for 1 min at 21° C with [^{125}I]T_3 and increasing amounts of unlabelled T_3 in 4 ml incubation medium.

Uptake experiments were terminated by removing the medium and washing the monolayer with 4 ml incubation medium without albumin. This procedure resulted in an almost complete removal of the medium (99.96%), as tested with ^{131}I-labelled albumin.

For counting of radioactivity and DNA determination (Burton, 1956) cells were removed after lysing with 0.1 M NaOH. After incubation with T_3, viability of cells, as determined by the exclusion of Trypan blue, was over 90%. At each concentration of unlabelled T_3 the uptake data of 4-6 experiments were combined after correction for varying DNA concentrations. For all doses used, free T_3 and T_4 concentrations in the incubation medium were estimated by equilibrium dialysis (Sterling and Brenner,1966).

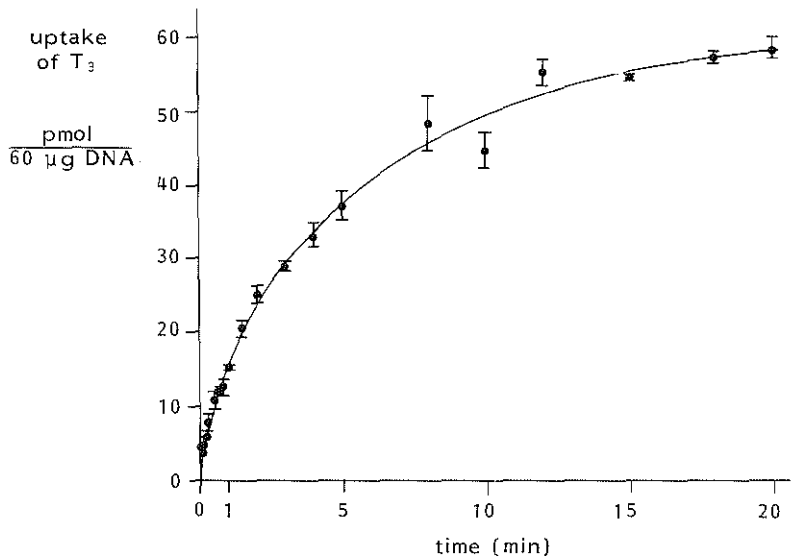

Fig. 2-1. Uptake of T_3 ([FT_3] 5.5 nM) by rat liver cells as function of time of incubation at 21°C. For details see section 2.

3. Results

As illustrated in fig. 2-1, uptake of T_3 was almost linearly related with time up to 1 min. Therefore all uptake studies were performed using 1 min incubations. The total uptake of T_3 (fig. 2-2) was not linearly related to the free concentration of T_3, indicating that in addition to diffusion (in our kinetic model adsorption can not be differentiated from diffusion) carrier-mediated processes were involved.

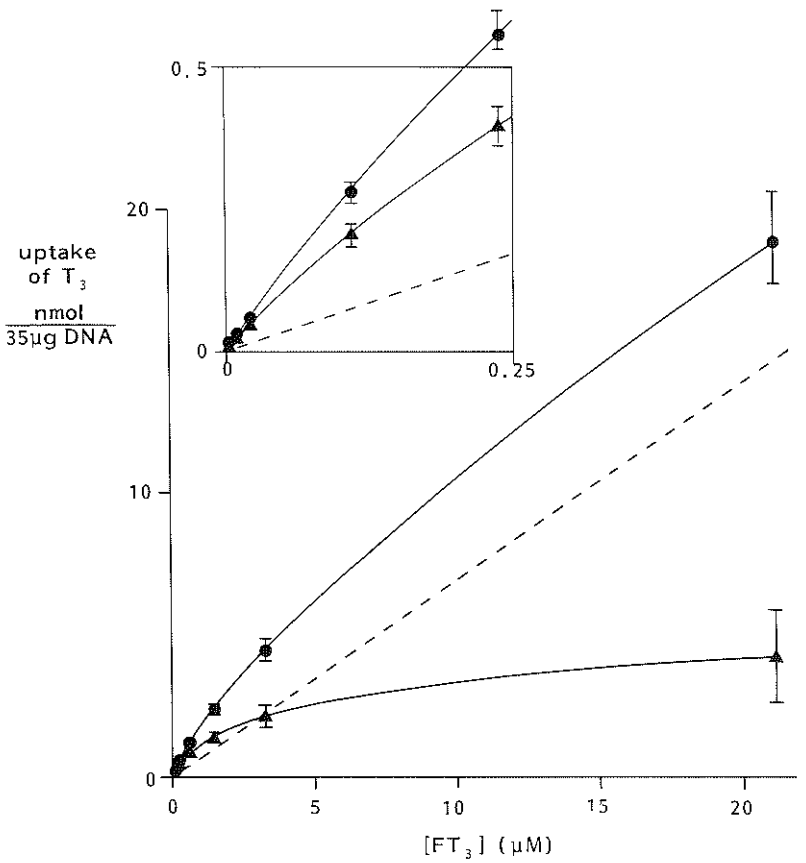

Fig. 2-2. Uptake of T_3 (mean ± SEM) by rat liver cells during incubation for 1 minute at 21°C. using increasing concentrations of T_3. The inset is a magnification of the area near the origin. (●——●) Represents total uptake, (- - - -) diffusion and (▲——▲) uptake by carrier-mediated processes (n=25)

The contributions of the saturable processes were negligible (<8.5%; taking account of these contributions resulted in minor differences of the calculated kinetic parameters) at very high concentrations of T_3 ([FT_3] 48 μM) and since diffusion is linearly related to the free hormone concentration the line, representing uptake by means of diffusion, was constructed through this highest measured uptake point and the origin. By subtracting the diffusion line from the total uptake curve, the uptake curve of the carrier-mediated processes was obtained.

By plotting these uptake data against the free T_3 concentration in a double reciprocal plot, fig. 2-3 was obtained. In fig. 2-3 the regression line for the 5 highest data points is shown. From the inset it can be seen that the five lowest data points are significantly ($p<0.001$) below this regression line, indicating that a second saturable uptake system is present. To calculate the K_m and V_{max} values of both systems the method of Spears et al. (1971) was used which corrects for the contribution of one system when the parameters of the other are calculated. In table 2-1 the results are summarized.

TABLE 2-1

CHARACTERISTICS OF THE UPTAKE OF T_3 BY RAT LIVER CELLS; THE EFFECT OF TEMPERATURE, ADDITION OF T_4 AND PREINCUBATIONS WITH METABOLIC INHIBITORS

Test		n	K_{m1} (μM)	V_{max1} (nmol/35 μg DNA/min)	K_{m2} (nM)	V_{max2} (pmol/35μg DNA/min)
21°C		25	1,8 (1.1-2.5)[a]	3.3 (2.0-4.8)[a]	21 (9-29)[a]	16 (6-25)[a]
0°C		4	1.5 (0.9-1.9)	2.8 (1.2-4.9)	-	-[b]
37°C		4	2.8 (2.5-3.1)	4.1 (3.1-5.0)	61 (34-108)	48 (26-91)[b]
(FT_4)	20 μM	6	1.9 (1.1-2.5)	2.4 (1.3-3.8)	-	-
(KCN)	2 mM	6	2.0 (1.9-2.7)	2.7 (2.0-4.5)	-	-
(DNP)	2 mM	4	2.3 (2.2-2.4)	4.2 (3.5-4.9)	-	-[b]
(Oligomycin)	100 μM	4	1.8 (1.6-1.9)	2.4 (2.0-2.7)	-	-[b]

[a] Mean (range)

[b] Zero V_{max} after cross-correction for uptake by the low affinity system

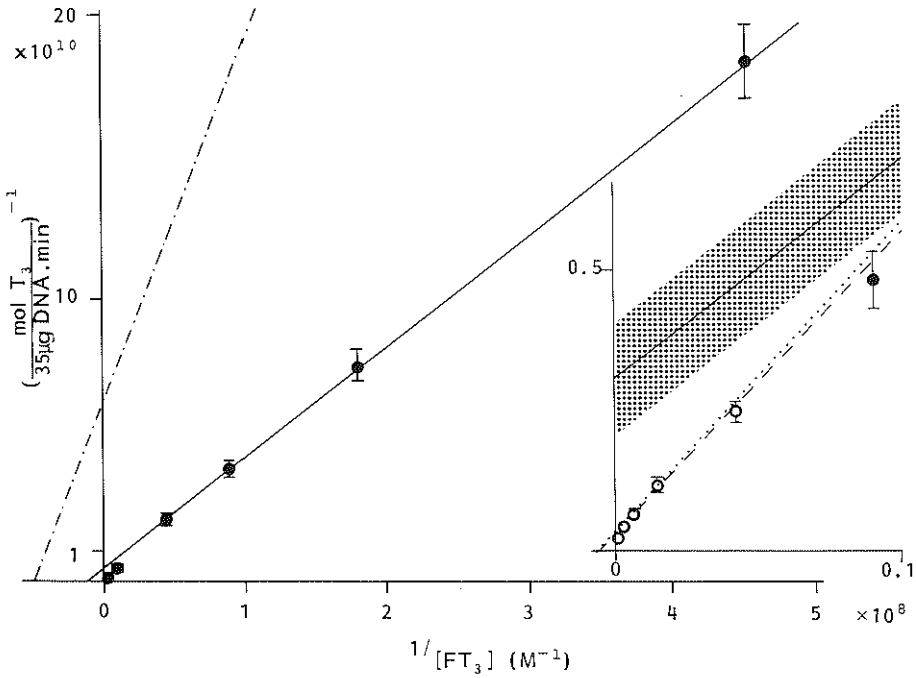

Fig. 2-3. Double reciprocal plot of the T_3 uptake by rat liver cells corrected for diffusion, against the free T_3 concentration. Data are pooled from 25 experiments. The lowest point in the left panel represents a cluster of the 5 data points (o) shown on a magnified scale in the inset. (———) Regression line for the data points (●) (mean ± SEM) representing the high affinity system, with 95% confidence limits (shaded area). (– – – –) Regression line for the data points (o) representing the low affinity system. (–·–·–) and (·····) Regression lines of the high and the low affinity systems, respectively, after cross-correction to Spears et al, 1971.

At 21° C a low affinity system with a K_m of 1.8 μM and a V_{max} of 3.3 nmol/35 μg DNA/min, and a high affinity system with a K_m of 21 nM and a V_{max} of 16 pmol/35 μg DNA/min were found. Increasing the temperature to 37° C had only a slight effect on the K_m of the low affinity system but increased both K_m and V_{max} of the high affinity system. If the temperature was lowered to 0° C the high affinity system was not measurable. When T_4 ([FT_4] 20 μM) was present during

the incubations, uptake of T_3 via the high affinity system was not measurable, indicating that T_4 interfered with T_3 uptake. The low affinity system remains unaffected by T_4. When the cells were preincubated with 2 mM KCN, 2 mM DNP or 0.1 mM oligomycin for 30 min at 37° C the high affinity system was blocked. These treatments resulted in a decrease of total intracellular ATP of ~80% (not detailed here).

4. Discussion

The results clearly show that there are two saturable uptake systems for T_3 in cultured rat liver parenchymal cells. The high affinity system is blocked by KCN, DNP and oligomycin, probably as a result of their effects on intracellular ATP concentration. The effects of temperature on this system could also support this energy dependency, although other explanations like changes in membrane fluidity are possible. The fact that KCN, DNP or oligomycin do not influence the uptake characteristics of the low affinity system makes it unlikely that in this case an energy-dependent process is involved. Since initial T_3 uptake kinetics have been analyzed, it is assumed that transport and/or binding at the level of the cell membrane is being measured. The energy dependency of the high affinity system strongly suggests that at least part of the transport through the cell membrane takes place via this pathway. The low affinity energy-independent system may represent binding at the membrane level. This is supported by the finding that temperature changes do not affect the 'V_{max}' of this system. Cytosolic thyroid hormone binding proteins are probably not involved, since

binding of T_3 to these sites is inhibited by T_4 (Visser et al., 1976) as opposed to the lack of effect of T_4 on the low affinity system.

FEBS LETTERS 107 (1979) 227-230

CHAPTER 3

THE ESSENTIAL ROLE OF ALBUMIN IN THE ACTIVE TRANSPORT OF THYROID HORMONES INTO PRIMARY CULTURED RAT HEPATOCYTES

E.P. Krenning, R. Docter, H.F. Bernard, T.J. Visser and G. Hennemann

1. Introduction

We have shown that both 3,5,3'-triiodo-L-thyronine (T_3) (Krenning et al., 1978) and L-thyroxine (T_4) (Docter et al., 1978) are taken up by primary cultured hepatocytes of adult rats by two saturable processes and by diffusion. One saturable uptake system shows a high affinity with a low capacity, while the second one has lower affinity and higher capacity. The high affinity systems of T_3 and T_4 are energy dependent in contrast with their low affinity systems (Krenning et al., 1978; Docter et al., 1978). T_4 inhibits the high affinity system of T_3 competitively (Docter et al., 1978) and vice versa (E.P.K., R.D., H.F.B., unpublished). We suggested that the high affinity systems represent a transport function while the uptake systems with low affinity may be involved in binding at the cell surface (Krenning et al., 1978). Our results were obtained from incubations of hepatocytes in a medium containing 10 g/l bovine serum albumin. Since similar studies (Sorimachi and Robbins, 1978) with cultured monkey hepatocarcinoma cells do not show saturable uptake of

T_3 in the absence or with a low concentration of albumin (2 g/l), we decided to study the effect of albumin on the uptake of T_3 in our system.

The results here reported show that at <5 g/l albumin, saturable uptake is not observed, in contrast with higher concentrations of albumin. By increasing the concentration of albumin from 5-20 g/l the V_{max} values of both affinity systems of T_3 increase, whereas the K_m values remain unchanged.

It is therefore suggested (a) that albumin is necessary for optimal diffusion through the unstirred water layer around the cell in a system of cultured rat hepatocytes in monolayer and (b) that increasing concentrations of albumin lead to augmentation of the uptake processes which are involved in transport and binding of T_3.

2. Materials and methods

The materials used and the procedure of isolation and culture of parenchymal rat liver cells has been described (Krenning et al., 1978).

Incubations were performed in quadruplicate for 1 min at 37° C with [^{125}I]T_3 and increasing amounts of unlabelled T_3 in 4 ml incubation medium with varying concentrations of bovine serum albumin as mentioned in section 3. The subsequent procedure for calculating the uptake kinetics is similar to that described previously (Krenning et al., 1978). Free T_3 concentrations at the different albumin concentrations used were estimated with equilibrium dialysis (Sterling and Brenner, 1966). The range of the free T_3 concentrations ([FT_3]) was 1.3 nM to 76 μM.

Correction for the contribution of diffusion was done essentially as by Christensen (1975).

3. results

3.1. Effect of albumin on diffusion

As illustrated in fig. 3-1, without albumin in the incubation medium, the velocity of diffusion at a $[FT_3]$=22.3 μM is 6.2 (SEM=1.0) nmol/35 μg DNA/min. The use of 1 g/l albumin leads to an increase ($p<0.01$) in the diffusion velocity to a level of 36.6 ($\pm$ 1.7) nmol/35 μg DNA/min, which does not increase further under conditions of higher albumin concentrations.

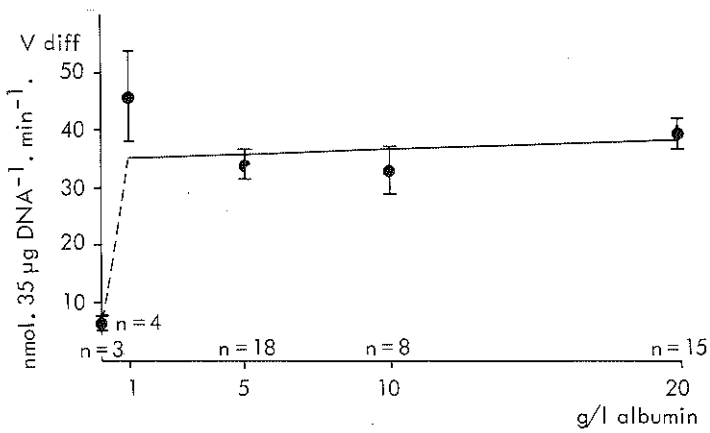

Fig. 3-1. Effect of albumin in the incubation medium on uptake of T_3 by diffusion by primary cultures of parenchymal rat liver cells, at 22,3 µM, free T_3 at 37^{0}C and with 1 minute incubation. Each point represents the mean ± SEM of n experiments (each carried out in quadruplicate).

3.2. Effect of albumin on the kinetics parameters of saturable uptake

At zero or 1 g/l albumin concentration in the incubation medium the V_{max} of both affinity systems was not measurable. Increasing albumin to 5 g/l allowed the measurement of the kinetics parameters of both systems. Upon further increase to 20 g/l albumin the V_{max} of the high affinity system increased significantly from 26-103 pmol/35 μg DNA/min, while the K_m value did not change significantly (fig. 3-2). Similar findings were obtained for the low affinity system, i.e., an increase of V_{max} from 5.8-10 nmol/35 μg DNA/min (fig. 3-3).

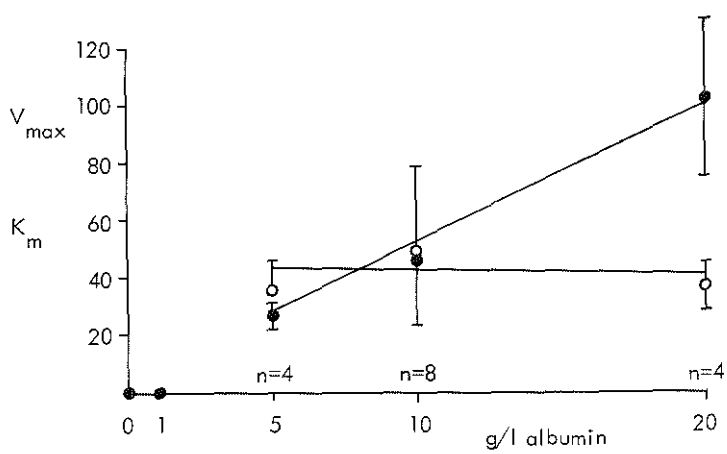

Fig. 3-2. Effect of albumin in the incubation medium on the uptake of T_3 by the high affinity system by primary cultures of parenchymal rat liver cells at 37°C. Each point represents the mean ± SEM of n experiments (each carried out in quadruplicate). (●) Represents V_{max} in pmol.35 µg $DNA^{-1}.min^{-1}$ ($r=0.50$; $p<0.05$) and (o) K_m in nmol/l.

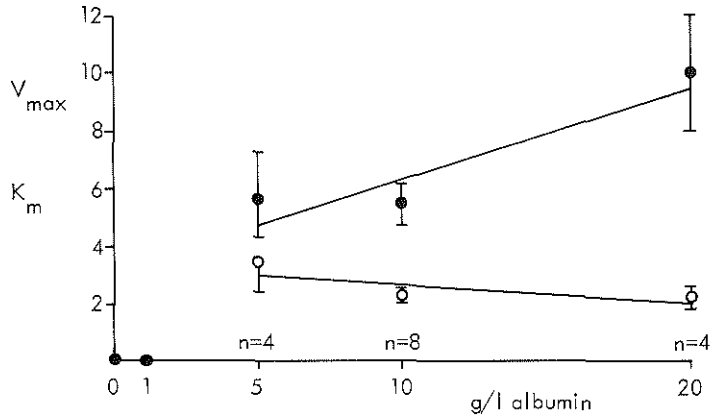

Fig. 3-3. Effect of albumin in the incubation medium on the uptake of T_3 by the low affinity system by primary cultures of parenchymal rat liver cells at 37°C. Each point represents the mean ± SEM of n experiments (each carried out in quadruplicate) (●) Represents V_{max} in nmol.35 µg $DNA^{-1}.min^{-1}$ ($r=0.53$; $p<0.05$) and (○) K_m in µmol/l.

3.3. Effect of 5 and 20 g/l albumin on the velocities of the high and low affinity systems

In addition to the experiment in section 3.2 the velocities of the high and low affinity systems were studied with 5 and 20 g/l albumin in the incubation medium. Uptake of T_3 was measured at two free T_3 concentrations, viz. a low concentration (~12 nM) below the K_m of the high affinity uptake system of T_3 and a high concentration (6.7 µM) above the K_m of the low affinity uptake system of T_3. The net uptake by the high affinity system was obtained by subtracting the contribution of the low affinity system from the total saturable uptake at the low free T_3 concentration. This contribution was measured at the same low free T_3 concentration by adding T_4 in sufficient amounts to block the high affinity system (Krenning et al., 1978).

As is shown in fig. 3-4 a significant increase in uptake of both the high (30.2 versus 13.7 pmol/35 μg DNA/min) and low (7.9 versus 5.9 nmol/35 μg DNA/min) affinity system is found at 20 g/l, as compared to 5 g/l albumin. Since the K_m does not change at the different albumin concentrations (see section 3.2), the differences in uptake between 5 and 20 g/l albumin represent changes in maximal velocity.

