ROLE OF CELLULAR TRANSPORT SYSTEMS IN THE REGULATION OF THYROID HORMONE BIOAVAILABILITY

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Voor Marianne

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate	
CRF	chronic renal failure	
Da	dalton	
DNA	deoxyribonucleic acid	
DNP	dinitrophenol	
FDH	familial dysalbuminemic hyperthyroxinemia	
FT_3	free (not protein bound) triiodothyronine	
FT_4	free (not protein bound) thyroxine	
GH	growth hormone	
HAS	high affinity system	
Hep G2	human hepatoma cell line	
K_a	equilibrium association constant	
kDa	kilodalton	
K_i	Michaelis constant of inhibition	
K_m	Michaelis constant	
LAS	low affinity system	
NTI	non-thyroidal illness	
PTU	6-propylthiouracil	
REP	rapidly equilibrating pool	
SEP	slowly equilibrating pool	
SHBG	sex hormone binding globulin	
TBG	thyroxine binding globulin	
TBPA	thyroxine binding prealbumin	
T_2	3,3'-diiodo-L-thyronine	
T_2S	3,3'-diiodo-L-thyronine sulfate	
rT_3	3,3',5'-triiodo-L-thyronine	(reverse T_3)
T_3	3,3',5-triiodo-L-thyronine	(triiodothyronine)
T_3S	3,3',5-triiodo-L-thyronine sulfate	
T_4	3,3',5,5'-tetraiodo-L-thyronine	(thyroxine)
V_{max}	maximal velocity	

Chapter I

Scope of the thesis

SCOPE OF THE THESIS

Thyroid hormone plays an important role in the development and in the regulation of metabolic processes in vertebrates. The hormone is produced by the thyroid, mainly as the biologically inactive product thyroxine (T_4). Only 20% of the biologically active form of thyroid hormone, 3,3',5-triiodothyronine (T_3), is produced by the thyroid, the remaining 80% stems from the peripheral deiodination of T_4 to T_3. In the latter conversion process, the deiodinase located in the liver plays an important role. Organs, like the brain, the pituitary and brown adipose tissue, produce T_3 from T_4 locally to meet (part of) their needs of active hormone. In contrast, muscle, and also liver, largely depends on plasma T_3 for biologic action (This means that most of the T_3 produced in the liver from T_4 is first secreted into the plasma before part of it is internalized again). Because the deiodinases are located in the endoplasmic reticulum, it is clear that T_4 has to cross the plasma membrane as a first and important step in its activation. Furthermore, plasma membrane transport of T_3 is necessary before it can regulate gene expression by binding to nuclear receptors found in a wide variety of cells and tissues, or before it can be degraded. Chapter II describes the general knowledge about thyroid hormone transport and metabolism accumulated by others and ourselves predominantly during the last decade.

In this thesis the relevance of the plasma and plasmamembrane transport of thyroid hormone in its activation and metabolism is described. All early studies in our laboratory on transport of thyroid hormone over the plasma membrane were performed with rat hepatocytes in primary culture, with a medium containing only 0.5 or 1% albumin, in fact in no way comparable with plasma or interstitial fluid, the physiological medium. Plasma contains three thyroid hormone binding proteins, which bind more than 99.97% of the circulating T_4. About 75% of this T_4 is bound to thyroxine binding globulin, 15% to thyroxine binding prealbumin and 10% to albumin. Because there existed (and still exists) a controversy in literature whether different concentrations of binding proteins influence uptake of hormone into tissue cells, we have tried to resolve this problem by comparing the results of tracer kinetic studies in subjects with an

autosomal dominant inherited elevated binding of T_4 to serum proteins with similar data obtained in normals. In chapter III it was shown that a strongly elevated binding of T_4 in serum does not lead to a change in free hormone concentration or a change in disposal rate of T_4, which finding makes a role of these binding proteins in the tissue uptake of thyroid hormone unlikely. Furthermore, it was shown that this biochemical syndrome, originally described by us, is caused by albumin with high affinity for T_4, and that this albumin is also present in low concentrations in normals. In later studies in the literature this syndrome is known as "familial dysalbuminemic hyperthyroxinemia" (FDH).

Other questions addressed in this thesis are: Is the thyroid hormone uptake system, present in rat hepatocytes, also operative in human cells (Chapters IV and VII); Is the efflux of thyroid hormone out of the cell an active, carrier mediated process (Chapter V); Does inhibition of the uptake of hormone results in a diminished metabolism (Chapters VI and VIII) or occupancy of the nuclear T_3 receptor (Chapter V); and finally, Does normal albumin play a role in cellular transport of iodothyronines (Chapter IX).

In chapter IV it is demonstrated that human erythrocytes, an easily obtainable human cell type, contain a carrier mediated transport system for T_3, which system is, in contrast to rat hepatocytes, not energy (ATP) dependent and cannot be inhibited by ouabain. Therefore, this system is not comparable to the energy dependent transport system of rat hepatocytes. On the other hand, chapter VI shows that human cultured fibroblasts contain both a high and a