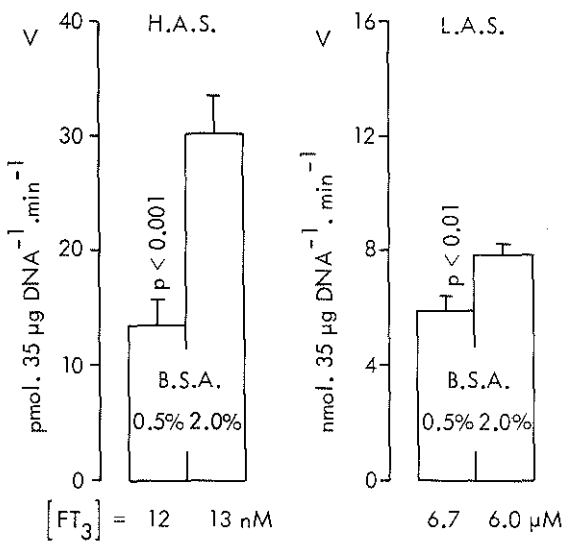

Fig. 3-4. Effect of bovine serum albumin (BSA) in the incubation medium on the uptake velocities of T_3 by the high affinity system (HAS)(at $[FT_3]$~12 nM, after correction for the low affinity system, see text) and low affinity system (LAS)(at $[FT_3]$~6.7 µM) by primary cultures of parenchymal rat liver cells at 37°C. Each bar represents mean ± SEM of 7 experiments (each carried out in quadruplicate).

3.4. Effect of wash procedure with 10% T_3-free serum

After incubation of the monolayer with T_3 in 5 g/l albumin at 37° C, the cells were washed with 10% T_3 free serum and results were compared with the usual procedure where incubation medium without albumin is used for washing the monolayer after incubation (Krenning et al., 1978).

Discrimination of the velocities of both uptake systems was performed as described in section 3.3.

The uptake (mean ± SEM,n=8) by the high affinity system ($[FT_3]$=12 nM) is 14.1 ± 1.7 versus 15.6 ± 3.0 pmol/35 µg DNA/min (control versus wash procedure with 10% T_3 free serum) and by the low affinity system ($[FT_3]$=6.7 µM) 6.2 ± 0.5 versus 4.8 ± 0.6 nmol/35 µg DNA/min ($p<0.05$, paired t-test).

4. Discussion

Two binding sites with different affinities at the plasma-membrane level of hepatocytes of rats have been described for T_3 both in intact cells either in cell suspensions (Rao et al., 1976) or in primary culture (Krenning et al., 1978), and in purified plasma-membranes (Pliam and Goldfine, 1977), and for T_4 both in intact cells (Docter et al., 1978) and in purified plasma-membranes (Gharbi and Torresani, 1979). According to the results in this study albumin influences both diffusion and the V_{max} of the two uptake systems of T_3, without significantly affecting the K_m values. If there is no albumin present in the medium diffusion is low, but increases to a constant level when ≥1 g/l albumin is present. These findings may be explained as follows. With albumin present in

the water layer around the cell, the free T_3 concentration is sufficiently stabilized to ensure optimal diffusion through the cell membrane. However, without albumin or at low albumin concentrations the water layer becomes depleted of T_3 during the uptake process so that the diffusion through the waterlayer becomes rate limiting.

At the two lowest concentrations of albumin studied (zero and 1 g/l) no V_{max} values of either uptake system could be measured, but 5 g/l albumin was sufficient to disclose both saturable uptake processes. The fact that Sorimachi and Robbins (1978) were unable to find a saturable uptake in a similar system as we used may be explained not only on the basis of differences in cell type studied but also by the low albumin concentrations used by these investigators (maximal $\sim$2 g/l). The observation that the K_m values of both uptake systems are not a function of the albumin concentration between 5 and 20 g/l indicates that diffusion through the waterlayer is not rate limiting (Lerner, 1978). The increase in V_{max} values with increasing albumin concentrations suggests that albumin has an additional role in the saturable uptake of T_3 by rat liver cells in this system. The exact mechanism by which albumin exerts this phenomenon is not known at present. A possible explanation for the change in V_{max} of the high affinity system might be the following. Removal by bovine serum albumin of fatty acids from membrane vesicles of Escherichia coli stimulated the active transport of proline (Goto and Mizushima, 1978). A similar effect of albumin could stimulate the high affinity system of T_3, which is suggested to represent a transport mechanism (Krenning et al., 1978).

It is noteworthy that the binding characteristics

of the low affinity systems of T_3 and T_4 (Docter et al., 1978) are similar as reported for their interaction with albumin (Nicoloff, 1978; Hennemann et al., 1979). Therefore, the low affinity system may represent binding of T_3 to membrane-bound or water layer-trapped albumin. From unpublished observations in this study it appeared that $\sim 0.3\%$ of albumin remains attached to the cells after washing with incubation medium without albumin, when the monolayer was pre-incubated with 5-20 g/l albumin medium. This indicates that with increasing amounts of albumin, more albumin remains at the outer cellular surface. The increase in V_{max} as reported for this system may be explained by this increase in the amount of albumin around the cell. Against this explanation is the fact that with the highest T_3 concentrations used in our experiments albumin was not found to be saturated (E.P.K., R.D., F.v.d. Does-Tobe, unpublished) whereas the low affinity system was. Further studies are needed to clarify this problem. Washing the monolayer with medium, containing (T_3 binding) protein, after incubation lowers the uptake via the low affinity system which is in agreement with the suggestion that this system represents binding of T_3 at the cell membrane. As the high affinity system is considered to be involved in active transport through the plasma membrane, it is not surprising that the washing procedure does not affect the amount of hormone taken up by this system.

If the postulate is correct that the high affinity system is indeed the pathway by which iodothyronines are actively transported through the plasma membrane then it should consequently be considered that a gradient with regard to the concentrations of the free hormone exists over the cell membrane. In other

words, that the intracellular free hormone concentration is higher than the extracellular, e.g., free plasma concentration. This possibility may explain the findings (Samuels, 1978) of an $\sim$1 order of magnitude higher affinity of T_3 for the nuclear binding site when this was measured in in vivo experiments in comparison with in vitro studies. The elevated K_a in the in vivo studies may be explained by the fact that for calculation of this parameter the assumption was made that the intracellular free T_3 concentration was equal to that in the plasma. If, however, the intracellular free T_3 concentration appears to be higher than that in the plasma a lower value of the K_a will be calculated.

FEBS LETTERS 119 (1980) 279-282

CHAPTER 4

REGULATION OF THE ACTIVE TRANSPORT OF 3,3',5-TRIIODOTHYRONINE (T_3) INTO PRIMARY CULTURED RAT HEPATOCYTES BY ATP

E.P. Krenning, R. Docter, H.F. Bernard, T.J. Visser and G. Hennemann

1. Introduction

Translocation of thyroid hormone over the plasma membrane of hepatocytes and other cells is an essential step for intracellular deiodination (Visser, 1978) and binding to nuclear receptors (Samuels, 1978) and other intracellular sites (Sterling and Milch, 1975; Visser et al., 1976). The nucleus of the target cell is supposed to be the site of initiation of thyroid hormone action (Tata and Widnell, 1966; Surks et al, 1973). Recent studies show that thyroid hormone binds to isolated plasma membranes of hepatocytes (Pliam and Goldfine, 1977; Gharbi and Torresani, 1979) and is actively transported into hepatocytes by means of a carrier-mediated process (Docter et al., 1978; Krenning et al., 1978; Eckel et al., 1979). Evidence has been presented that T_3 and thyroxine (T_4) are translocated into the cells by different high-affinity, energy-dependent mechanisms which can be blocked by ouabain (Docter et al., 1978). This suggests that a sodium gradient over the cell membrane is essential for transport. In addi-

tion, T_3 and T_4 bind with low affinity to the plasma membrane at different sites (Krenning et al., 1978; 1979). Studies with human erythrocytes (Holm and Jacquemin, 1979) showed similar kinetics of T_3 tranport and ouabain sensitivity as our previously published observations with hepatocytes (Docter et al., 1978; Krenning et al., 1978).

We report that (1) pre-exposure of hepatocytes in monolayer to increasing amounts of T_3 results in a progressive decrease in the active transport of T_3 into the cell. The extent of this diminution is dependent on time and hormone concentration and not on de novo protein synthesis, as cycloheximide does not interfere with this phenomenon. (2) Pre-exposure of the cells to T_3 or fructose effected a decrease in total cellular ATP content. The positive correlation between the transport of T_3 and total cellular ATP content suggests a causative relationship. We postulate that uptake in vivo of thyroid hormone by target cells is dependent on intracellular ATP levels. In pathophysiologic conditions, such as non-thyroidal illness, fasting and hyperthyroidism, low cellular ATP concentrations may counteract further energy expenditure by decreasing cellular T_3 levels and peripheral production of T_3 from T_4.

2. Materials and methods

The procedure of the experiments and calculation of the active transport has been described (Krenning et al., 1978; 1979). After isolation, cells suspended in culture medium (Krenning et al., 1978) with or without added T_3 were inoculated into plastic dishes. Four types of experiments were performed.

Firstly, cells were allowed to attach to the plastic dishes without added T_3. Cells were then pre-exposed to increasing concentrations of T_3 for 15 min. Secondly, cells were pre-exposed to T_3 directly following isolation during the 4h attachment period. Thirdly, cells were cultured for 22h (with a change of culture medium after 7h) in the absence of added T_3 followed by a pre-exposure period of 4h to T_3. Culture medium normally contains 15% fetal calf serum (FCS). In this medium total T_4 concentration is 26 nM and T_3 is not detectable, however at the end of 4h incubation due to intracellular deiodination of T_4 the medium contains 0.55 nM T_3 corresponding to a free concentration of 14 pM. The free T_3 concentration due to the addition of T_3 to this medium amounts to 0, 39, 190 and 1700 pM, respectively. In addition, medium free of T_4 and T_3 was used by replacing FCS by hypothyroid calf serum (HCS) (Rockland, Gilbertsville, PA, USA). Fourthly, after attachment, cells were pre-incubated with modified incubation medium, containing varying concentrations of glucose or fructose. After the pre-exposure period, incubations (for 1 min, at 37° C) were performed essentially as described (Krenning et al., 1978) earlier to measure the transport of T_3. The free T_3 concentration in the incubation medium (Krenning et al., 1978) containing 1% bovine serum albumin, was 9.6 nM, which is 5- to 6-fold lower than the K_m of the transport system (Krenning et al., 1978; 1979). To test whether T_3 is degraded during the uptake experiments, cells were extracted with ethanol after incubation for 1 min with [^{125}I]T_3. The extract was subjected to HPLC (Hearn et al., 1978) and it was found that 93% of total radioactivity eluted in the position of T_3.

For the measurement of ATP, monolayers were treated for 20 min with 2 M perchloric acid at room temperature. Supernatants were stored at -22° C until assayed by the method of Buchner (1947), using the kit of Boehringer Mannheim GmbH.

Statistical evaluation of the difference with group II was performed according to the one way analysis of variance (Snedecor and Cochran, 1967).

3. Results and discussion

As is shown in fig. 4-1 (left panel) a significant progressive decrease in active transport from 21 to 11 pmol/35 μg DNA/min is observed after 4 h pre-exposure of hepatocytes to free T_3 concentrations from zero to 190 pM. Similar results (fig. 4-2, left panel) are obtained if the hepatocytes were cultured for 22h before the 4h pre-exposure to T_3 was started. Very similar results were obtained by adding the given amounts of T_3 to medium containing HCS (not shown).

To our knowledge this is the first report indicating that T_3 regulates its own entry into rat hepatocytes. Such a regulation is a well-known phenomenon for membrane receptors for several hormones, e.g., insulin and glucagon (for a review see Tell et al., 1978).

To study the possible mechanism of the here described auto-regulation of T_3 entry, the following experiments were performed. Instead of a pre-exposure period of 4h, hepatocytes were pre-incubated for 15 min with the same increasing free T_3 concentrations (fig. 4-1, right panel). The here described auto-regulation appears to be time dependent since an insigni-

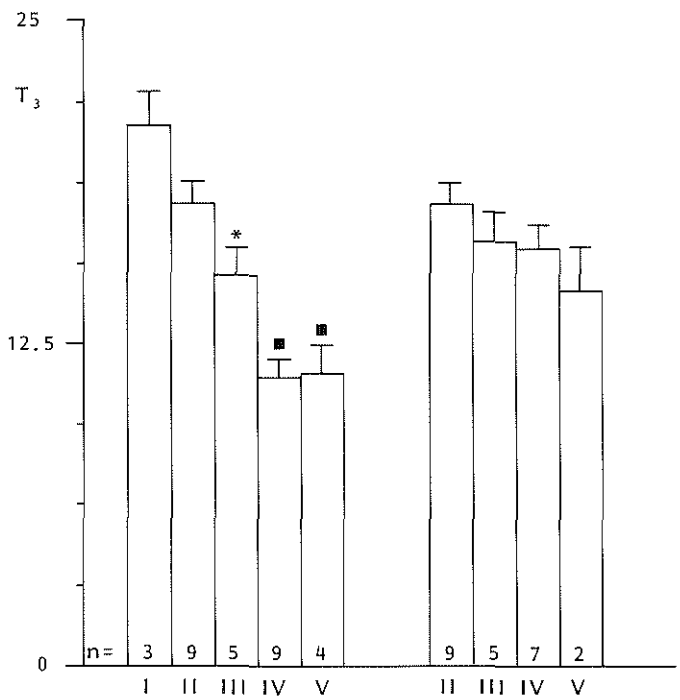

Fig. 4-1. Effect on the active transport of T_3 (in pmol.35µg DNA^{-1}. min^{-1}) into primary cultured rat hepatocytes by 4 hours (left panel) or 15 minutes (right panel) pre-exposure of these cells to increasing amounts of T_3 (medium without T_3 and T_4)(I),II-V represent free T_3 concentrations in culture medium of 0; 39; 190 and 1700 pM, respectively (see Materials and methods). Each bar represents mean ± SEM of n experiments (each carried out in quintuplicate). (* $P<0.05$; and ■ $P<0.001$).

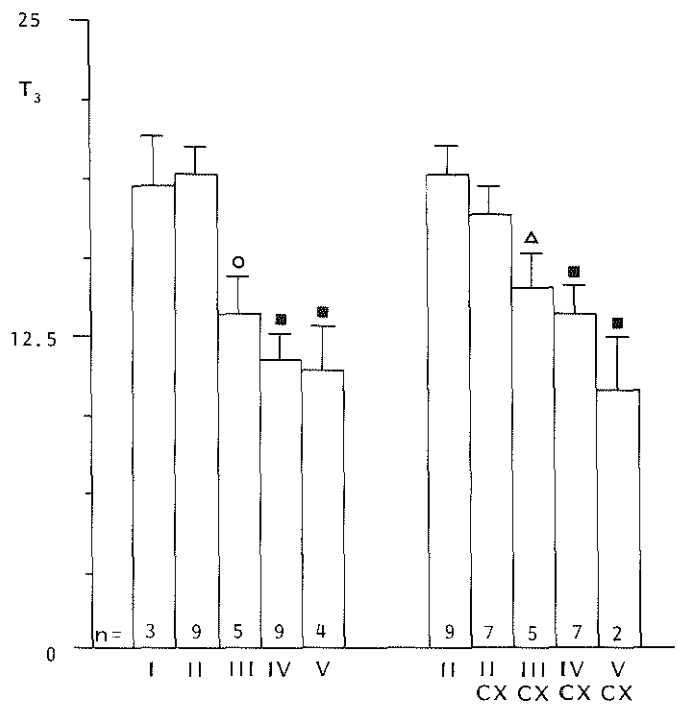

Fig. 4-2. Auto-regulation of the active transport of T_3 in pmol.35µg $DNA^{-1}.min^{-1}$ into primary cultured rat hepatocytes by 4 hours pre-exposure of these cells to increasing amounts of T_3 (see legend of fig. 4-1) in the absence (left panel) or presence (right panel) of 10 µg/ml cycloheximide (CX). This pre-exposure period followed a 22 hours culture period of hepatocytes (obtained from the same rats as used in fig. 4-1) in medium without added T_3. Each bar represents mean ± SEM of n experiments (each carried out in quintuplicate). (Δ $P<0.025$; o $P<0.005$ and ■ $P<0.001$).

ficant decrease (19%) is observed after 15 min compared with up to 50% after 4h pre-exposure. This excludes a direct (e.g., conformational change of the carrier) effect at the membrane level which could lead to desensitization.

Studies with cycloheximide (fig. 4-2, right panel) were performed to investigate whether this autoregulation is dependent on de novo protein synthesis (Pariza et al., 1976). No effect on T_3 transport is observed in cells not pre-exposed to T_3, if protein synthesis is blocked by cycloheximide. This indicates that the turnover of the carrier is very slow with a half-life far beyond 4h. In addition, cycloheximide does not change the effect by pre-exposure to T_3 which suggests that T_3 does not regulate its own transport by the induction of certain proteins.

Previous studies (Docter et al., 1978; Krenning et al., 1978) showed that pre-incubations of the hepatocytes with metabolic inhibitors (KCN, dinitrophenol and oligomycin) blocked the transport of thyroid hormone. Under these conditions a substantial decrease of intracellular ATP concentrations has been found (Krenning et al., 1978) suggesting that ATP is needed for thyroid hormone transport into hepatocytes. Since we could not find an effect by the inhibition of protein synthesis on the auto-regulation reported here we decided to study the effect of pre-exposure to T_3 on the cellular ATP content. Concomitant with the diminution in transport of T_3, a decrease of ATP is observed. A corollary of this study is shown in fig. 4-3. A positive, statistically significant correlation is observed between the cellular ATP content and transport of T_3.

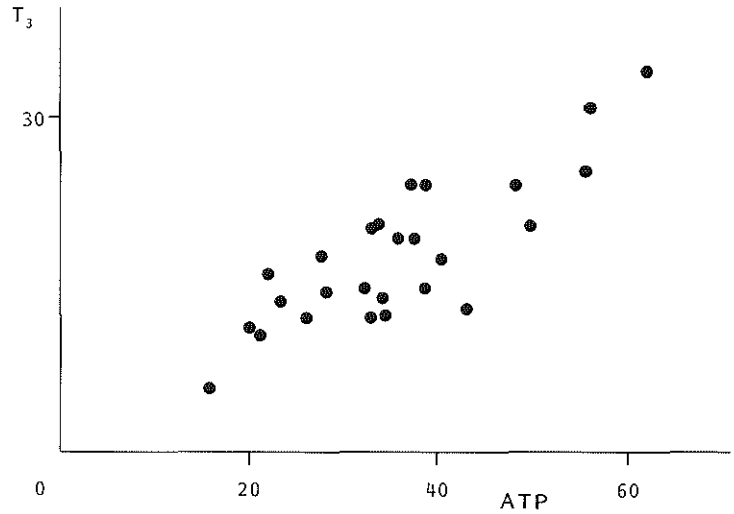

Fig. 4-3. T_3 transport (pmol.35µg DNA^{-1}.min^{-1}) into rat hepatocytes in primary culture as function of cellular ATP content (nmol.35µg DNA^{-1}) (2 experiments; r=0.83, n=26, P<0.001). Both parameters are varied by the pre-exposure of the cells to T_3 as described in Materials and methods.

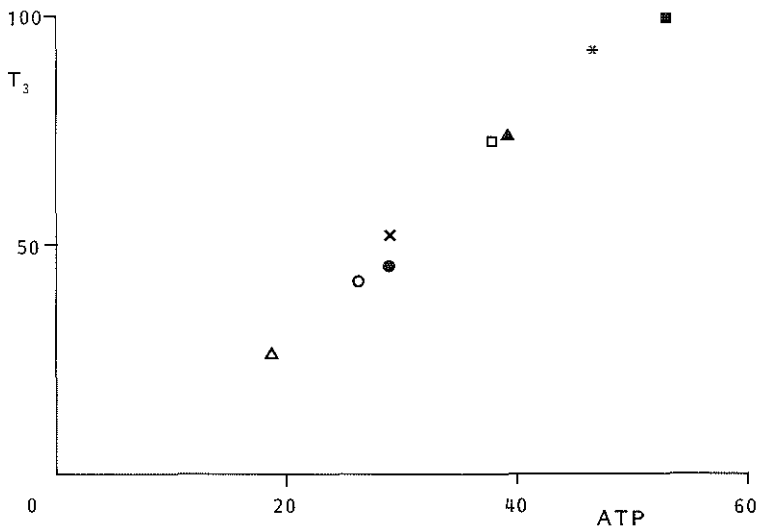

Fig. 4-4. T_3 transport (% of control, i.e., 6.7mM glucose) into rat hepatocytes in primary culture as function of cellular ATP content (nmol.35µg DNA^{-1}) (4 experiments; r=0.99, n=8, P<0.001). Both parameters are varied by the pre-incubation of the cells with fructose (△=10 mM; ○=2 mM; ●=1mM) and glucose (×=0 mM; □=1 mM; ▲=2 mM; ✳=4 mM and ■=6.7 mM) as described in Materials and methods.

If the ATP content of the hepatocytes is varied by pre-incubations with different glucose and fructose concentrations a similar relationship is found (fig. 4-4). Since changes in intracellular ATP levels induced by separate mechanisms are associated with concomitant alterations of T_3 uptake, this strongly suggests that cytoplasmic ATP levels regulate thyroid hormone transport through the plasma membrane.

Our studies (Docter et al., 1978) involving pre-incubations of hepatocytes with ouabain suggested that thyroid hormone transport is coupled to that of sodium by $Na^+ + K^+$-dependent adenosine triphosphatase (Na-K-ATPase). The activity of the latter is a function of the number of enzyme units and the concentration of ATP in the cell. Both rapid (Blanchard and Davis, 1978) and long-term (Edelman, 1974) stimulatory effects of T_3 on Na-K-ATPase have been described. This stimulation cannot be the explanation of our observations of a decreased cellular ATP content since in that case one would expect an augmented sodium gradient and therefore, increased T_3-transport.

The here described (auto-)regulation of T_3 plasma membrane transport is observed in in vitro experiments and its significance in the homeostasis of intracellular thyroid hormone (dependent) metabolism in vivo has to be established. However, in view of decreased intracellular ATP levels in pathophysiologic conditions, a regulatory role of ATP in vivo seems likely. Hyperthyroidism can lead to a negative cellular energy balance in vivo, as determined by the reported decreased hepatic ATP contents in thyroxine-injected rats (Chatagner and Gautheron, 1960) and running down of stores of energy-rich substances in skeletal muscle of thyrotoxic patients (Satoyoshi et al., 1963). Also, in non-thyroidal illness like

shock, anemia and the diabetic state and also during fasting, decreases in cellular ATP concentrations can be found. A decrease of intracellular T_3 in these circumstances will lead to diminution of energy expenditure which may be of protective value in these states of stress. In this context it is worth mentioning that, in critically ill patients, cellular uptake of thyroid hormone appears to be diminished (Kaptein et al., 1980).

Low cellular concentrations of ATP will effect a decrease of intracellular T_3 concentrations in two ways. Firstly, by depressing T_3 transport into the cell and secondly by decreasing cellular production of T_3 from T_4 since T_4 uptake, being also ATP dependent (Docter et al., 1978), would be diminished as well.

The decreased peripheral production of T_3 from T_4 in non-thyroidal illness known as the 'low T_3 syndrome' may not only be explained on the basis of decreased concentrations of reduced glutathione which is the cofactor in the enzymic conversion of T_4 into T_3 (Visser, 1978), but also by reduced cellular ATP levels.

BIOCHIM. ET BIOPHYS. ACTA 676 (1981) 314-320

CHAPTER 5

CHARACTERISTICS OF ACTIVE TRANSPORT OF THYROID HORMONE INTO RAT HEPATOCYTES

E.P. Krenning, R. Docter, H.F. Bernard, T.J. Visser and G. Hennemann

1. Introduction

3,3',5,5'-Tetraiodothyronine (thyroxine) may be considered primarily as a prohormone which will acquire biological activity only after intracellular conversion into 3,3',5-triiodothyronine (triiodothyronine). Biological response to triiodothyronine is initiated by the binding of the hormone to nuclear receptor proteins of the target cell (Samuels, 1978). To reach the cellular compartment thyroxine and triiodothyronine have to cross the membrane of the tissue cell.

In recent studies with purified rat liver plasma membranes the existence of specific binding sites with different affinities for triiodothyronine and thyroxine have been demonstrated (Pliam and Goldfine, 1977; Gharbi and Torresani, 1979). Studies on the uptake of triiodothyronine by freshly isolated rat hepatocytes and by these cells in primary cultures have indicated the presence of an active transport mechanism (Krenning et al., 1978; 1979; Eckel et al., 1979). Parameters concerning the kinetics of triiodothyronine uptake by rat hepatocytes (Krenning et al., 1978) and human red cell ghosts (Holm and

Jacquemin, 1979) appeared to be similar and revealed two saturable sites. One is characterized by high affinity and low capacity (representing the active transport mechanism) and the other by low affinity and high capacity (representing binding to the cell surface). Active triiodothyronine transport into hepatocytes in primary culture is inhibited by thyroxine (Krenning et al., 1978). It is also blocked by ouabain and metabolic inhibitors, pointing to the important role of the ($Na^+ + K^+$) adenosine triphosphatase (($Na^+ + K^+$)-ATPase) and of intracellular ATP (Docter et al., 1978; Krenning et al., 1978). Pre-exposure of hepatocytes to triiodothyronine causes a dose-dependent decrease in cellular ATP levels and in the ability to take up triiodothyronine as assessed by subsequent incubations. Transport activity and cellular ATP content were found to be strictly correlated. When intracellular ATP content was decreased by incubation with different concentrations of fructose and glucose, a similar parallel decrease in triiodothyronine transport was observed. These results suggest a key role of ATP in the translocation of triiodothyronine into the cells (Krenning et al., 1980).

In the light of the above, it seems unlikely that thyroid hormone enters the target cell by diffusion only. In the present work we report on (1) the characteristics of the uptake of thyroxine by rat hepatocytes in monolayer; (2) the mutual inhibition by the substrates for the triiodothyronine and thyroxine transport systems; (3) the effect of ouabain on the uptake processes. A preliminary account of this work has been published (Docter et al., 1978).

2. Experimental

2.1. Materials

The sources of most materials are mentioned in a previous paper (Krenning et al., 1978). Ouabain was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.. The purity of [3',5'-^{125}I]thyroxine and tri[3'-^{125}I]iodothyronine (from the Radiochemical Centre, Amersham, Bucks., U.K.), specific radioactivity over 1200 μCi/μg, was verified by cellulose thin-layer chromatography (Zappi, 1967) using acetone/0.5 M acetic acid, 30 : 70 (v/v), as the solvent.

2.2. Animals and isolation of hepatocytes

Adult male Wistar rats were used in all experiments: the animals had free access to tap water and food. Perfusion of the liver under pentobarbital anaesthesia started between 8 and 8.30 a.m.. The procedure of the perfusion was essentially according to Berry and Friend (1969). In brief, a pre-perfusion was carried out via the portal vein with calcium-free Hanks' solution (Hanks and Wallace, 1949), pH 7.4, for 10 min. This was followed by a perfusion with 0.05% collagenase and 2 mM Ca^{2+} in Hanks' solution for 15 min at 37° C. The pH was continuously monitored and maintained at 7.4 by gassing with a mixture of 5% CO_2 and 95% O_2. The flow rate of the perfusate was 25 ml/min. For preparation of monolayers, about 2x 10^6 cells (approx. 35 μg DNA) in 4 ml culture medium (Ham's F10 (Ham, 1965)/10.6 mM Pipes/11.2 mM Bes/8.9 mM Hepes/12 mU/ml insulin/15% fetal bovine

serum/2 mM $CaCl_2$/10 U/ml penicillin and streptomycin, pH 7.4) were inoculated into 60-mm dishes. After 4 h culture at 37° C in air, the non-attached cells (plating efficiency is 80%, as assessed by DNA determinations) were removed with the medium and the monolayer was washed with 4 ml incubation medium (136.9 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$/6.7 mM glucose, pH 7.4).

Before and after the uptake studies viability was checked by trypan blue exclusion (Mapes and Harris, 1975). Only those experiments are reported in which over 85% of the cells excluded the dye.

2.3. Measurement of thyroid hormone uptake

Incubations of the 4 h cultured monolayers were performed at least in triplicate for 1 min at 21° C with ^{125}I-labelled thyroid hormone and additional amounts of unlabelled hormone in 4 ml incubation medium containing 1% bovine serum albumin. During this incubation the dishes were situated on a tableau rotating at a speed of 10 rev./min at 15°. Incubations were terminated by aspiration of the supernatant and washing the cells with 4 ml ice-cold incubation medium.

For counting of cell-associated radioactivity and DNA determination (Burton, 1956) cell contents were removed after lysis with 0.1 M NaOH, which resulted in recovery of over 99% radioactivity.

Free triiodothyronine and thyroxine concentrations in incubation medium plus 1% bovine serum albumin were estimated by equilibrium dialysis (Hennemann et al., 1971).

2.4. Experimental design

Two types (I and II) of experiments were carried out. In the first, to calculate the kinetic parameters of the saturable uptake of thyroxine, monolayers were incubated with various concentrations of thyroxine (range of free hormone concentration, 0.2 nM-19.2 μM). The nature of inhibition of thyroxine uptake by triiodothyronine was studied by analyzing uptake of thyroxine at the above concentrations in the absence or presence of 110 nM (free) triiodothyronine. Vice-versa, the effect of 4, 13 and 47 nM thyroxine was studied on the uptake of triiodothyronine (range of free concentrations, 2.2 nM - 52.8 μM). The data were analysed by means of a Dixon plot.

In experiment II, uptake was preceded by preincubations with ouabain or metabolic inhibitors (potassium cyanide and oligomycin) in incubation medium for 30 min at 37° C. Preincubations were terminated by aspiration of the supernatant and washing the monolayer with 4 ml incubation medium. Uptake measurements were performed using two concentrations of substrate, i.e., 90 pM and 2.3 μM free thyroxine or 11.1 nM and 1.5 μM free triiodothyronine. To discriminate between high and low-K_m systems, uptake of thyroxine was investigated in the absence or presence of triiodothyronine and vice-versa (see Calculations).

2.5. Calculations

2.5.1. Experiments type I

Uptake of triiodothyronine (Krenning et al., 1978) and thyroxine (this study) as a function of the free

hormone concentration shows a typical curvature indicative of both saturable and non-saturable processes. Correction for non-saturable processes and calculation of the kinetic parameters of the saturable uptake systems of thyroxine and triiodothyronine was done as described previously (Krenning et al., 1978). In brief, saturable or carrier-mediated uptake was calculated by subtracting the rate of non-saturable uptake (approximately equal to the area below the line constructed between the rate of uptake at the highest external substrate concentration and the origin) from the rate of the total uptake. The highest free thyroxine and triiodothyronine concentrations amounted to 19.2 and 52.8 μM, repectively. Analysis of carrier-mediated uptake of thyroid hormone in a Lineweaver-Burk plot revealed two saturable processes (Krenning et al. 1978 and this Chapter). Kinetic parameters of these processes were calculated using a cross-correction method. This involved correction for the contribution of the high-K_m system to the uptake at low substrate concentrations and vice-versa. This was repeated four times to attain constant values of the parameters.

2.5.2. Experiments type 2

In the experiments concerning the effects of metabolic inhibitors and ouabain, the discrimination between the saturable uptake processes was carried out in a different way. The principle of the mutual inhibition by the substrates for the high affinity systems of triiodothyronine (Krenning et al., 1978 and this Chapter) and thyroxine (this Chapter) was used. After preincubation with the metabolic inhibitors or ouabain, two different hormone concentrations

were used to assess uptake: one below the low K_m, the other above the high K_m. To parallel incubations sufficient amounts of triiodothyronine (9.2 μM) or thyroxine (0.72 μM) were added to inhibit the low K_m systems of thyroxine and triiodothyronine, respectively. The difference in uptake by cells exposed to substrate only and those exposed to substrate plus analogue represents uptake via the high affinity system. Uptake at the higher substrate concentration is an estimate of the activity of the high-K_m system, which is not inhibited by the analogue.

Statistical analysis was performed with Student's t-test. Data are presented as the mean $\pm$ S.E.

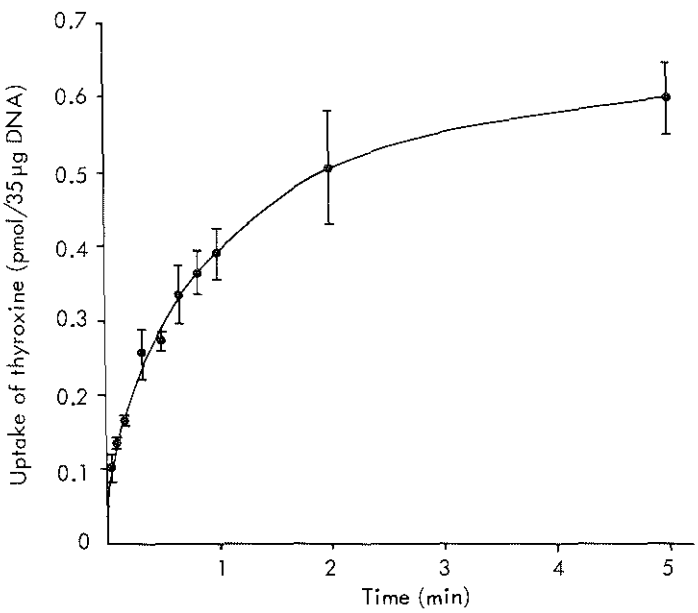

Fig. 5-1. Uptake of thyroxine into primary cultured rat hepatocytes as function of incubation time. For the indicated periods, 4 hours cultured hepatocytes in monolayers were exposed to thyroxine at a free concentration of 39 pM at 21 ^{0}C. After the exposure period monolayers and supernatant were separated. After washing the cells were lysed in 0.1 M NaOH and uptake of thyroxine was measured. Results are from 4 experiments (± SE).

3. Results

3.1. Kinetics of thyroxine uptake by hepatocytes

As is shown in Fig. 5-1, uptake of thyroxine is a rapid phenomenon and a plateau is reached after approx. 5 min. In the following experiments initial velocity has been studied using 1-min incubations.

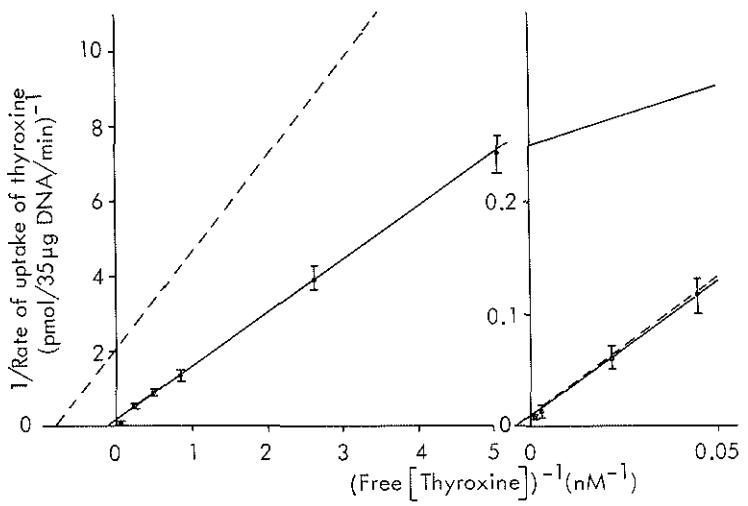

Fig. 5-2. Lineweaver-Burk plot of the uptake of thyroxine into rat hepatocytes in primary culture. The indicated rates of uptake are obtained by subtracting non-saturable uptake from the observed total uptake (see Methods). The lowest point in the left panel represents a cluster of five data points of a second saturable uptake process, shown on a magnified scale at the right. The dashed Lineweaver-Burk plot of the high-affinity system at the left is obtained after cross-correction of the indicated rates of uptake at the left for the contribution of the low-affinity system. At the right, the top line represents the non-cross-corrected uptake via the high-affinity system and the lower two lines the non-corrected and cross-corrected (dashed line) uptake of the low affinity system of thyroxine. Number of experiments is eight (± SE).

Uptake of thyroxine by hepatocytes appears to be a non-linear function of free thyroxine concentration in the medium (not illustrated). After correction for non-saturable uptake a Lineweaver-Burk plot is constructed (Fig. 5-2) which indicates two sites. One is saturated at low concentrations (apparent K_m=1.2 (range 0.5-1.9; n=8)nM) and has low capacity (V=4.7 (1.8-7.6) pmol/35 μg DNA/min) and a second has low affinity (apparent K_m=1.0 (0.4-1.6) μM) and high capacity (V=3.5 (1.85-5.3) nmol/35 μg DNA/min) (Table 5-1).

3.2. Effect of thyroxine on triiodothyronine uptake and vice-versa

Triiodothyronine uptake by the high-affinity system was studied in the presence of 0-47 nM free thyroxine and the data were analysed using the Dixon plot (Fig. 5-3). The results demonstrate that inhibition by thyroxine is competitive. The apparent value for K_i amounts to 46 nM (Table 5-1). Similarly, triiodothyronine is a competitive inhibitor of thyroxine uptake by the high-affinity system, with an apparent K_i of 90 nM (Fig. 5-4 and Table 5-1). Uptake via the high-K_m systems is not affected by the analogue.

TABLE 5-1

KINETIC PARAMETERS OF THYROID HORMONE UPTAKE BY RAT HEPATOCYTES IN PRIMARY CULTURES FOR 1 MIN AT 21°C

For experimental details see Methods and legends to Figs. 5-2, 3 and 4.

	Low-K_m system		High-K_m system	
	K_m (nM)	V (pmol/35 µg DNA per min)	K_m (µM)	V (nmol/35 µg DNA per min)
Thyroxine: n=8	1.2 (0.5-1.9)[a]	4.7 (1.8-7.6)[a]	1.0 (0.4-1.6)[a]	3.5 (1.8-5.3)[a]
K_i of the inhibition by triiodothyronine 90 nM			no inhibition	
Triiodothyronine: n=25	21[b] (9 -29)	16[b] (6 -25)	1.8[b] (1.1-2.5)	3.3[b] (2.0-4.8)
K_i of the inhibition by thyroxine 46 nM			no inhibition	

[a] Representing mean (range); n, number of experiments.
[b] From Krenning et al, 1978.

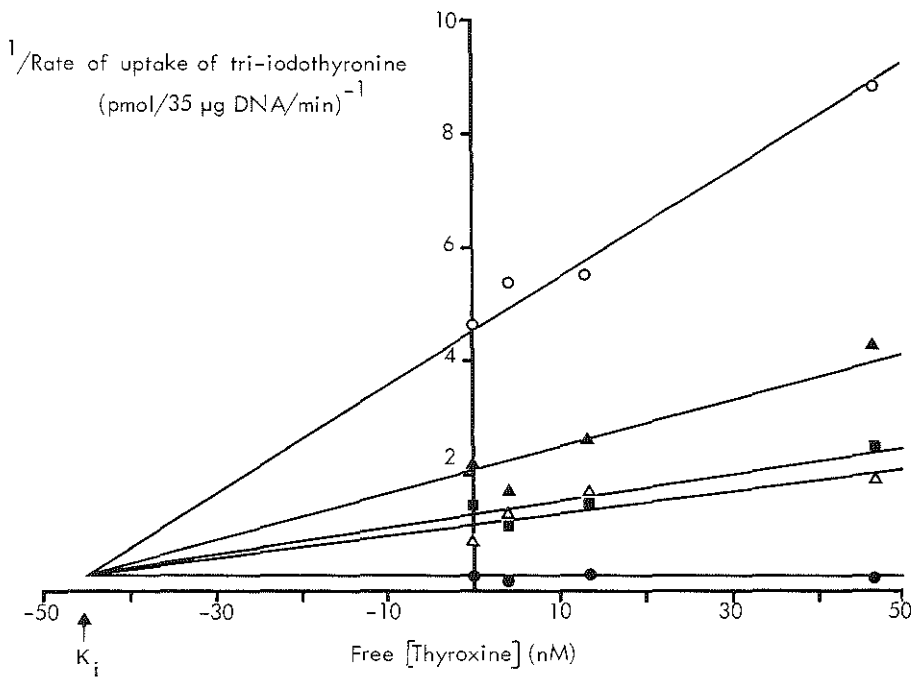

Fig. 5-3. Dixon plot of the inhibition of uptake of triiodothyronine (free concentrations: 2.2(○), 5.5(▲), 11.1(■), 22.1(△) and 111(●) nM) into primary cultured rat hepatocytes by thyroxine (free concentration: 4, 13, and 47 nM). Hepatocytes were incubated with thyroid hormone for 1 minute at 21°C. Data are corrected for non-saturable uptake and cross-corrected for the influence of the two saturable uptake processes on eachother's kinetic data (see Methods). Results are means of at least four experiments.

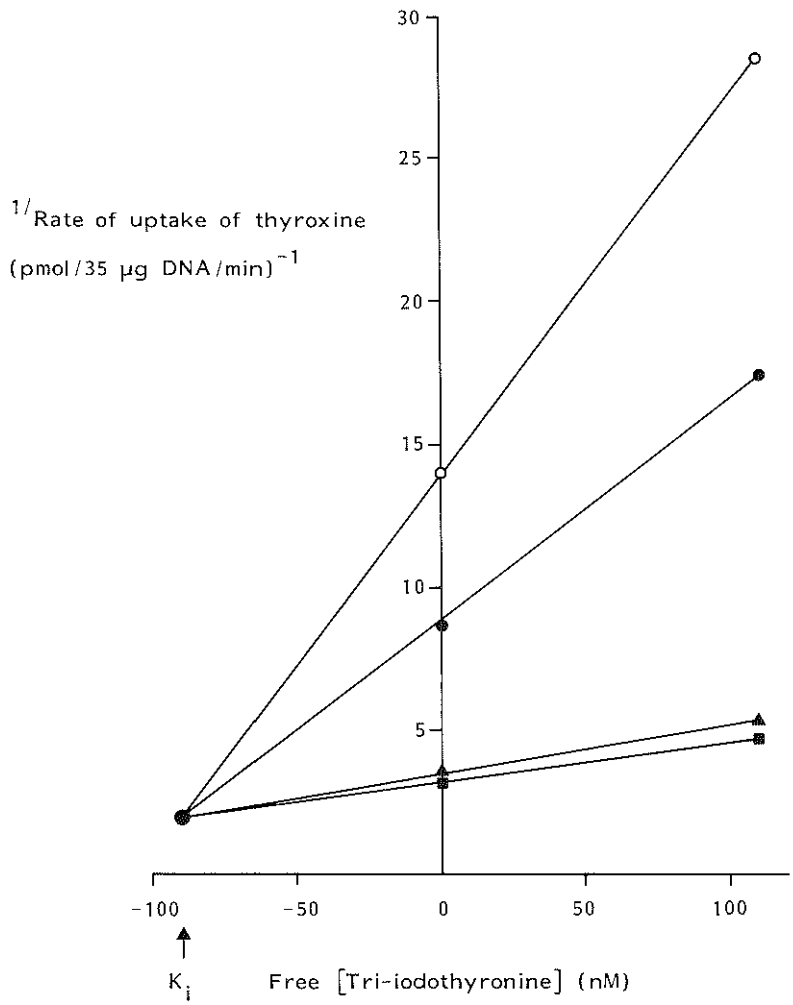

Fig. 5-4. Dixon plot of the inhibition of uptake of thyroxine (free concentrations: 0.2(○), 0.4(●), 1.2(▲), 2.0(■) nM) into primary cultured rat hepatocytes by triiodothyronine (free concentration is 110 nM). Hepatocytes were incubated with thyroid hormone for 1 minute at 21°C. Data are corrected for non-saturable uptake and cross-corrected for the influence of the two saturable uptake processes on eachother's kinetic data (see Methods). Results are means of three experiments.

TABLE 5-2

EFFECT OF PREINCUBATION WITH 2 mM POTASSIUM CYANIDE 0.1 mM OLIGOMYCIN AND 0.5 mM OUABAIN ON THE UPTAKE VELOCITIES OF THYROXINE AND TRIIODOTHYRONINE VIA THEIR HIGH- AND LOW-AFFINITY SYSTEMS OF RAT HEPATOCYTES IN PRIMARY CULTURE

After preincubation of monolayers for 30 min at 37°C, uptake for 1 min at 21°C was carried out at different hormone concentrations. Uptake via the high-affinity system was measured at free concentrations of 90 pM thyroxine and 11.1 nM triiodothyronine, and via the low-affinity system at 2.3 and 1.5 µM, respectively, n.s., statistically not significant (P>0.05), n, number of experiments.

Agent in preincubation	n	Uptake of thyroid hormone via:			
		High-affinity system		Low-affinity system	
		Uptake-low substrate (pmol/35 µg DNA per min)	P	Uptake - high substrate (nmol/35 µg DNA per min)	P
Thyroxine					
Control	9	0.34±0.04		5.4±0.6	
2 mM KCN	6	0.08±0.07	<0.01	5.1±0.9	n.s.
0.1 mM oligomycin	5	0.06±0.08	<0.01	5.4±0.7	n.s.
0.5 mM ouabain	6	0 ±0.07	<0.005	5.1±0.4	n.s.
Triiodothyronine					
Control	4	16.7 ±3.8		1.2±0.1	n.s.
0.5 mM ouabain	8	8.3 ±1.2	<0.025	1.2±0.2	n.s.

3.3. Effect of metabolic inhibitors and ouabain

Preincubation of the monolayers with 2 mM KCN or 0.1 mM oligomycin resulted in a decrease of thyroxine uptake by the low-K_m system by 73% and 80%, respectively (Table 5-2), without affecting uptake by the high-K_m system.

Pretreatment of monolayers with 0.5 mM ouabain (Schwartz et al., 1975), resulted in a decrease in triiodothyronine uptake by 50% and in thyroxine uptake by 100% via their respective high-affinity systems (Table 5-2). The low-affinity systems of both triiodothyronine and thyroxine are unaffected by ouabain.

4. Discussion

This study demonstrates that uptake of thyroxine by primary cultures of rat hepatocytes involves two saturable processes. One system is characterized by high affinity and low capacity, and the second by low affinity and high capacity. Only the high-affinity system is energy dependent, as revealed by the depressed uptake of thyroxine after treatment of hepatocytes with metabolic inhibitors (KCN or oligomycin). Uptake of triiodothyronine by monolayers of rat hepatocytes obeyed similar kinetics (Krenning et al., 1978; 1979). As discussed previously (Krenning et al., 1978; 1979), the low-affinity system probably represents a binding component of the cell membrane. This supposition was based on the following findings: (1) changes in incubation temperature do not affect the V of this system and (2) washing the monolayer with medium, containing triiodothyronine-

binding protein, after incubation with thyroid hormone, removed only that hormone taken up through the low-affinity system. In analogy, the high K_m system of thyroxine may represent binding at the membrane level. In the remainder of this discussion only uptake of thyroid hormone by the high-affinity system will be dealt with and is suggested to represent active transport through the plasma membrane (vide infra).

In general, evidence for active transport is provided by the finding of movement of solutes against concentration gradients (Rosenberg, 1954). In the case of hepatocytes, being rich in intracellular binding proteins and active in the metabolism of thyroid hormone, it is not feasible to estimate the actual free thyroid hormone concentration in the cell and, therefore, an eventual uphill gradient. On the other hand, because of intracellular metabolism, an uphill gradient may be absent despite the presence of active transport. However, in the human red cell ghost, lacking binding proteins and degrading enzymes, an uphill gradient for triiodothyronine has been found with about 10-fold higher concentration inside (Holm and Jacquemin, 1979). Considering the similar kinetic parameters of triiodothyronine uptake, it is very likely that active transport has indeed been studied in hepatocytes. Active transport probably involves the low-K_m system. This is substantiated by the finding that the activity of this system is (1) energy-dependent (i.e. inhibited by oligomycin and KCN); (2) positively correlated with incubation temperature; (3) unaffected by subsequent washing with binding proteins (Krenning et al., 1978); (4) inhibited by ouabain.

Inhibition of the activity of (Na^+ + K^+)-ATPase,

an enzyme localized in the plasma membrane, by ouabain effected decreased transport, pointing to the importance of a sodium gradient, analogous to the transport of other amino acids (Le Cam and Freychet, 1977). A similar inhibition has been found using human red cell ghosts (Holm and Jacquemin, 1979).

Triiodothyronine and thyroxine inhibit each other's transport into rat hepatocytes in a competitive way. The apparent K_i value of inhibition of triiodothyronine transport by thyroxine was found to be 46 nM, whereas the apparent K_m value of thyroxine transport is 1.2 nM. The apparent K_i for triiodothyronine inhibition of thyroxine transport is 90 nM and its apparent K_m value 21 nM These large differences between the apparent K_m and K_i values for both iodothyronines exclude the possibility that triiodothyronine and thyroxine enter the hepatocyte via a common, active transport mechanism. The results suggest that rat liver plasma membranes contain at least two sites for the translocation of thyroid hormone (besides low-affinity binding sites not involved with transport). Thus, it would appear that one carrier preferentially transports thyroxine into the cell, which binds triiodothyronine but transports it not at all or only poorly. The other carrier would then be preferentially involved with the active transport of triiodothyronine, binding thyroxine without appreciably facilitating its entry. However, studying the uptake of triiodothyronine, binding to the thyroxine carrier was not detectable. In the absence of an appreciable triiodothyronine-transport activity, this site may escape detection because of a low (binding) capacity. The same may hold for the apparent lack of binding of thyroxine to the triiodothyronine carrier.

Recent studies have demonstrated that in critical-

ly ill patients cellular uptake of thyroid hormone is diminished (Kaptein, 1980). We have found that membrane transport of iodothyronines is correlated with cellular levels of ATP under a variety of conditions (Krenning et al., 1980). ATP concentrations in the cell are decreased in pathological situations such as the diabetic state, anaemia and shock. We, therefore, propose that in acute and chronic illness the impaired cellular uptake of thyroid hormone is secondary to the reduction of ATP levels. This may serve a dual protective mechanism: firstly, by preventing thyroxine from entering the cell with subsequent conversion into triiodothyronine, and secondly, by interfering with the interaction of triiodothyronine with intracellular receptors, because of diminished uptake of circulating triiodothyronine. This reduction of intracellular triiodothyronine may serve as an energy-conserving process in sick patients.

Fasting may also result in diminished ATP levels in the cell (Knoche and Hartmann, 1961; Jeejeebhoy et al., 1980). Interestingly, using rat liver perfusions, Jennings et al. (1979) have demonstrated that hepatic thyroxine uptake is decreased in fasting by 40%. In illness and during fasting conversion of thyroxine into triiodothyronine is decreased (Braverman and Vagenakis, 1979). Apparently, besides the tissue deiodinase activity, the membrane transport mechanism may play an important role in the regulation of thyroid hormone action and metabolism.

5. Summary

Thyroid hormone uptake into primary cultured rat hepatocytes was studied using 1-min incubations with

radioiodine-labelled iodothyronines. (1) Uptake of thyroxine indicates two saturable sites with apparent K_m values of 1.2 nM and 1.0 μM, and non-saturable uptake. Similar kinetics of triiodothyronine uptake have been observed. (2) The high-affinity systems of both hormones are energy-dependent (i.e. inhibited by KCN and oligomycin). It is postulated that these systems represent active transport of thyroid hormone into the cell. (3) Analysis of mutual inhibition by the substrates for the triiodothyronine and thyroxine transport systems indicates that triiodothyronine and thyroxine cross the cell membrane via separate transport systems. (4) Preincubation with ouabain resulted in a decrease in uptake of both triiodothyronine and thyroxine, suggesting that a sodium gradient is essential for this transport.

FEBS LETTERS 140 (1982) 229-233

CHAPTER 6

DECREASED TRANSPORT OF THYROXINE (T_4), 3,3',5-TRIIODOTHYRONINE (T_3) AND 3,3',5'-TRIIODOTHYRONINE (rT_3) INTO RAT HEPATOCYTES IN PRIMARY CULTURE DUE TO A DECREASE OF CELLULAR ATP CONTENT AND VARIOUS DRUGS

E.P. Krenning, R. Docter, H.F. Bernard, T.J. Visser and G. Hennemann

1. Introduction

T_4, the main secretory product of the thyroid gland, is deiodinated and conjugated in peripheral tissues (Schimmel en Utiger, 1977; Visser, 1980a). Phenolic ring deiodination of T_4 accounts for $\sim$80% of the total body production of T_3, the most biologically active iodothyronine (Chopra, 1978). The remainder is produced by the thyroid gland. The other main product of peripheral deiodination is rT_3, which is biologically inactive (Jorgensen, 1976). Initiation of biological activity by T_3 occurs after binding to nuclear receptors (Samuels, 1978). At least 70% of the liver nuclear bound T_3 is derived from the extracellular compartment and the remainder from local, intracellular deiodination of T_4 (Larsen, 1982). The liver, which contains $\sim$30% of the total T_4 pool and 80% of all intracellularly located T_4, is an important organ for the production of thyroid hormone metabolites (Hennemann, 1981). Studies related to membranal transport of iodothyronines into hepatic cells

are important since they may increase our understanding of regulatory mechanisms involved in the ultimate delivery of thyroid hormone to intracellular active sites like metabolizing enzymes and receptors.

Uptake of T_3 and T_4 by rat hepatocytes in primary culture is mediated by distinct, ATP-dependent processes (Krenning et al., 1981). Additionally, they are saturably bound to the surface of the plasma membrane (Krenning et al., 1978; 1979; 1981). Study of the binding of T_3 and T_4 to purified rat liver plasma membranes has revealed 2 sets of saturable sites for both hormones of which the highest affinity components do not seem to reflect a common binding site (Pliam and Goldfine, 1977; Gharbi and Torresani, 1979;1981). In harmony with the concept of an active transport mechanism for the uptake of thyroid hormone by cells is the visualization by video intensification microscopy of receptor-mediated endocytosis of fluorescent T_3 in cultured fibroblasts (Cheng et al., 1980; Maxfield et al., 1981). We report here on a difference in ATP-dependency of the uptake of T_3 on the one hand and that of T_4 and rT_3 on the other by rat hepatocytes in primary culture. T_4 and rT_3 showed the most remarkable diminution of transport by small decreases in cellular ATP content. In addition, effects of propranolol, X-ray contrast agents, amiodarone and cytoskeleton-disrupting agents have been studied. The results indicate that besides changes in T_4 deiodination (Schimmel and Utiger, 1977; Visser, 1980) and sulfoconjugation (Sato and Robbins, 1981; Otten et al., 1981), decrease in cellular uptake of T_4 by tissues secondary to decreased cellular ATP concentrations or to the effects of some compounds may be a contributing factor to the clinical condition known as low T_3 syndrome. A prelimina-

ry account of this work has been published (Krenning et al., 1981a)

2. Materials and methods

The sources of most materials are mentioned previously (Krenning et al., 1978; 1981). The purity of 3,[3',5'-^{125}I]-triiodothyronine (The Radiochemical Centre, Amersham), spec.act. >1200 μCi/μg, was verified by HPLC (Hearn et al., 1978). The following drugs were generous gifts: sodium ipodate (Schering, Berlin), sodium tyropanoate and iopanoic acid (Sterling-Winthrop Labs, NY), D- and L-propranolol (ICI, Rotterdam) and amiodarone (La Baz, Maassluis). Inhibitors of the cytoskeleton (vinblastine, colchicine, cytochalasin B) were purchased from Sigma (St. Louis, MO).

The experimental details of isolation (collagenase perfusion technique), culture and incubations of rat hepatocytes with thyroid hormone are described in a previous paper (Krenning et al., 1981). Uptake was studied in rat hepatocytes in monolayer culture (2 x 10^6 cells/dish) and were performed in quadruplicate/expt by incubations for 1 min at 37^{o} C with ^{125}I-labelled thyroid hormone and additional amounts of unlabelled hormone in 4 ml incubation medium (Krenning et al., 1981) containing 1% (for uptake of T_3 and rT_3) or 0.5% (T_4) bovine serum albumin. In some experiments, uptake studies were preceded by incubations in incubation medium for 30 min with various concentrations of glucose or fructose to decrease the cellular ATP content (Krenning et al., 1980), 1 and 10 μM propranolol, 11 μM vinblastine, 25 μM colchicine or 25 μM cytochalasin B. In other experi-

ments uptake was studied in the presence of iopanoic acid, sodium tyropanoate, sodium ipodate (10 and 100 μM) or amiodarone (1 μM).

The procedure of calculation of transport was based on the principle of the mutual inhibition by substrates for their energy-dependent uptake systems as described previously (Krenning et al., 1981). Unpublished observations (E.P.K., R.D., H.F.B.) showed transport of reverse T_3 with K_m 6 nM at 37° C, which can be inhibited by T_3. To parallel uptake studies of T_4, T_3 and rT_3 the following free amounts of iodothyronines were added to inhibit transport of the particular iodothyronine (in parentheses): 6.8 μM T_3 (T_4); 0.8 μM T_4 (T_3); and 2.9 μM T_3 (rT_3). The difference in total uptake and uptake in the parallel incubation yields a measure of the active transport. Measurements of free hormone concentrations and ATP content was done as mentioned in a previous paper (Krenning et al., 1981).

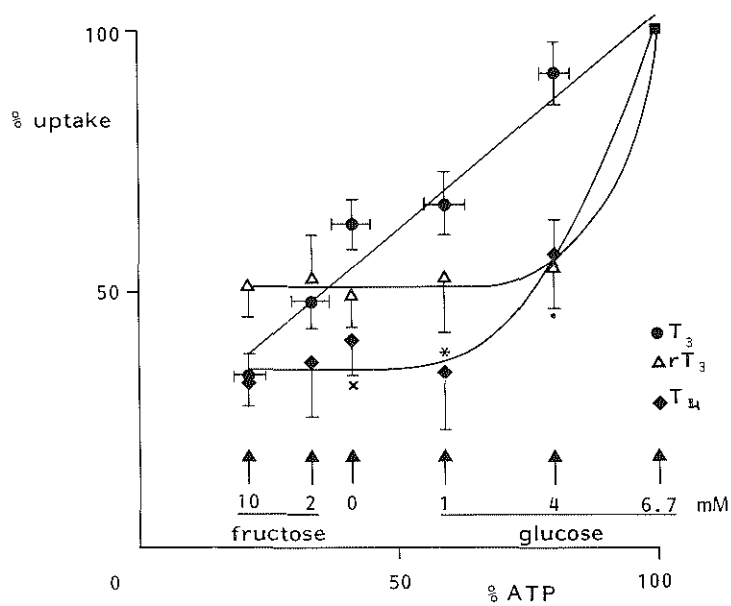

Fig. 6-1. Active transport (in % of control) of iodothyronines into rat hepatocytes in primary culture as function of cellular ATP content (in % of control). Values from incubations with 6.7 mM glucose are expressed as 100 %. The absolute control values amounted to 0.67 T_4, 36 T_3 and 3.8 rT_3 pmol.35 µg $DNA^{-1}.min^{-1}$ and 64 nmol ATP/35 µg DNA. ATP content was varied by pre-exposure of the cells to the indicated concentrations of glucose or fructose. Thereafter the monolayers were exposed to the following free hormone levels for 1 minute at 37^0C: 0.1 nM T_4, 9 nM T_3 and 1.2 nM rT_3. Each uptake value represents the mean ± SEM of ≥6 expts (in quadruplicate) and ATP values are from ≥16 expts (at least in duplicate). Statistical evaluation of T_3 and T_4 transport with Student's t-test results in: •$p<0.005$; ∗$p<0.025$; ×$p=0.025$.

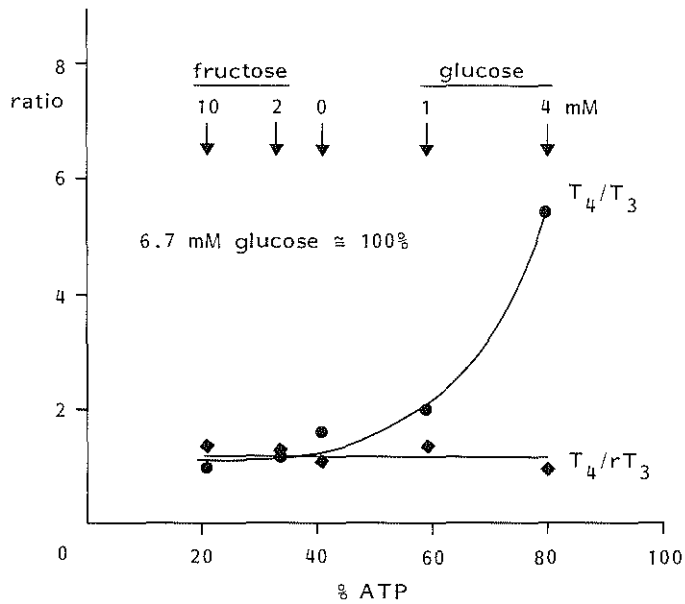

Fig. 6-2. Ratios of the amount of inhibition of T_4-T_3 transport and T_4-rT_3 transport as funtion of cellular ATP content. Data are taken from fig. 6-1.

3. Results

3.1. Effect of changes in ATP content on T_4, T_3 and rT_3 transport

Monolayers of hepatocytes were preincubated for 30 min with different concentrations of glucose or fructose to vary the ATP content of the liver cells (fig. 6-1). A curvilinear relationship is found between transport of T_4 or rT_3 and ATP, in contrast to a linear correlation with T_3. This transport was studied with hormone concentrations which are below the K_m of the transport system of the particular iodothyronine (Krenning et al., 1981; this paper) to study transport in the absence of saturation of the uptake mechanism. The interrelationships between T_4 and T_3 transport and between T_4 and rT_3 transport are shown in fig. 6-2. In fig. 6-2 the ratios of the percentage of inhibition of T_4 transport relative to that of T_3 or rT_3 are calculated and related to the ATP content. For instance, with a preincubation with 4 mM glucose, the fall in ATP content compared to control is 20%. This resulted in $\sim$5-times more inhibition of T_4 transport than of T_3 (ratio is 5), whereas the inhibition of T_4 and rT_3 transport is about equal (ratio is 1.2, which value does not change with lower ATP levels). Thus, it seems that T_4 and rT_3 transport are in a similar way dependent on ATP concentration in contrast to T_3 transport and it is the small decrease in cellular ATP content, that results in a greater inhibition of T_4 and rT_3 transport compared to T_3.

3.2. Effects of structurally related compounds on T_4 and T_3 transport

Uptake of thyroid hormone in the presence of 10 or 100 μM sodium ipodate, sodium tyropanoate, iopanoic acid or 1 μM amiodarone results in a statistically significant decrease in transport of T_3 and T_4 (table 6-1). A lower concentration of bovine serum albumin in the incubation medium is used for T_4 uptake studies to increase the percentage uptake, as the free fraction of T_4 is much lower compared to T_3 or rT_3. This may have implications for the free amounts (not tested) of the above compounds, and therefore comparison of the decreases in T_3 and T_4 transport by these compounds in this experiment is not opportune.

3.3. Effects of D- and L-propranolol and inhibitors of the cytoskeleton function on T_3 and T_4 transport

Pretreatment of monolayers for 30 min with vinblastine, colchicine or cytochalasin B leads to a diminished transport of T_3 and T_4 (table 6-1). Similarly, the isomers of propranolol show this effect (table 6-1). These agents are able to decrease the cellular ATP content markedly (table 6-1). When similar ATP-decreases were compared between the experiments as illustrated in fig. 6-1 and table 6-1 no significant differences in inhibition of T_4 transport were present. However, T_3 transport is significantly more diminished (one way analysis of variance (Snedecor and Cochran, 1967), all p levels <0.025) in the experiments from table 6-1.

TABLE 6-1

EFFECT OF PREINCUBATION FOR 30 MIN WITH D, L-PROPRANOLOL, VINBLASTINE, COLCHICINE AND CYTOCHALASIN B AND OF INCUBATION FOR 1 MIN WITH SODIUM TYROPANOATE, SODIUM IPODATE, IOPANOIC ACID AND AMIODARONE ON THE ACTIVE TRANSPORT OF T_4 AND T_3 INTO RAT HEPATOCYTES IN PRIMARY CULTURE

Agent	µM	T_4			T_3			ATP		
		% control	(n)	SEM	% control	(n)	SEM	% control	(n)	SEM
Sodium tyropanoate	10	51	(5)	8[a]	53	(4)	21[a]			
Sodium tyropanoate	100	19	(5)	13[a]	39	(6)	11[a]	n.s.		
Iopanoic acid	10	58	(5)	11[b]	57	(3)	10[a]			
Iopanoic acid	100	22	(5)	14[a]	44	(5)	14[a]	n.s.		
Sodium ipodate	10	49	(5)	9[a]	53	(3)	13[a]			
Sodium ipodate	100	32	(4)	10[a]	45	(5)	4[a]	n.s.		
Amiodarone	1	20	(3)	9[a]	31	(3)	8[a]	n.s.		
Vinblastine	11	26	(3)	13[a]	45	(4)	7[a]	43	(4)	5
Colchicine	25	14	(3)	2[a]	34	(5)	5[a]	37	(4)	8
Cytochalasin	25	17	(3)	2[a]	23	(4)	9[a]	37	(4)	7
D-Propranolol	1	43	(6)	7[a]	45	(4)	8[a]	42	(3)	5
D-Propronalol	10	19	(6)	7[a,c]	23	(7)	6[a,d]	41	(4)	6
L-Propranolol	1	44	(6)	7[a]	53	(4)	13[a]	45	(3)	12
L-Propranolol	10	21	(6)	4[a]	30	(5)	7[a,e]	39	(4)	8

[a,b]p-Values for difference with control ([a] $p < 0.001$, [b] $p < 0.005$)
[c,d]Difference with 1 µM D-isomer ([c] $p < 0.05$; [d] $p < 0.001$)
[e]Difference with 1 µM L-isomer ([e] $p < 0.01$); n.s., not significant

The free hormone concentrations used are mentioned in fig.6-1, and are below the K_m of the transport system. Statistical evaluation was performed according to the one way analysis of variance. (Snedecor and Cochran, 1967) n, no expt (in quadruplicate)

4. Discussion

By kinetic analysis of their mutual inhibition of transport, active transport of T_3 and T_4 were shown to take place via different pathways (Krenning et al., 1981). According to these results, the ATP dependency of these transport mechanisms is different, such that small decreases in ATP content result in a larger inhibition of T_4 transport and after a certain amount of decrease in ATP, T_4 transport becomes ATP-independent. Unpublished observations (E.P.K., R.D., H.F.B.) have shown that similarly to T_4 (Krenning et al., 1981) and T_3 (Krenning et al., 1978), rT_3 is taken up by a high affinity system ($K_m \sim 6$ nM), which is energy-dependent (this study). Transport of T_4 and rT_3 are in a similar way dependent on ATP, which might indicate that T_4 and rT_3 share a common transport pathway. Apparently, in addition to the intracellularly located deiodinase activity (Schimmel and Utiger, 1977; Visser, 1980) and conjugating enzymes (Sato and Robbins, 1981; Otten et al., 1981), rat hepatocytes in primary culture are able to regulate the peripheral metabolism of iodothyronines by influencing the rate of their entry. Diminished entry of T_4 and rT_3 by a lowered ATP content in (patho-)physiological conditions results in decreased production of T_3 and degradation of rT_3, respectively. In concert with a diminished activity of the 5'-deiodinase activity in various conditions (Braverman and Vagenakis, 1979), these differences in transport can play a role in the genesis of the alterations of serum iodothyronine concentrations in the low T_3 syndrome. In T_4 perfusion of livers from fasted rats, T_4 uptake was diminished up to 40% (Jennings et al., 1979). Many compounds (Vagenakis,

1981) (e.g. X-ray contrast agents, propranolol, amiodarone) can induce a low T_3 syndrome, which has been explained by inhibition of the activity of 5'-deiodinase. However, 50% inhibition of this activity in isolated hepatocytes (Van Noorden et al., 1979) or homogenates (Fekkes et al., 1982) has been achieved with $\sim$ 500 μM D,L-propranolol, $\sim$ 3 orders of magnitude higher than in vivo plasma levels of subjects given the usual therapeutic dosage (Singh, 1973). Furthermore, a membrane-stabilizing effect seems to be achieved with concentrations of propranolol, which are $\sim$ 1 order of magnitude higher than the usual therapeutic plasma level (Singh, 1973). This makes it less probable that the observed effect of therapeutic amounts of D-propranolol (which is devoid of β-blocking activity) on T_3 formation from T_4 in T_4 substituted subjects is merely by this membrane stabilizing action, as suggested by Heyma et al. (1980;1980a). This suggestion was based on the fact that quinidine inhibited the formation of T_3 in a similar way. The ATP-lowering effect of local anaesthetics (including quinidine) is described by Montecucco et al.(1981). Pretreatment of monolayers of hepatocytes with D- and L-propranolol in the therapeutic concentration range, results in a marked lowering of the ATP content and in an inhibition of T_4 and T_3 entry by $\geqslant$ 50%. Consequently, at first sight it seems that the common denominator of the observed similar effects of both isomers is diminished active transport of iodothyronine by reduced ATP levels. However, at similar ATP levels, inhibition of T_3 transport by propranolol is more pronounced as compared to control. The reason for this finding is unclear.

Similarly, X-ray contrast agents and amiodarone

added to the incubation medium with T_4 or T_3 lead to a decreased uptake in our system. Because of the structural similarities between these compounds and thyroid hormone, their effect can be explained by competitive inhibition of uptake, as has been shown for T_4 conversion (Fekkes et al., 1982a).

X-ray contrast agents are able to inhibit uptake of T_4 and T_3 by rat liver slices (Green and Bellamy, 1977). In vivo, sodium tyropanoate can displace T_4 from the liver (Felicetta et al, 1980) and provoke an acute increase in serum T_4 (Suzuki et al., 1979). In addition to displacement from liver cytosol binding proteins, competitive inhibition of inward transport of T_4 through the plasma membrane may be another effect of tyropanoate (Felicetta et al., 1980).

In many plasma-membrane processes, like carrier-mediated transport, the cytoskeleton plays an essential role, and can be perturbed by agents like colchicine, vinblastine and cytochalasin B. In our system these agents decreased thyroid hormone transport too, suggesting a direct relation of the uptake mechanism and the cytoskeleton. However, these compounds decreased the ATP content as well, which in part may also explain our findings. Both effects are in agreement with Cheng et al.(1980) and Maxfield et al.(1981), that the mechanism by which thyroid hormone is taken up is endocytosis, which is an energy-, cytoskeleton-dependent (and time-consuming) process (Pastan et al., 1981).

J. ENDOCRINOL. INVEST. 6 (1983) 59-66

CHAPTER 7

PLASMA MEMBRANE TRANSPORT OF THYROID HORMONE: ITS POSSIBLE PATHOPHYSIOLOGICAL SIGNIFICANCE

E.P. Krenning, R. Docter, T.J. Visser
and G. Hennemann

1. Introduction

Thyroid hormone has a wide spectrum of activities on several tissues, most importantly those related to the economy of energy production and expenditure. The main secretory product of the thyroid, thyroxine (T_4) has little, if any, intrinsic metabolic activity (Surks and Oppenheimer, 1977). Most of the in vivo effects of T_4 can be explained by its conversion to 3,3',5-triiodothyronine (T_3) in peripheral tissues (Braverman et al., 1970). Another product of the peripheral deiodination of T_4 is the biologically inert 3,3',5'-triiodothyronine (reverse T_3, rT_3) (Jorgensen, 1976). Normally, extrathyroidal conversion accounts for approx. 80% of total T_3 production and an even greater fraction of total rT_3 production in humans as well as in rats (Chopra, 1976; Chopra et al., 1978a). Most of the action of T_3 is initiated by binding to a nuclear, chromatin-associated receptor (Oppenheimer et al., 1976), although direct effects on mitochondria (Sterling and Milch, 1975) and plasma membranes (Goldfine et al., 1976; Segal et al., 1977; Blanchard and Davis, 1978) have also been reported. Recent studies, primarily by the

group of Larsen, have pointed to differences between tissues with regard to the origin of nuclear T_3 (Larsen, 1982). Thus, in the pituitary 50% and in the central nervous system 80% of nuclear T_3 is derived from local $T_4 \rightarrow T_3$ conversion, the remainder being taken up from the circulation. Probably due to the rapid exchange between the plasma and tissue compartments, a larger proportion of nuclear T_3 in the liver and the kidneys is derived from the circulation with a negligible contribution from local $T_4 \rightarrow T_3$ conversion. That does not alter the fact that the latter tissues are more important sites for the total body production of T_3 (Chopra, 1977). It follows that passage of T_4 and T_3 through the plasma membrane plays an important part in the ultimate delivery of T_3 to nuclear receptors. It has been postulated that thyroid hormone enters cells by passive diffusion (Freinkel et al., 1957; Lein and Dowben, 1961). This assumption was borne in by the lipophilicity of the hormone which would therefore easily pass the lipid bilayer of the plasma membrane. Increasing evidence has been presented from different laboratories that, at least in part, thyroid hormone is transported through the plasma membrane by an energy dependent, saturable process (Rao et al., 1976; Parl et al., 1977; Krenning et al., 1978; 1981; 1982; Cheng et al., 1980; Horiuchi et al., 1982; Halpern and Hinkle, 1982). It is the purpose of this review to discuss recent studies with regard to transport of T_4, T_3 and rT_3 across the cell membrane. The results of in vitro and in vivo studies suggest a regulatory role of plasma membrane transport in the metabolism of thyroid hormone. In this review it will be attempted as well to indicate the possible implications of an altered plasma membrane transport in relation

to the pathophysiology of thyroid hormone metabolism.

Clinical conditions, like non-thyroidal illness, starvation or the use of compounds such as propylthiouracil (PTU), dexamethasone, propranolol and X-ray contrast agents, may induce the so-called low T_3 syndrome (Vagenakis, 1981). This syndrome is characterized by elevated rT_3 and decreased T_3 serum levels, while serum total T_4 concentrations usually are normal. Studies of the turnover kinetics of injected tracers, if performed, have revealed that the production of T_3 is decreased, while its clearance is unchanged (Vagenakis, 1981). Conversely, production of rT_3 is normal, but its clearance is diminished. This has been explained on the basis of a diminished 5'-deiodinase activity of the tissues resulting in decreased T_3 production from T_4 and reduced deiodinative breakdown of rT_3 (Vagenakis, 1981). It seems , however, quite possible that changes in transport of thyroid hormone across the plasma membrane may be of relevance in the development of this syndrome.

2. In vitro studies

2.1. Thyroid hormone binding to purified plasma membranes

Plasma membrane fractions of the liver, kidney and testis of the rat possess two sets of binding sites for both T_4 (Gharbi and Torresani, 1979; 1981) and T_3 (Pliam and Goldfine, 1979; Gharbi-Chihi and Torresani, 1981), except for a single site for T_3 in the testis. In purified plasma membranes of the spleen no binding sites are detectable (Gharbi-Chihi and Torresani, 1981). As testis and especially spleen

function are not greatly influenced by thyroid hormone (Oppenheimer et al., 1974) there appears to be a relationship between the presence of membrane binding sites in a given tissue and its responsiveness to thyroid hormone (Gharbi-Chihi and Torresani, 1981). The results of these binding studies are summarized in Table 7-1. Based on mutual competition experiments high affinity binding of T_4 and T_3 seems to occur to different sites (Gharbi-Chihi and Torresani, 1981). It was suggested that these binding sites are involved in transport of thyroid hormone into the cell and/or hormone action (Pliam and Goldfine, 1979; Gharbi-Chihi and Torresani, 1981).

TABLE 7-1
THYROID HORMONE BINDING TO PURIFIED PLASMA MEMBRANES

Tissue	Dissociation constants of binding (K_d)		
	Thyroxine	3,3',5-Triiodothyronine	Ref.
rat liver	n.t.	3.2 and 220 nM	Pliam and Goldfine, 1979
rat liver	0.4 and 23 nM	n.t.	Gharbi and Torresani, 1979
rat liver	0.6 and 23 nM	9.7 and 237 nM	Gharbi and Torresani, 1981
rat spleen	n.d.	n.d.	ibid.
rat kidney	4.5 and 127 nM	16 and 270 nM	ibid.
rat testis	28 and 286 nM	266 nM	ibid.

n.t. = not tested
n.d. = not detectable

3. Thyroid hormone uptake into isolated cells of animal tissues

3.1. Triiodothyronine

Kinetics of the initial uptake velocities of tracer T_3 into rat liver parenchymal cells in primary culture reveals the presence of 2 saturable sites designated as a high-affinity system ($HAST_3$) and a low affinity system ($LAST_3$), respectively (Table 7-2) (Krenning et al., 1978).

$HAST_3$ is blocked when cells are preincubated with metabolic inhibitors such as KCN, dinitrophenol or oligomycin pointing to the energy-dependence of this system. $HAST_3$ appears to be strongly influenced by temperature. An increase in incubation temperature induces an increase in the apparent dissociation constant and capacity of this system. Both energy and temperature dependence strongly suggests that $HAST_3$ represents a transport process. Finally, it has been shown that $HAST_3$ is inhibited by ouabain, a specific inhibitor of Na^+, K^+ - ATPase activity, indicating that a sodium gradient over the plasma membrane may be of importance, analogous to the membrane transport of other aminoacids (Krenning et al., 1981). No energy or important temperature dependence of the $LAST_3$ is observed (Table 7-2) and it has been suggested that $LAST_3$ represents binding of T_3 to albumin either bound to the cell membrane or trapped in the surrounding water layer (Krenning et al., 1978; 1979). Incubations with increasing albumin concentrations (5-20 g/l) results in a linear increase of the "V_{max}" of both $HAST_3$ and $LAST_3$. This finding supports the role of albumin in the binding process and also suggests a function in the transport mechanism (Krenning

TABLE 7-2

CHARACTERISTICS OF INITIAL UPTAKE OF 3,3',5-TRIIODOTHYRONINE (T_3), 3,3',5'- TRIIODOTHYRONINE (RT_3) AND THYROXINE (T_4), BY RAT HEPATOCYTES IN PRIMARY CULTURE

	LAS_{T_3}		HAS_{T_3}	
	K_m (μM)	V_{max} (nmol/35μg DNA/min)	K_m (nM)	V_{max} (pmol/35μg DNA/min)
0° C	1.5	2.8		absent
21° C	1.8	3.3	21	16
37° C	2.8	4.1	61	48
KCN 2 mM		unchanged		inhibition
DNP 2 mM		"		"
oligomycin 0.1 mM		"		"
ouabain 0.5 mM		"		"
	LAS_{rT_3}		HAS_{rT_3}	
37° C		not tested	6	14
	LAS_{T_4}		HAS_{T_4}	
21° C	1.0	3.5	1.2	4.7
KCN 2 mM		unchanged		inhibition
oligomycin 0.1 mM		"		"
ouabain 0.5 mM		"		"

Data adapted from Krenning et al. (1978, 1981, 1982)

LAS = low affinity system
HAS = high affinity system

et al., 1979). In a recent report (Weisiger et al., 1981) albumin has been shown to bind specifically to a saturable binding site on rat liver cells. The putative receptor appeared to mediate uptake of albumin-bound fatty acids. A similar mechanism may be operative for thyroid hormone uptake.

Investigations with rat liver cell suspensions (Rao et al., 1976; 1981; Eckel et al., 1979) and isolated rabbit adipocytes (Parl et al., 1977), also show saturable and energy dependent (Eckel et al., 1979) T_3 uptake. Of particular interest are the stu-

dies in which uptake of T_3-rhodamine conjugate by mouse fibroblasts was investigated using video intensification fluorescence microscopy (Cheng et al., 1980; Maxfield et al., 1981). It was shown that T_3 is bound to a saturable protein on the cell surface and subsequently internalized in endocytotic vesicles, which is in harmony with an energy-dependent process. Very recently, evidence for a similar process for T_3 uptake by GH3 or GH4C1 cells (rat pituitary cell lines) has been reported (Horiuchi et al., 1982; Halpern and Hinkle, 1982).

3.2. Thyroxine

T_4 tracer kinetics of initial uptake into cultured rat hepatocytes show two saturable uptake systems: a high affinity system ($HAST_4$) and a system with lower affinity ($LAST_4$) (Table 7-2) (Krenning et al. 1981). $HAST_4$ is energy dependent and ouabain sensitive, suggesting a transport function. $LAST_4$ is not dependent on energy and insensitive to ouabain, analogous to $LAST_3$. The effects of temperature on the uptake have not been tested in these studies.

3.3. Reverse triiodothyronine

Similar to T_3 and T_4, initial uptake of rT_3 into rat hepatocytes in primary culture occurs at least in part by an energy-dependent high-affinity system (Krenning et al., 1982) (Table 7-2). No studies have been performed to reveal the possible excistence of a lower affinity system or a temperature effect on transport.

3.4. Interaction between T_3 and T_4 transport

Since both T_3 and T_4 are actively transported into the cell it was interesting to study if both hormones are translocated via the same or different pathways. To this purpose kinetic studies of the inhibition of T_3 uptake by T_4 and of T_4 uptake by T_3 have been performed (Krenning et al., 1981). It appears that T_3 and T_4 inhibit each others transport in a competitive way. The apparent K_i value of inhibition of T_3 transport by T_4 is 46 nM, whereas the apparent K_m value of T_4 transport is 1.2 nM. The apparent K_i for T_3 inhibition of T_4 transport is 90 nM and its apparent K_m value 21 nM. These large differences between apparent K_m and K_i values for both iodothyronines exclude the possibility that T_3 and T_4 enter the hepatocyte via a common pathway. Thus, it would appear that one carrier preferentially transports T_4 into the cells, which binds T_3 but does not or only poorly transport it. The other carrier would then be preferentially involved with the active transport of T_3, binding T_4 without appreciably facilitating its entry.

Although not studied as extensively as the above mentioned interaction between T_3 and T_4 transport, T_3 and T_4 do inhibit rT_3 transport (Krenning et al., 1982).

3.5. Regulation of uptake of thyroid hormones: importance of intracellular ATP

Regulation of transport of T_3 into rat liver cells has been studied by preincubation of monolayers with increasing amounts of T_3 (free concentrations 0-1.7 nM) for 4 h. (Krenning et al., 1980). In a subse-

quent incubation, initial T_3 uptake was found to be decreased. This phenomenon is time dependent since preincubation for 15 min is without significant effect. Studies with cycloheximide reveal that de novo protein synthesis is not involved in the effect of T_3 on subsequently assessed transport activity. However, it was found that preincubation with increasing amounts of T_3 results in a progressive decrease of intracellular ATP. A positive and significant correlation is found between T_3 transport and intracellular ATP content (Fig. 4-3). Furthermore, if a progressive decrease of intracellular ATP is induced using varying glucose and fructose concentrations during cell culture a similar linear relationship between T_3 transport and intracellular ATP is observed (Fig. 6-1) (Krenning et al., 1980; 1982).

Extension of this kind of experiments to T_4 and rT_3 transport, however, reveals a curvilinear relationship (Fig. 6-1) (Krenning et al., 1982). A decrease in ATP content to about 50% results in a statistically significant greater inhibition of T_4 or rT_3 transport as compared to that of T_3. T_4 and rT_3 transport are in a similar way dependent on ATP. These findings are compatible with different transport pathways for T_4 and rT_3 on one hand, and T_3 on the other.

ATP depleted rat hepatocytes in monolayer, also show a diminished steady-state content of thyroid hormone (Krenning et al., 1982a).

3.6. Interaction with various compounds

Two kinds of experiments with cultured rat hepatocytes are mentioned (Krenning et al., 1980; 1982).

First, cells were pre-exposed to cycloheximide (35 μM), D- or L-propranolol (1 and 10 μM), cholchicine (25 μM), vinblastine (11 μM) and cytochalasin B (25 μM) followed by measurements of transport activity, and in the other experiments uptake was studied in the presence of amiodarone (1 μM) or X-ray contrast agents such as sodium tyropanoate, sodium ipodate and iopanoic acid (10 and 100 μM). With the exception of cycloheximide (Krenning et al., 1980) all these compounds decrease T_4 and T_3 transport significantly (Krenning et al., 1982). Parallel studies showed that D- and L-propranolol and the inhibitors of the cytoskeleton (vinblastine, cytochalasin and colchicine) decrease the cellular ATP content (Krenning et al., 1982), which might at least partly explain their effect on thyroid hormone transport. The effects of X-ray contrast agents and of amiodarone are explained by competitive inhibition of transport, because of the structural similarities with the substrate.

In a rat liver perfusion study (Jennings et al., 1979), it has been shown that PTU and methimazole do not alter uptake of T_4. Only PTU inhibits 5'-deiodinase activity. The effects of these compounds on thyroid hormone uptake in the primary liver cell culture have not been studied. Surprisingly, methimazole has been reported to inhibit T_3 uptake by suspended liver cells (Rao et al., 1981).

Specific T_3 binding to nuclear receptors in intact rat pituitary tumour cells was inhibited by cytochalasin B, chloroquine (a lysosomal inhibitor) or monodansylcadaverine (a potent inhibitor of receptor-mediated endocytosis in fibroblasts). This inhibition appears to be related to a diminished T_3 entry into cells (Halpern and Hinkle, 1982; Horiuchi et al., 1982).

4. Thyroid hormone uptake by isolated human cells

Studies with cultured IM-9 lymphocytes show an energy-dependent saturable uptake system for T_3 with a Km of 110 nM measured under equilibrium conditions (Holm et al., 1980). Maximal uptake is obtained between 15 and 45 min. T_3 uptake by red cells is saturable (Km 100 nM) and non-energy dependent (Docter et al., 1982). Maximal uptake is reached after 60 min. This uptake is not dependent on the activity of Na^+, K^+-ATPase as revealed by the lack of effect of ouabain and vanadate, whereas some X-ray contrast agents inhibit T_3 uptake in a dose dependent fashion.

5. Studies with liver perfusion techniques

One h perfusions of livers from 3 days fasted rats with T_4 result in a maximal decrease of T_3 formation by 50% compared to control levels (Jennings et al., 1979). As total liver T_4 content is diminished but percentage of intrahepatic $T_4 \rightarrow T_3$ conversion is unchanged, decreased hepatic uptake of T_4 may be responsible for the diminished T_3 formation during fasting. It was hypothesized that decreased hepatic uptake was caused by loss of intracellular T_4 binding proteins. Another explanation might be that active uptake is decreased due to a diminished intracellular ATP concentration (vide supra), which is known to be decreased during fasting (Schwenke et al., 1981).

In this and another study (Pardridge and Mietus, 1980) using liver perfusions no saturability of thyroid hormone uptake could be demonstrated. This finding is in sharp contrast to the kinetic studies with isolated hepatocytes (Krenning et al., 1978;

1981) and the saturable endocytosis of T_3 in fibroblasts (Cheng et al., 1980; Maxfield, 1981), demonstrated by video intensification fluorescence microscopy. Possible explanations for this discrepancy may be as follows. In the first study the maximal free thyroxine concentration (0.8 μM dissolved in 4% bovine serum albumin (BSA)) is below the apparent Km of T_4 transport into rat hepatocytes as studied in the primary culture system and may therefore not be sufficient to detect saturation of uptake. Lack of saturation in the second study (Pardridge and Mietus, 1980) may be inherent to the technique used. In this investigation hepatic uptake of T_4 has been studied by using a bolus injection technique allowing only a very short period of time for hormone-tissue interaction. This time period is probably much too short for the time required for hormone transport over the cell membrane and may only reflect transcapillary movement. In such studies it is difficult to distinguish between T_4 bound to the cell surface and intracellulalry located T_4. Moreover, the concentration of BSA (0.1%) used in this liver perfusion study has shown to be inadequate, when used in kinetic studies with rat hepatocytes in primary cultures (Krenning et al., 1979). In this latter system a BSA concentration of at least 0.5% is necessary for the detection of saturable transport processes for thyroid hormone.

6. Indications of alterations in thyroid hormone transport in vivo

In man, it has been shown that prednisone slows the acute disappearance of injected labelled T_4 (Kumar et al., 1968) from plasma and decreases T_4

distribution space. A similar defect in the rapid phase of the equilibration of injected T_4 and especially rT_3 has been reported in patients with severe non-thyroidal illness (Kaptein et al., 1982), which could indicate a diminished uptake by peripheral tissues. This may be caused by a circulating inhibitor, which not only interferes with the binding of thyroid hormone to serum transport protein, but also with the entry of iodothyronines into hepatocytes (Oppenheimer et al., 1982).

Rats receiving a nutritionally deficient Remington diet with normalized iodine intake display elevated serum T_4 and free T_3 levels, while no increase in oxygen consumption is demonstrable (Okamura et al., 1981). Kinetic data suggest that cellular entry of thyroid hormone is decreased. The way by which this diet provokes this effect is unknown.

7. Possible implications of altered plasma membrane transport in relation to the pathophysiology of thyroid hormone metabolism

As discussed in the previous sections, inhibition of plasma-membrane transport of T_4, T_3 and rT_3 may be effected on the basis of competition by structurally related compounds or by decreased intracellular ATP concentrations. The latter mechanism may play a role in the changes in thyroid hormone transport in pathophysiological conditions. Thus in non-thyroidal illness like shock, anemia and in the diabetic state low cellular ATP concentrations are present. Also during fasting a decrease in hepatic ATP content has been observed (Knoche and Hartmann, 1961; Schwenke et al., 1981).

Evidence for decreased T_4 entry into peripheral tissue in rats and men has been obtained from the following studies. In T_4 substituted rats serum T_4 increased and plasma disappearance of radiolabelled T_4 decreased during fasting (Ingbar and Galton, 1975). However, in other reports no effects of dietary manipulations on serum T_4 levels are observed in T_4 substituted rats (Chopra, 1980; Gavin et al., 1981). In T_4 substituted humans serum concentrations of T_4 increased during starvation (Vagenakis et al., 1975). The fact that serum total T_4 concentrations do not change appreciably in starvation and non-thyroidal illness in non-substituted subjects, may be explained by the fact that in addition to diminution of tissue extraction of T_4, thyroidal secretion of T_4 is diminished as well (Kaplan and Utiger, 1978). Consequently, because of diminished uptake of T_4 in peripheral tissues, T_3 production and serum T_3 levels will be lowered. From the inhibition of T_4 transport into the liver one would expect that rT_3 production would be diminished as well. Studies in humans with the low T_3 syndrome notably due to liver cirrhosis have shown however that rT_3 production is normal (Chopra, 1976). This would suggest that rT_3 production takes place mainly outside the liver. These same studies have demonstrated that rT_3 clearance is decreased, which may be the result of reduced rT_3 uptake in the liver and/or rT_3 deiodination in this tissue. If the here described changes in thyroid hormone transport contribute significantly to the generation of the low T_3 syndrome it is necessary to postulate the following:

a) transport into different tissues is not inhibited to the same extent, but is most pronounced in the liver,

b) $T_4 \rightarrow rT_3$ conversion takes place mainly outside the liver in tissues not or to a lesser extent subject to inhibition of transport of thyroid hormone, and
c) the liver is quantitatively important in the production of T_3 from T_4 and degradation of rT_3.

In a number of situations such as fasting, diabetes, dexamethasone treatment, liver 5'-deiodinase activity is decreased as tested in tissue homogenates (Balsam et al., 1978; Kaplan and Utiger, 1978; Gavin et al., 1981a). However, it is not clear to what extent this decrease contributes to the low T_3 syndrome, as it is unknown whether the actual deiodination reaction is the rate-limiting step in the tissue conversion of iodothyronines. Therefore, it seems not unreasonable to assume that effects on tissue uptake also lead to alterations in thyroid hormone metabolism. The in vivo effect of propranolol could be explained on the basis of a reduced plasma membrane transport of T_4 and rT_3 in the liver (Krenning et al., 1982). A direct effect of propranolol on liver 5'-deiodinase activity is unlikely as inhibition of the enzyme is only observed at concentrations which are 3 orders of magnitude higher than the therapeutic levels (Fekkes et al., 1982a). However, studies on the effects of propranolol pretreatment of rats on $T_4 \rightarrow T_3$ conversion by tissue homogenates such as has been done for other drugs (Balsam et al., 1978; Kaplan and Utiger, 1978) have not been reported. Such an indirect effect of propranolol on enzyme activity, for instance by influencing enzyme synthesis, can therefore not be excluded.

In stresss conditions like acute or chronic disease or fasting a decrease in intracellular T_3 may act as an energy-conserving mechanism. A decrease in intracellular ATP may promote diminished cellular T_3

in two ways. First, decreased entry of T_4 into the liver leads, in addition to other mechanisms interfering with $T_4 \rightarrow T_3$ conversion, to diminished T_3 production. Second, T_3 transport from the circulation into body cells is attenuated, albeit to a lesser extent than T_4, at least when ATP is only moderately lowered.

REFERENCES

Adamson, L.F. and S.H. Ingbar (1967). Some properties of the stimulatory effect of thyroid hormones on amino acid transport by embryonic chick bone. Endocrinology 81: 1372-1378.

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

Bellabarba, D., Inada, M., Varsano-Aharon, N. and K. Sterling (1968). Thyroxine transport and turnover in major nonthyroidal illness. J. Clin. Endocr. Metab. 28: 1023-1030.

Bernal, J. and S. Refetoff (1977). The action of thyroid hormone. Clin. Endocrinol. 6: 227-249.

Bernstein, G., Hasen, J. and J.H Oppenheimer (1967). Turnover of ^{131}I-thyroxine in patients subjected to surgical trauma. J. Clin. Endocrinol. Metab. 27: 741-744.

Berry, M.N and D.S. Friend (1969). High-yield preparation of isolated rat liver parenchymal cells. J. Cell. Biol. 43: 506-520.

Bissel, D.M., Hammaker, L.E. and U.A. Meyer (1973). Parenchymal cells from adult rat liver in nonproliferating monolayer culture. Functional studies. J. Cell. Biol. 59: 722-734.

Blanchard, R.F. and P.J. Davis (1978). Dependence of renal Na,K-adenosine triphosphatase on subphysiologic levels of thyroid hormone in vitro. Endocrinology 103 (suppl), T-19.

Blouin, A. (1977). Morphometry of liver sinusoidal cells. In: Kupffer cells and other liver sinusoidal cells. (E. Wisse, D.L. Knook, eds). Amsterdam. Elsevier/ North-Holland Biomedical Press, 61-71.

Braverman, L.E., Ingbar, S.H. and K. Sterling (1970). Conversion of thyroxine (T_4) to triiodothyronine (T_3) in athyreotic human subjects. J. Clin. Invest. 49: 855-864.

Braverman, L.E. and A.G. Vagenakis (1979). The thyroid. Clin. Endocrinol. Metab. 8: 621-639.

Bucher, T. (1947). Uber ein phosphatubertragendes garungsferment. Biochem. Biophys. Acta 1: 292-314.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonuclei acid. Biochem. J. 62: 315-323.

Chatagner, F. and D. Gautheron (1960). Influence des hormones thyroidiennes sur la teneur en adenosine triphosphate du foie du rat. Biochim. Biophys. Acta 41: 544-545.

Cheng, S.-Y., Maxfield, F.R., Robbins, J., Willingham, M.C. and I.H. Pastan (1980). Receptor-mediated uptake of 3,3',5-triiodo-L-thyronine by cultured fibroblasts. Proc. Natl. Acad. Sci. USA 77: 3425-3429.

Chopra, I.J., Chopra, U., Smith, S.R., Reza, M. and D.H. Solomon (1975). Reciprocal changes in serum concentrations of reverse triiodothyronine and triiodothyronine in systemic illnesses.
J. Clin. Endocrinol. Metab. 41: 1043-1049.

Chopra, I.J. (1976). An assessment of daily production and significance of thyroidal secretion of 3,3',5'-triiodothyronine (reverse T_3) in man. J. Clin. Invest. 58: 32-40.

Chopra, I.J. (1977). A study of extrathyroidal conversion of thyroxine (T_4) to 3,3 ,5'-triiodothyronine (T_3) in vitro. Endocrinology 101: 453-463.

Chopra, I.J. (1978). Sulfhydryl groups and the monodeiodination of thyroxine to triiodothyronine. Science 199: 904-906.

Chopra, I.J., Solomon, D.H., Chopra, U., Wu, S.Y., Fisher, D.A. and Nakamura, Y. (1978a). Pathways of metabolism of thyroid hormones. Recent Prog. Horm. Res. 34: 521-567.

Chopra, I.J. (1980). Alterations in monodeiodination of iodothyronines in the fasting rat: Effects of reduced nonprotein sulfhydryl groups and hypothyroidism. Metabolism 29: 161-167.

Christensen, H.N. (1975). Kinetics in transport. In: Biological transport 107-165, W.A. Benjamin, Massachusetts.

Dickson, A.J. and C.I. Pogson (1977). The metabolic integrity of hepatocytes in sustained incubations. FEBS Letters 83: 27-32.

Docter, R., Visser, T.J., Stinis, J.T., v.d. Hout-Goemaat, N.L. and G. Hennemann (1976). Binding of L-triiodothyronine to isolated rat liver nuclei under various circumstances. Acta Endocr. 81: 82-95.

Docter, R., Krenning, E.P., Bernard, H.F., Visser, T.J. and G. Hennemann (1977). Uptake of triiodothyronine by isolated rat liver cells. Endocrinology 100: T-16.

Docter, R., Krenning, E.P., Bernard, H.F., Visser, T.J. and G. Hennemann (1978). Uptake of triiodothyronine and thyroxine by cultured rat liver parenchymal cells. Ann. Endocrinol. 39, 44^{A}.

Docter, R., Krenning, E.P., Bos, G., Fekkes, D. and G. Hennemann (1982). Evidence that the uptake of triiodo-L-thyronine by human erythrocytes is carrier-mediated but not energy-dependent. Biochem. J. 208: 27-34.

Doorn van, J., Roelfsema, F. and D. van der Heide (1982). Contribution from local conversion of thyroxine to 3,5,3 -triiodothyronine to intracellular 3,5,3'-triiodothyronine in several organs in hypothyroid rats at isotope equilibrium. Acta Endocrinol. 101: 386-396.

Eckel, J., Rao, G.S., Rao, M.L. and H. Breuer (1979). Uptake of L-triiodothyronine by isolated rat liver cells. Biochem. J. 182: 473-491.

Edelman, I.S. (1974). Thyroid thermogenesis. N. Engl. J. Med. 290: 1303-1308.

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

Erlinger, S. (1982). Editorial review: Does Na^+-K^+-ATPase have any role in bile secretion? Am. J. Physiol. 243: G243-G247.

Farer, L.S., Robbins, J., Blumberg, B.S. and J.E. Rall (1962). Thyroxine-serum protein complexes in various animals. Endocrinology 70: 686-696.

Fekkes, D. (1982). Isolation and characterization of enzyme actvities deiodinating thyroid hormone. Thesis, Rotterdam.

Fekkes, D., Hennemann, G. and T.J. Visser (1982a). One enzyme for the 5'-deiodination of 3,3',5'-triiodothyronine and 3',5'-diiodothyronine in rat liver. Biochem. Pharmacol. 31: 1705-1709.

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

Freinkel, N., Ingbar, S.H. and J.T. Dowling (1957). The influence of extracellular thyroxine-binding protein upon the accumulation of thyroxine by tissue slices. J. Clin. Invest. 36: 25-38.

Gavin, L.A., McMahon, F.A. and M. Moeller (1981). Carbohydrate in contrast to protein feeding increases the hepatic content of active thyroxine-5'-deiodinase in the rat. Endocrinology 109: 530-536.

Gavin, L.A., McMahon, F.A. and M. Moeller (1981a). The mechanism of impaired T_3 production from T_4 in diabetes. Diabetes 30: 694-699.

Gharbi, J. and J. Torresani (1979). High affinity thyroxine binding to purified rat liver plasma membranes. Biochem. Biophys. Res. Commun. 88: 170-177.

Gharbi-Chihi, J. and J. Torresani (1981). Thyroid hormone binding to plasma membrane preparations; studies in different thyroid states and tissues. J. Endocrinol. Invest. 4: 177-183.

Goldfine, I.D., Smith, G.J., Simons, C.G., Ingbar, S.H. and E.C. Jorgensen (1976). Activities of thyroid hormones and related compounds in an in vitro thymocyte assay. J. Biol. Chem. 251: 4233-4238.

Goto, K. and S. Mizushima (1978). Removal by bovine serum albumin of fatty acids from membrane vesicles and its effect on proline transport activity in Escherichia coli. J. Biochem. 84: 251-258.

Green, W.L. and G. Bellamy (1977). Role of organic anion transport systems in uptake and metabolism of thyroid hormones by rat liver slices. Proceedings of the 53rd Annual Meeting of the American Thyroid Association, Cleveland, Ohio, TR-16.

Gross, J. (1981). The thyromimetic effect of triiodothyronine and its modulation. In: The low T_3 syndrome (R.-D. Hesch, ed.) 40: 83-87, Academic Press, New York.

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

Ham, R.G. (1965). Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc. Natl. Acad. Sci. USA 53: 288-293.

Hanks, J.H. and R.E. Wallace (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med. 71: 196-200.

Hasen, J., Bernstein, G., Volpert, E. and J.H. Oppenheimer (1968). Analysis of the rapid interchange of thyroxine between plasma and liver and plasma and kidney in the intact rat. Endocrinology 82: 37-46.

Hearn, M.T.W., Hancock, W.S. and C.A. Bishop (1978). High-pressure liquid chromatography of amino acids, peptides and proteins. V. Separation of thyroidal amino acids by hydrophilic ion-paired reversed-phase high-performance liquid chromatography. J. Chromatogr. 157: 337-344.

Hennemann, G., Docter, R. and A. Dolman (1971). Relationship between total thyroxine and absolute free thyroxine and the influence of absolute free thyroxine on thyroxine disposal in humans. J. Clin. Endocr. 33: 63-67.

Hennemann, G , Docter, R., Krenning, E.P., Bos, G., Otten, M. and T.J. Visser (1979). Raised total thyroxine and free thyroxine index but normal free thyroxine. A serum abnormality due to inherited increased affinity of iodothyronines for serum binding protein. Lancet i: 639-642.

Hennemann, G. (1981). Transport and distribution of thyroid hormones. In: The low T_3 syndrome (R.-D. Hesch, ed) 40: 41-47, Academic Press, New York.

Heyma, P., Larkins, R.G. and D.G. Campbell (1980). Inhibition by propranolol of 3,5,3'-triiodothyronine formation from thyroxine in isolated rat renal tubules: an effect independent of β-adrenergic blockade. Endocrinology 106: 1437-1441.

Heyma, P., Larkins, R.G., Higgenbotham, L. and H.G.K. Wah (1980a). D-propranolol and DL-propranolol both decrease conversion of L-thyroxine to L-triiodothyronine. Brit. Med. J. i: 24-25.

Holm, A.-C. and C. Jacquemin (1979). Membrane transport of L-triiodothyronine by human red cell ghosts. Biochem. Biophys. Res. Commun. 89: 1006-1017.

Holm, A.-C., Wong, K.Y., Pliam, N.B., Jorgensen, E.C. and I.D. Goldfine (1980). Uptake of L-triiodothyronine into human cultured lymphocytes. Acta Endocrinol. 95: 350-358.

Horiuchi, R., Cheng, S.-Y., Willingham, M. and I. Pastan (1982). Inhibition of the nuclear entry of 3,3',5-triiodo-L-thyronine by monodansylcadaverine in GH_3 cells. J. Biol. Chem. 257: 3139-3144.

Ichihara, A., Nakamura, T., Tanaka, K., Tomita, Y., Aoyama, K., Kato, S. and H. Shinno (1980). Biochemical functions of adult rat hepatocytes in primary culture. Ann. New York Acad. Sci. 349: 77-84.

Inada, M. and K. Sterling (1967). Thyroxine turnover and transport in Laennec's cirrhosis of the liver. J. Clin. Invest. 46: 1275-1282.

Ingbar, D.H. and V.A. Galton (1975). The effect of food deprivation on the peripheral metabolism of thyroxine in rats. Endocrinology 96: 1525-1532.

Izumi, M. and P.R. Larsen (1977). Triiodothyronine, thyroxine and iodine in purified thyroglobulin from patients with Graves' disease. J. Clin. Invest. 59: 1105-1121.

Jeejeebhoy, K.N., Ho, J., Mehra, R. and A. Bruce-Robertson (1980). Hepatotrophic effects of insulin on glucose, glycogen and adenine nucleotides in hepatocytes isolated from fasted adult rats. Gastroenterology 78: 556-570.

Jennings, A.S., Ferguson, D.C. and R.D. Utiger (1979). Regulation of the conversion of thyroxine to triiodothyronine in the perfused rat liver. J. Clin. Invest. 64: 1614-1623.

Jorgensen, E.C. (1976). Structure activity relationships of thyroxine analogs. Pharmacol. Ther. (B) 2: 661-682.

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

Kaptein, E.M., Grieb, D.A. and W.S. Wheeler (1980). Peripheral thyroid hormone kinetics in critical illness. Proceedings of the 62nd annual meeting of the endocrine society, abstract no. 459.

Kaptein, E.M., Robinson, W.J., Grieb, D.A. and J.T. Nicoloff (1982). Peripheral serum thyroxine, triiodothyronine and reverse triiodothyronine kinetics in the low thyroxine state of acute non-thyroidal illnesses: A non-compartmental analysis. J. Clin. Invest. 69: 526-535.

Kato, S., Aoyama, K., Nakamura, T. and A. Ichihara (1979). Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. J. Biochem. 86: 1419-1425.

Keller, N., Richardson, U.I. and F.E. Yates (1969). Protein binding and the biological activity of corticosteroids: in vivo induction of hepatic and pancreatic alanine aminotransferases by corticosteroids in normal and estrogen-treated rats. Endocrinology 84: 49-62.

Knoche, I. and F. Hartmann (1961). Influence of starvation and choline deficiency on adenosine-phosphate and glycolytic enzymes in rat liver. Biochem. Zeitschr. 334: 269-278.

Knook, D.L. and E.C. Sleyster (1980). Isolated parenchymal, Kupffer and endothelial rat liver cells characterized by their lysosomal enzyme content. Biochem. Biophys. Res. Commun. 96: 250-257.

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

Krenning, E.P., Docter, R., Bernard, H.F., Visser, T.J. and G. Hennemann (1979). The essential role of albumin in the active transport of thyroid hormone into primary cultured hepatocytes. FEBS Letters 107: 227-230.

Krenning, E.P., Docter, R., Bernard, H.F., Visser, T.J. and G. Hennemann (1980). Regulation of the active transport of 3,3',5-triiodothyronine (T_3) into primary cultured rat hepatocytes by ATP. FEBS Letters 119: 279-282.

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

Krenning, E.P., Bernard, H.F. and G. Hennemann (1981a). Alterations in transport of T_3 and T_4 into rat hepatocytes in primary culture by decrease of cellular ATP content and by various drugs. Ann. Endocrinol. 42: 33A

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

Krenning, E.P., Bernard, H.F., van Loon, M. and G. Hennemann (1982a). Thyroid hormone (TH) in- and efflux kinetics of rat liver cells in primary culture: implications for intracellular bioavailability. Ann. Endocinol. 43: 54a.

Kumar, R.S., Musa, B U., Appleton, W.G. and J.T. Dowling (1968). Effect of prednisone on thyroxine distribution. J. Clin. Endocr. 28: 1335-1340.

Larsen, P.R. (1982). Thyroid-pituitary interaction. Feedback regulation of thyrotropin secretion by thyroid hormones. N. Engl. J. Med. 306: 23-32.

Le Cam, A. and P. Freychet (1977). Neutral amino acid transport. Characterization of the A and L systems in isolated rat hepatocytes. J. Biol. Chem. 252: 148-156.

Lein, A. and R.M. Dowben (1961). Uptake and binding of thyroxine and triiodothyronine by rat diaphragm in vitro. Am. J. Physiol. 200: 1029-1032.

Lerner, J. (1978). Organ and tissue absorption of amino acids. In: A review of amino acid transport processes in animal cells and tissues, 1-77, University of Maine at Orono press, Orono, Maine.

Mapes, J.P. and R.A. Harris (1975). On the oxidation of succinate by parenchymal cells isolated from rat liver. FEBS Letters 51: 80-83.

Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P. and S.-Y. Cheng (1981). Binding and mobility of the cell surface receptors for 3,3',5-triiodo-L-thyronine. Science 211: 63-65.

Montecucco, C., Ballardin, S., Zaccolin, G.P. and T. Pozzan (1981). Effect of local anaesthetics on lymphocyte capping and energy metabolism. Biochem. Pharmacol. 30: 2989-2992.

Munniksma, J., Noteborn, M., Kooistra, T., Stienstra, S., Bouma, J.M.W., Gruber, M., Brouwer, A., Praaning-van Dalen, D. and D.L. Knook (1980). Fluid endocytosis by rat liver and spleen. Biochem. J. 192: 613-621.

Nicoloff, J.T (1978). Thyroid hormone transport and metabolism: pathophysiologic implications. In: The Thyroid (Werner S.C. and Ingbar S.H., eds) 88-99, Harper and Row, Hagerstown, MD.

Obregon, M.J., Roelfsema, F., Morreale de Escobar, G., Escobar del Rey, F. and A. Querido (1979). Exchange of triiodothyronine derived from thyroxine with circulating triiodothyronine as studied in the rat. Clin. Endocrinol. 10: 305-315.

Okamura, K., Taurog, A. and J.J. DiStefano (1981). Elevated serum levels of T_3 without metabolic effect in nutritionally deficient rats, attributable to reduced cellular uptake of T_3. Endocrinology 109: 673-675.

Oppenheimer, J.H., Schwartz H.L. and M.I. Surks (1974). Tissue differences in the concentration of triiodothyronine nuclear binding sites in the rat: liver, kidney, pituitary, heart, brain, spleen and testis. Endocrinology 95: 897-903.

Oppenheimer, J.H., Schwartz, H.L , Surks, M.I., Koerner, D. and W.H. Dillman (1976). Nuclear receptors and the initiation of thyroid hormone action. Recent Prog. Horm. Res. 32: 529-557.

Oppenheimer, J.H., Schwartz, H.L., Mariash, C.N. and F.E. Kaiser (1982). Evidence for a factor in the sera of patients with non-thyroidal disease which inhibits iodothyronine binding by solid matrices, serum proteins and rat hepatocytes J. Clin. Endocrinol. Metab. 54: 757-766.

Otten, M.H., Blom, J., Bernard, H.F., van Koetsveld, P. and T.J. Visser (1981). Sulfo-conjugation of 3,3'-diiodothyronine in thiouracil treated rat liver cells. Ann. Endocrinol. 42, 75A.

Oxender, D.L. (1972). Membrane transport. Ann. Rev. Biochem. 41: 777-814.

Pardridge, W.M. and L.J. Mietus (1980). Influx of thyroid hormones into rat liver in vivo. J. Clin. Invest. 66: 367-374.

Pariza, M.W., Butcher, F.R., Kletzien, R.F., Becker, J.E. and V.R. Potter (1976). Induction and decay of glucagon-induced amino acid transport in primary cultures of adult rat liver cells: paradoxical effects of cycloheximide and puromycin. Proc. Natl. Sci. USA 73: 4511-4515.

Parl, F., Korcek, L., Siegel, J.S. and M. Tabachnick (1977). Uptake of triiodothyronine and thyroxine by isolated rabbit adipocytes. FEBS Letters 83: 145-147.

Pastan, I.H. and M.C. Willingham (1981). Receptor-mediated endocytosis of hormones in cultured cells. Ann. Rev. Physiol. 43: 239-250.

Pliam, N.B. and I.D. Goldfine (1977). High affinity thyroid hormone sites on purified rat liver plasma membranes. Biochem. Biophys. Res. Commun. 79: 166-173.

Praaning-van Dalen, D.P., Brouwer, A. and D.L. Knook (1981). Clearance capacity of rat liver Kupffer, endothelial, and parenchymal cells. Gastroenterology 81: 1036-1044.

Rao, G.S., Eckel, J., Rao, M.L. and H. Breuer (1976). Uptake of thyroid hormone by isolated rat liver cells. Biochem. Biophys. Res. Commun. 73: 98-104.

Rao, G.S., Rao, M.L., Thillmann, A. and H.D. Quednau (1981). Study of fluxes at low concentrations of L-triiodothyronine with rat liver cells and their plasma-membrane vesicles. Biochem. J. 198: 457-466.

Ray, T.K., Lieberman, I. and A.I. Lansing (1968). Synthesis of the plasma membrane of the liver cell. Biochem. Biophys. Res. Commun. 31: 54-58.

Reeve, E.B. and A.Y. Chen (1970). Regulation of interstitial albumin. In: Plasma Protein Metabolism. (M.A. Rothschild and T. Waldmann, eds), 89-109. Academic Press, London and New York.

Robbins, J. and J.E. Rall (1957). The interaction of thyroid hormones and protein in biological fluids. Recent Prog. Horm. Res. 13: 161-208.

Robbins, J. and M.L. Johnson (1982). Possible significance of multiple transport proteins for the thyroid hormones. In: Free hormones in blood. (A. Albertini and R.P. Ekins, eds) 53-64. Elsevier Biomedical Press, Amsterdam.

Rosenberg, T. (1954). The concept and definition of active transport. Symp. Soc. Exp. Biol. 8: 27-41.

Samuels, H.H. (1978). In vitro studies on thyroid hormones receptors. In: Receptors and hormone action. (L. Birnbaumer and B.W. O'Malley, eds) 3: 35-75. Academic Press, New York.

Sato, K. and J. Robbins (1981). Thyroid hormone metabolism in primary cultured rat hepatocytes. Effects of glucose, glucagon, and insulin. J. Clin. Invest. 68: 475-483.

Satoyoshi, E., Murakami, K. and J. Toni (1963). Thyrotoxic myopathy (letter). Lancet ii: 843.

Schimmel, M. and R.D. Utiger (1977). Thyroidal and peripheral production of thyroid hormones. Review of recent findings and their clinical implications. Ann. Int. Med. 80: 760-768.

Schreiber, G. and M. Schreiber (1973). The preparation of single cell suspensions from liver and their use for the study of protein synthesis. Subcell. Biochem. 2: 321-383.

Schwartz, A., Lindenmayer, G.E. and J.C. Allen (1975). The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. Pharmacol. Rev. 27: 3-134.

Schwenke, W.-D., Soboll, S., Seitz, H.J. and H. Sies (1981). Mitochondrial and cytosolic ATP/ADP ratios in rat liver in vivo. Biochem. J. 200: 405-408.

Segal, J., Schwartz, H. and A. Gordon (1977). The effect of triiodothyronine on 2-deoxy-D[1-^{3}H]glucose uptake in cultured chick embryo heart cells. Endocrinology 101: 143-149.

Segal, J. and S.H. Ingbar (1979). Stimulation by triiodothyronine of the in vitro uptake of sugars by rat thymocytes. J. Clin. Invest. 63: 507-515.

Segal, J. and S.H. Ingbar (1982). Specific binding sites for triiodothyronine in the plasma membrane of rat thymocytes. Correlation with biochemical responses. J. Clin. Invest. 70: 919-926.

Seglen, P.O. (1973). Preparation of rat liver cells. Exp. Cell. Res. 82: 391-398.

Seglen, P.O. (1977). Protein-catabolic state of isolated rat hepatocytes. Biochim. Biophys. Acta 496: 182-191.

Seglen, P.O. and J. Fossa (1978). Attachment of rat hepatocytes in vitro to substrata of serum protein, collagen, or concanavalin A. Exp. Cell. Res. 116: 199-206.

Singh, B.N. (1973). Clinical aspects of the anti-arrhythmic actions of beta-receptor blocking drugs: part 2, clinical pharmacology. N.-Z. Med. J. 78: 529-535.

Sips, H.J. (1981). The plasma membrane of the liver parenchymal cell. Structure and function. Thesis, Amsterdam.

Snedecor, G.W. and W.G. Cochran (1967). One-way classifications. Analysis of variance. In: Statistical Methods, 6th ed, 258-298. The Iowa State Univ. Press, Ames, Iowa, USA.

Sorimachi, K. and J. Robbins (1978). Uptake and metabolism of thyroid hormones by cultured monkey hepatocarcinoma cells. Biochim. Biophys. Acta 542: 515-526.

Spears, G., Sneyd, J.G. and E.G. Loten (1971). A method for deriving kinetic constants for two enzymes acting on the same substrate. Biochem. J. 125: 1149-1151.

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

Sterling, K. and P.O. Milch (1975). Thyroid hormone binding by a component of mitochondrial membrane. Proc. Natl. Acad. Sci. USA 72: 3225-3229.

Sterling, K., Brenner, M.A. and T. Sakurada (1980). Rapid effect of triiodothyronine on the mitochondrial pathway in rat liver in vivo. Science 210: 340-343.

Stitzer, K. and J.A. Jacquez (1975). Neutral amino acid transport pathways in uptake of L-thyroxine by Ehrlich ascites cells. Am. J. Physiol. 229: 172-178.

Surks, M.I., Koerner, D., Dillman, W. and J.H. Oppenheimer (1973). Limited capacity binding sites for L-triiodothyronine in rat liver nuclei. Localization to the chromatin and partial characterization of the L-triiodothyronine-chromatin complexes. J. Biol. Chem. 248: 7066-7072.

Surks, M.I. and J.H. Oppenheimer (1977). Concentration of L-thyroxine and L-triiodothyronine specifically bound to nuclear receptors in rat liver and kidney. Quantitative evidence favoring a major role of T_3 in thyroid hormone action. J. Clin. Invest. 60: 555-562.

Sutherland, R.L. and M.W. Simpson-Morgan (1975). The thyroxine-binding properties of serum proteins. A competitive binding technique employing Sephadex G-25. J. Endocrinol. 65: 319-332.

Suzuki, H., Kadena, N.,Takeushi, K. and S. Nakagawa (1979). Effects of three-day oral cholecystography on serum iodothyronines and TSH concentrations: comparison of the effects among some cholecystographic agents and the effects of iopanoic acid on the pituitary-thyroid axis. Acta Endocrinol. 92: 477-488.

Tait, J.F. and S. Burnstein (1964). In vivo studies of steroid dynamics in man. In: The Hormones (G. Pincus, K.V. Thimann, E.B Astwood, eds) 5: 441-557. Academic Press, New York.

Tata, J.R. (1964). Distribution and metabolism of thyroid hormones. In: The Thyroid Gland (R. Pitt-Rivers and W.B. Trotter, eds) p. 163-168, Butterworths, London.

Tata, J.R. and C.C. Widnell (1966). Ribonucleic acid synthesis during early action of thyroid hormones. Biochem. J. 98: 604-620.

Tell, G.P., Haour, F. and J.M. Saez (1978). Hormonal regulation of membrane receptors and cell responsiveness: a review. Metabolism 27: 1566-1592.

Vagenakis, A.G., Burger, A., Portnay, G.I., Rudolph, M., O'Brian, J.T., Azizi, F., Arky, R.A., Nicod, P., Ingbar, S.H. and L.E. Braverman (1975). Diversion of peripheral thyroxine metabolism from activating to inactivating pathways during complete fasting. J. Clin. Endocrinol. Metab. 41: 191-194.

Vagenakis, A.G. (1981). Non-thyroid diseases affecting the thyroid hormone metabolism. In: The low T_3 syndrome (R.-D. Hesch, ed) 40: 128-139. Academic Press, New York.

Van Noorden, C.J.F., Wiersinga, W.M. and J.L. Touber (1979). Propranolol inhibits the in vitro conversion of thyroxine into triiodothyronine by isolated rat liver parenchymal cells. Horm. Metab. Res. 11: 366-370.

Visser, T.J., Bernard, H.F., Docter, R. and G. Hennemann (1976). Specific binding sites for L-triiodothyronine in rat liver and kidney cytosol. Acta Endocrinol. 82: 98-104.

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

Visser, T.J. (1980). Thyroid hormone deiodination. Thesis, Rotterdam.

Visser, T.J. (1980a). Deiodination of thyroid hormone and the role of glutathione. Trends Biochem. Sci. 5: 222-224.

Wagle, S.R. (1975). Minireview: critical evaluation of methods used for the isolation of rat liver hepatocytes for metabolic studies. Life Sciences 17: 827-836.

Weissel, M., Stummvoll, H.K., Wolf, A. and H. Fritzsche (1977). Thyroid hormones in chronic renal failure. Ann. Int. Med. 86: 664-665.

Weissel, M. and H.K. Stummvoll (1981). Effects of T_3-administration in patients with chronic renal failure on maintenance hemodialysis. In: The low T_3-syndrome. (R.-D. Hesch, ed) 40: 167-169. Academic Press, New York.

Weisiger, R., Gollan, J. and R. Ockner (1981). Receptor for albumin on the liver cell surface may mediate uptake of fatty acids and other albumin-bound substances. Science 211: 1048-1050.

Zappi, E. (1967). Application of the FFCA-reaction for the detection of thyroid hormones and iodinated derivatives to thinlayer chromatography. J. Chromatogr. 31: 241-242.

SUMMARY

Thyroxine, which is produced only by the thyroid, attains its metabolic activity after conversion to 3,3',5-triiodothyronine in many other tissues. Especially the liver and the kidneys are thought to be important tissues for the total body production of triiodothyronine in healthy subjects. About 20% of the total body production of triiodothyronine is derived directly from the thyroid. Another metabolite of thyroxine, 3,3',5'-triiodothyronine (reverse triiodothyronine), is also produced predominantly from thyroxine outside the thyroid but is metabolically inert. The conversion of thyroxine to triiodothyronine and reverse triiodothyronine is enzymatically catalyzed by socalled iodothyronine deiodinases. Deiodination is one of the most important routes for the degradation of thyroxine and its metabolites.

From the foregoing it follows that uptake of thyroxine by, among others, the liver is obligatory for the intracellular production of the metabolic active thyroid hormone, triiodothyronine, and degradation. Similarly, reverse triiodothyronine, produced elsewhere is taken up and metabolized in the liver.

Recent studies have shown, that most triiodothyronine, associated with its receptors in the hepatocyte, is not derived directly from intracellular conversion of thyroxine, but rather from the extracellular compartment. Thus, before initiating its metabolic activity, triiodothyronine has to be taken up by the hepatocyte.

The aim of this thesis is to study the mechanism, by which thyroxine, triiodothyronine and reverse tri-

iodothyronine are taken up by the hepatocyte. The experiments have been carried out by using primary cultures of rat hepatocytes, as explained in CHAPTER 1. The influence of many variables on initial kinetics of tracer uptake has been studied.

As described in CHAPTER 2, the kinetics of triiodothyronine uptake show characteristics of saturation after adding increasing amounts of unlabelled hormone. In addition to a nonspecific component, two saturable systems have been found, viz. a high-affinity system (h.a.s.) with relatively low capacity and a low-affinity system (l.a.s.) with relatively high capacity. Uptake via the h.a.s. is inhibited by preincubation of hepatocytes with metabolic blockers (KCN, DNP and oligomycin) and by the presence of thyroxine. This system is also temperature dependent. These variables did not affect the l.a.s.. The energy dependency of the h.a.s. suggests a transport function. Since the maximum capacity of the l.a.s. is energy-independent and only moderately affected by changes in temperature, this system may represent binding to the plasma membrane.An other argument in favour of this supposition is mentioned in chapter 3.

In CHAPTER 3 the influence of the extracellular albumin concentration on uptake of triiodothyronine is described. The motive for this particular study was the apparent lack of a saturable uptake process for triiodothyronine in monkey hepatocarcinoma cells as reported in the literature. This uptake had been studied with a relatively low extracellular concentration of albumin. Also in the rat hepatocyte system, saturation of triiodothyronine uptake was not detected when the albumin concentration was below 5 g/l. The effects of albumin in the medium were found to be twofold. First, below 5 g/l, the velocity of

the nonspecific component (diffusion) increased with albumin, while above 5 g/l diffusion remained constant. It is postulated that at very low levels of albumin diffusion of hormone through the waterlayer around the cell is rate-limiting, whereas above 5 g/l albumin it is not. However, between 5 and 20 g/l albumin also increased the maximum capacities of the saturable systems without affecting their affinity constants. The possible mechanisms underlying this second effect of albumin are described in chapters 3 and 7.

In CHAPTER 3, evidence is presented which supports the view that the l.a.s. represents binding to the plasma membrane. The contribution of this system in contrast to the h.a.s. decreases after washing with thyroid hormone-free serum.

In CHAPTER 4 it is described, that pre-exposure of rat hepatocytes for 4 h to increasing amounts of triiodothyronine diminishes the subsequent uptake of triiodothyronine via the h.a.s.. This effect is dependent on the time of exposure, but not on protein synthesis, as tested with cycloheximide. In addition, this pre-exposure to triiodothyronine resulted in a diminution of the cellular ATP content. A positive correlation was observed between the active uptake of triiodothyronine and the cellular ATP content. This correlation was also found, if changes in the cellular ATP content were induced by pre-exposure of the cells to various concentrations of glucose or fructose. The magnitude of the correlation coefficient of the relationship between uptake of triiodothyronine and intracellular ATP content suggests that at least in vitro ATP has a regulatory role in the active uptake of triiodothyronine.

In CHAPTER 5 the uptake of thyroxine by isolated

hepatocytes is described. In concert with the uptake of triiodothyronine, a nonspecific component as well as two saturable systems have been demonstrated. The h.a.s. is inhibited by pre-exposure of the cells to metabolic inhibitors, in contrast to the l.a.s.. The active transport of both thyroxine and triiodothyronine was inhibited by pre-incubation of the cells with ouabain. This indicates, that the sodium gradient over the plasma membrane is of importance for the active uptake of thyroid hormone.

After demonstrating the active uptake of triiodothyronine and thyroxine by isolated rat hepatocytes, experiments were performed to study whether both iodothyronines are taken up via the same membrane system or via separate systems. Analysis of competition experiments, using both iodothyronines as ligand or competitor, indicated that uptake occurs separately.

An extension of this aspect is described in CHAPTER 6, which concerns the detailed analysis of the ATP-dependency of uptake of triiodothyronine, reverse triiodothyronine and thyroxine. Evidence is presented for the uptake of reverse triiodothyronine via a high-affinity, ATP-dependent process. Both the uptake of thyroxine and reverse triiodothyronine are curvilinearly related to the cellular ATP content, in contrast to the uptake of triiodothyronine, as described in chapter 4. The difference in ATP-dependency of triiodothyronine and thyroxine uptake is also in favour of distinct transport mechanisms, whereas thyroxine and reverse triiodothyronine may share a single transport mechanism.

In this chapter, the effect of various compounds on the active uptake of thyroxine and triiodothyronine is described too.

Substances which disrupt the cytoskeleton appeared

to induce a significant decrease in thyroid hormone uptake. However, these inhibitors also diminished the cellular ATP content to an extent sufficient by itself to explain the diminished transport of thyroxine. The decrease in triiodothyronine transport affected by these inhibitors is more pronounced than expected from the diminished cellular ATP content. Studies by others have shown that triiodothyronine is taken up by mouse fibroblasts via a saturable endocytotic process also implying energy dependency and the involvement of the cytoskeleton.

Compounds, like D- and L-propranolol, amiodarone and X-ray contrast agents, which are able to induce the so-called "low T_3 syndrome" in humans (vide infra), inhibited the active transport of triiodothyronine and thyroxine as well. Both isomers of propranolol also caused a diminished cellular ATP content. For the decrease in triiodothyronine transport, the ATP diminution forms only a partial explanation. The reason for the additional effect is unknown. Inhibition by amiodarone and X-ray contrast agents, which are structurally related to iodothyronines, may be explained by competition for the carrier.

In CHAPTER 7, a review of the literature is given, concerning the translocation of iodothyronines over the plasma membrane and the possible pathophysiological significance of an energy-dependent uptake. The increased interest in this aspect of thyroid hormone metabolism in recent years is remarkable. Information from different laboratories, using various types of isolated cells, have indicated that:

1. "thyroid hormone responsive" cells have thyroid hormone binding sites on the plasma membrane,
2. translocation over the plasma membrane is not

merely determined by diffusion,

3. the uptake mechanism is "carrier mediated" and requires ATP. In pituitary cells and fibroblasts, this transport is of the "carrier mediated endocytosis" type,
4. this adsorptive endocytosis of thyroid hormone is of physiological importance, since inhibition of this process leads to a diminished binding of triiodothyronine to intracellular receptors as is shown for pituitary cells.

Of pathophysiological significance are the findings obtained with thyroxine-perfusion of livers from fasted rats. A diminished production of triiodothyronine was measured, which may be ascribed to a decreased uptake of thyroxine. In chapter 7 several studies are cited, which suggest that in vivo too, transport of thyroid hormone through the plasma membrane is of importance in the regulation of the peripheral thyroid hormone metabolism. In this way, this transport may play a role in the genesis of the "low T_3 syndrome", which in general is characterized by normal serum thyroxine, decreased triiodothyronine and increased reverse triiodothyronine levels.

This syndrome is encountered in various illnesses and is also induced by many compounds such as propylthiouracil, dexamethasone and the before-mentioned propranolol, amiodarone and X-ray contrast agents.

Up to now, this syndrome has been explained by a selective decrease in the 5'-deiodinase activity, resulting in a diminished conversion of thyroxine to triiodothyronine and degradation of reverse triiodothyronine. As it has recently been shown that in the liver both 5- (e.g. thyroxine $\longrightarrow$ reverse triiodothyronine conversion) and 5'-deiodinations are catalyzed by the same enzyme, the situation seems more compli-

cated. In view of indications that circulating triiodothyronine is mainly produced by the liver and circulating reverse triiodothyronine by tissues other than the liver, an alternative explanation may be put forward. The "low T_3 syndrome" is a reflection of an abnormal hepatic metabolism of thyroid hormone. The reduced thyroxine → triiodothyronine conversion and reverse triiodothyronine degradation may be explained both by a decreased uptake of thyroxine and reverse triiodothyronine, and by a decreased deiodination of these compounds. The result in both cases is a decrease in serum triiodothyronine and increase in serum reverse triiodothyronine. Considering the high rate of reverse triiodothyronine 5'-deiodination in the liver, most reverse triiodothyronine produced in this tissue by 5-deiodination of thyroxine will be degraded before it is released into circulation. A decreased hepatic production of reverse triiodothyronine would not be reflected, therefore, in analysis of serum values.

Factors, which may play a role in an alteration of active transport through the plasma membrane, can be summarized as follows:

1. the cellular ATP content,
2. the sodium gradient over the plasma membrane,
3. direct inhibition by various exogenous compounds, e.g. compounds with structural similarities with iodothyronines, and
4. a circulating, endogenous inhibitor, affecting both binding of iodothyronines to thyroid hormone-binding serum proteins and cellular uptake of iodothyronines as suggested by others.

Advanced in vivo studies have to be carried out to test the importance of regulation of transport at the plasma membrane level.

Adaptive changes in peripheral thyroid hormone metabolism, as occur in the "low T_3 syndrome" during stress, may have a protective function for the organism during these situations. A diminished triiodothyronine production and nuclear occupancy during stress may act for instance as an energy-conserving mechanism.

SAMENVATTING

Het uitsluitend door de schildklier geproduceerde thyroxine wordt biologisch werkzaam na omzetting in 3,3',5-trijodothyronine in andere organen. Met name aan de lever en nieren wordt een belangrijke rol toegeschreven voor de totale lichaamsproduktie van trijodothyronine bij gezonde mensen. Ongeveer 20% van de totale lichaamsproduktie van trijodothyronine komt direkt uit de schildklier. Een andere metaboliet van thyroxine, 3,3',5'-trijodothyronine (reverse trijodothyronine) wordt ook bijna geheel buiten de schildklier gevormd, maar is biologisch niet werkzaam. De omzetting van thyroxine in trijodothyronine en reverse trijodothyronine wordt enzymatisch gekatalyseerd door zogenaamde jodothyronine dejodasen. De jodering neemt een van de voornaamste plaatsen in bij de afbraak van thyroxine en van thyroxine-metabolieten.

Uit het bovenstaande volgt dat opname van thyroxine door onder andere de lever noodzakelijk is voor de intracellulaire produktie van het biologisch aktieve schildklierhormoon en voor afbraak. Zo kan ook het elders gevormde reverse trijodothyronine in de lever opgenomen en gemetaboliseerd worden.

Recente studies hebben aangetoond dat trijodothyronine dat gelokaliseerd is op zijn receptoren in de levercel, niet direkt afkomstig is van de intracellulaire omzetting van thyroxine, maar voor het grootste deel vanuit het extracellulaire compartiment. Dus, voordat trijodothyronine zijn biologische aktiviteit kan initieren, dient dit hormoon eveneens door de levercel opgenomen te worden.

Het doel van dit proefschrift behelst nadere bestudering van het mechanisme, waarmee thyroxine, trijodothyronine en reverse trijodothyronine door de levercel opgenomen worden. Deze studie is verricht met gebruikmaking van primaire kweken van rattelevercellen zoals uiteengezet is in HOOFDSTUK 1. De invloed van velerlei variabelen op de initiele kinetiek van tracer-opname werd nagegaan.

Zo wordt in HOOFDSTUK 2 beschreven, dat de kinetiek van trijodothyronineopname verzadigingskenmerken toont bij toevoeging van opklimmende hoeveelheden niet-radioactief hormoon. Naast een aspecifieke component worden twee verzadigbare systemen waargenomen n.l. een hoog-affiniteitssystem (h.a.s.) met relatief lage capaciteit en een laag-affiniteitssystem (l.a.s.) met relatief hoge capaciteit. Opname via het h.a.s. kan geremd worden door pre-incubatie van de levercellen met metabole remmers (KCN, DNP en oligomycine) en door de aanwezigheid van thyroxine. Tevens is dit systeem temperatuursafhankelijk. Deze variabelen bleken geen effekt te hebben op het l.a.s.. De energie-afhankelijkheid van het h.a.s. suggereert een transportfunktie. Daar de "V_{max}" van het l.a.s. weinig beinvloed wordt door temperatuursveranderingen en niet energie afhankelijk is, kan dit systeem binding aan de buitenzijde van de plasmamembraan voorstellen.Een andere argument hiervoor wordt gegeven in hoofdstuk 3.

In HOOFDSTUK 3 wordt de invloed van de extracellulaire albumine concentratie op de opname van trijodothyronine beschreven. De aanleiding tot deze studie waren literatuurgegevens, waarin melding gemaakt wordt van de afwezigheid van een verzadigbare opname van trijodothyronine door levercarcinoomcellen, afkomstig van de aap. Deze opname werd bestudeerd

met een relatief lage extracellulaire albumine concentratie. In het rattelevermodel kon ook geen verzadiging van de trijodothyronineopname gevonden worden indien de albumineconcentratie lager was dan 5 g/l. De effecten van albumine in het medium waren tweeledig. Allereerst, bij concentraties lager dan 5 g/l, neemt de snelheid van de aspecifieke component (diffusie) toe met het albumine, terwijl bij hogere concentraties dan 5 g/l, de diffusiesnelheid niet verandert. Verondersteld wordt, dat alleen bij een albumineconcentratie van minder dan 5 g/l, diffusie door de watermantel om de cel een snelheidsbepalende factor is. Ten tweede, tussen 5 en 20 g/l, veroorzaakt albumine ook een toename van de maximale capaciteiten van de verzadigbare systemen zonder beinvloeding van de affiniteitsconstanten. De mogelijkheden waardoor albumine het tweede effect bewerkstelligt, worden besproken in de hoofdstukken 3 en 7.

In HOOFDSTUK 3 wordt het idee, dat het l.a.s. binding aan de buitenzijde van de cel voorstelt, ondersteund, doordat de bijdrage van dit systeem wel afneemt na een wasprocedure met schildklierhormoonvrij serum in tegenstelling tot de bijdrage van het h.a.s.

In HOOFDSTUK 4 wordt beschreven dat voorbehandeling van de rattelevercellen met toenemende hoeveelheden trijodothyronine gedurende 4 uur een negatief effekt heeft op de opname van trijodothyronine via het h.a.s.. Dit effekt is afhankelijk van de duur van de voorbehandeling, terwijl remming van de eiwitsynthese door cycloheximide geen invloed had. Deze voorbehandeling met trijodothyronine bleek tevens een negatief effekt te hebben op de intracellulaire ATP hoeveelheid. Een positieve relatie was aantoonbaar tussen de aktieve opname van trijodothyronine en de ATP hoe-

veelheid. Deze relatie werd ook gevonden, nadat veranderingen in de ATP hoeveelheid werden aangebracht door de cellen met verschillende concentraties glucose of fructose voor te behandelen. De hoogte van de correlatiecoefficient van de relatie tussen de opname van trijodothyronine en de intracellulaire ATP hoeveelheid suggereert dat ATP, tenminste in vitro, een regulerende funktie heeft bij de aktieve opname van trijodothyronine.

In HOOFDSTUK 5 wordt de opname van thyroxine door geisoleerde rattelevercellen beschreven. Vergelijkbaar met de opname van trijodothyronine worden zowel een aspecifieke component als twee affiniteitssystemen aangetoond. Het h.a.s. is te remmen door voorbehandeling van de cellen met metabole remmers in tegenstelling tot het l.a.s.. Ook beschreven wordt, dat de h.a.s. van zowel trijodothyronine als thyroxine negatief te beinvloeden zijn door voorbehandeling met ouabaine, in tegenstelling tot de l.a.s.. Dit is een aanwijzing, dat de natriumgradient over de plasmamembraan een rol speelt bij de aktieve opname van schildklierhormoon.

Nadat aangetoond was, dat trijodothyronine en thyroxine aktief opgenomen worden door geisoleerde rattelevercellen, werd bestudeerd of beide jodothyronines via hetzelfde opnamesysteem of via gescheiden systemen worden opgenomen. Analyse van kompetitie experimenten, gebruik makend van beide jodothyronines als ligand en competitor, hebben aangetoond dat trijodothyronine en thyroxine gescheiden opgenomen worden.

In HOOFDSTUK 6 wordt dit aspekt verder beschreven, gebruik makend van de ATP-afhankelijkheid van trijodothyronine- en thyroxine-opname. Tevens werd dit bestudeerd voor reverse trijodothyronine-opname, nadat

bleek dat voor deze jodothyronine ook een h.a.s. aanwezig is. De opname van zowel thyroxine als reverse trijodothyronine zijn curvilineair afhankelijk van de cellulaire ATP hoeveelheid in tegenstelling tot opname van trijodothyronine, zoals beschreven is in hoofdstuk 4. Het verschil in ATP-afhankelijkheid van trijodothyronine- en thyroxine-opname pleit ook voor gescheiden opnamemechanismen, terwijl thyroxine en reverse trijodothyronine mogelijk via hetzelfde systeem worden getransporteerd.

Tevens wordt de invloed van verschillende stoffen op de aktieve opname van thyroxine en trijodothyronine in dit hoofdstuk beschreven.

Remmers van de funktie van het cytoskelet blijken ook een duidelijke vermindering van deze opname te geven. Echter, er werd ook een aanzienlijke daling van de cellulaire ATP hoeveelheid gekonstateerd, die de daling van het thyroxine-transport volledig kan verklaren. De daling van het trijodothyronine-transport onder invloed van deze remmers is echter meer uitgesproken dan veroorzaakt wordt door de daling van de cellulaire ATP hoeveelheid. Studies, verricht door anderen, hebben aangetoond, dat trijodothyronine door muizefibroblasten opgenomen wordt via een verzadigbaar endocytotisch proces. Dit impliceert, dat opname in deze cellen ook afhankelijk is van energie en bovendien van de funktie van het cytoskelet.

Stoffen, zoals D- en L-propranolol, amiodarone en rontgenkontrastmiddelen, die het zogenaamde "laag T_3 syndroom" (vide infra) kunnen veroorzaken, remmen het aktieve transport van trijodothyronine en thyroxine eveneens. Echter, beide isomeren van propranolol induceerden ook een daling van de cellulaire ATP hoeveelheid, hetgeen slechts een gedeeltelijke verklaring vormt voor het effekt op het trijodothyronine-

transport. De reden voor het additionele effekt is niet bekend. De remming door amiodarone en rontgenkontrastmiddelen, die struktuurverwantschap bezitten met jodothyronines, kan verklaard worden door kompetitie voor de carrier.

In HOOFDSTUK 7 wordt een overzicht gegeven van de literatuur, betreffende de passage van jodothyronines door de plasmamembraan en de mogelijke pathofysiologische betekenis van een energie-afhankelijke opname. Opvallend is de toegenomen belangstelling voor dit aspekt van het schildklierhormoon-metabolisme tijdens de laatste jaren. Zo blijkt uit gegevens van verschillende laboratoria, waarbij gebruik gemaakt werd van verschillende typen van geisoleerde cellen, dat:

1. "schildklierhormoongevoelige" cellen bindingsplaatsen voor deze hormonen bezitten op de plasmamembraan,
2. passage door deze membraan niet louter door diffusie bepaald wordt,
3. het opnamemechanisme "carrier mediated" is en ATP vergt. Dit actieve transport blijkt bij hypofysecellen en fibroblasten volgens "carrier mediated endocytosis" te verlopen,
4. deze adsorptie endocytose van schildklierhormoon van fysiologisch belang is, daar remming van dit proces tot een verminderde binding van trijodothyronine aan de intracellulaire receptor leidt, zoals aangetoond is met hypofyse cellen.

Van pathofysiologisch belang zijn de bevindingen verkregen met thyroxineperfusie van levers, afkomstig van gevaste ratten. Hierbij werd een verminderde trijodothyronineproduktie gemeten, die toegeschreven kan worden aan een verminderde opname van thyroxine. In hoofdstuk 7 worden enkele studies aangehaald, die

een aanwijzing vormen dat ook in vivo transport van schildklierhormoon door de plasmamembraan van belang is bij de regulatie van het perifere schildklierhormoon-metabolisme. Aldus kan dit transport een rol spelen bij de genese van het "laag T_3 syndroom", dat in het algemeen gekenmerkt wordt door normale serum thyroxine, verlaagde trijodothyronine en verhoogde reverse trijodothyronine spiegels.

Dit syndroom treedt bij allerlei ziekten op en tijdens of na gebruik van vele stoffen, zoals propylthiouracil, dexamethason en de eerder vermelde propranolol, amiodarone en rontgenkontrastmiddelen.

Tot op heden werd dit syndroom verklaard door een selektieve vermindering van de 5`-dejodase aktiviteit, waardoor thyroxine minder in trijodothyronine wordt omgezet en reverse trijodothyronine minder wordt gemetaboliseerd. Sinds recent aangetoond is, dat in de lever 5- (b.v. thyroxine → reverse trijodothyronine omzetting) en 5'-dejoderingen door hetzelfde enzym gekatalyseerd worden, is de verklaring gecompliceerder. Gezien de aanwijzingen, dat het circulerende trijodothyronine voor een groot deel geproduceerd wordt door de lever en het circulerende reverse trijodothyronine door andere organen dan de lever, kan de volgende hypothese opgesteld worden. Het "laag T_3 syndroom" is een uiting van een abnormaal schildklierhormoon-metabolisme in de lever. De verminderde thyroxine → trijodothyronine omzetting en reverse trijodothyronine afbraak kunnen verklaard worden door zowel een verminderde opname van thyroxine en reverse trijodothyronine als een verminderde dejodering van deze iodothyronines. In beide situaties resulteert dit in een verlaging van de trijodothyronine serumspiegel en een verhoging van de reverse trijodothyronine serumspiegel. De hoge snel-

heid van de 5`-dejodering van reverse trijodothyronine in de lever in beschouwing nemend, zal het merendeel van het reverse trijodothyronine, dat in de lever gevormd wordt door 5-dejodering van thyroxine, afgebroken worden voordat het in de circulatie kan komen. Een verminderde produktie van reverse trijodothyronine in de lever zal daardoor niet tot uiting komen door meting van de serumspiegels. Faktoren die een rol kunnen spelen bij een verandering van de aktieve passage door de plasmamembraan zijn:

1. de cellulaire ATP hoeveelheid,
2. de natrium gradient over de plasmamembraan,
3. een direkte remming door allerlei exogene stoffen, b.v. die met strukturele verwantschap met jodothyronines, en
4. een circulerende endogene remstof, die zowel de binding aan schildklierhormoon-bindende serumeiwitten als de cellulaire opname beinvloedt, zoals door anderen is gepostuleerd.

Geavanceerde in vivo studies zullen verricht moeten worden om het belang te testen van de regulatie van het transport door de plasmamembraan.

Het nut van adaptieve veranderingen van het perifere schildklierhormoonmetabolisme, zoals tot uiting komt door het "laag T_3 syndroom" tijdens stress, kan een bescherming van het organisme zijn tijdens deze omstandigheden. Immers, een verminderde trijodothyronine produktie tijdens stress zal b.v. een energiebesparend effect hebben.

NAWOORD

Velen ben ik dank verschuldigd bij de totstandkoming van dit proefschrift.

Allereerst mijn ouders voor alle inspanningen, die zij zich getroost hebben, om de basis te leggen, waarop dit mogelijk werd.

Ondanks het feit, dat zij eronder hebben moeten lijden, waren Ellen, Boudewijn en Andrea een stimulans bij het schrijven van dit proefschrift.

De inhoud is een voortvloeisel van een nauwe, vruchtbare en vriendschappelijke samenwerking met Jorg Hennemann, Roel Docter en Theo Visser. Zonder de specifieke eigenschappen en wetenschappelijke hobby's van een ieder zou naar mijn mening dit resultaat nooit verkregen zijn. Deze samenwerking heeft blijkbaar tevens onontgonnen gebieden bij mij blootgelegd. Zonder compleet te kunnen zijn, ben ik Jorg, mijn promotor, erkentelijk voor zijn altijd stimulerende invloed en de grote bewegingsvrijheid, die hij zijn medewerkers gunt. Roel, met zijn grote kennis van laboratorium-techniek en computer- programmering, is onder meer op dit gebied van onschatbare waarde geweest. Van de waardevolle kritiek van Theo werd altijd dankbaar gebruikgemaakt en aan de vele discussies met hem heb ik de beste herinneringen. Tevens ben ik erkentelijk voor het redactionele werk, dat hij voor dit proefschrift verricht heeft. Ik apprecieer het ten zeerste, dat Roel en Theo ook tijdens mijn promotie mijn paranimfen willen zijn.

Wim Hulsmann ben ik zeer dankbaar voor zijn belangstelling en de discussies tijdens de verscheidene besprekingen betreffende de energie-huishouding.

Verder ben ik hem erkentelijk voor de kritische opmerkingen, die hij als co-referent bij het proefschrift heeft gemaakt.

Het merendeel van de experimenten zijn op voortreffelijke wijze uitgevoerd door Bert Bernard, die hierbij zeker niet te klagen had over koude handen. Vele bepalingen van de vrije hormoon-concentraties zijn verricht door Ineke van der Does-Tobé . Analytische steun werd verder verleend door Wim klootwijk en Marla van Loon. Tevens ben ik het gehele laboratorium dankbaar voor de plezierige samenwerking.

Jan Lindemans heeft ons aanvankelijk ingewijd in de isolatie-techniek van rattelevercellen, zodat, na een korte inwerkingsperiode, het verkrijgen van het benodigde aantal levercellen geen probleem vormde.

Problemen met een variantie-analyse waren na het inwinnen van adviezen bij Johan Koerts snel opgelost.

Corry Boot-Timmer heeft het merendeel van de tekst in de tekstverwerker ingevoerd, waarbij zij steun gehad heeft van Anke de Graaff. Tevens ben ik Henk van Beek erkentelijk voor de plezierige steun bij problemen met de tekstverwerker, die natuurlijk bijna altijd een menselijke grondslag hadden. Marco Roede heeft de grafiek op de omslag vormgegeven. De grafische vormgeving van figuren en tabellen werd op snelle wijze verzorgd door de audio visuele dienst.

Dit onderzoek werd verricht met steun van FUNGO en T.N.O.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 11 december 1946 te `s-Gravenhage geboren, alwaar hij het H.B.S.-B diploma behaalde. De studie in de geneeskunde werd in 1966 aan de Medische Faculteit te Rotterdam aangevangen. In 1971 werd het doctoraal examen en in 1972 het artsexamen afgelegd. Vanaf begin 1973 genoot hij een opleiding tot internist aan de afdeling inwendige geneeskunde III (hoofd Prof. Dr. J.C. Birkenhäger) van het Academisch Ziekenhuis Rotterdam-Dijkzigt. In 1978 vond inschrijving in het specialisten register plaats. Sedertdien is hij als internist werkzaam bij bovenvermelde afdeling, alwaar dit proefschrift bewerkt werd onder leiding van Prof. Dr. G. Hennemann.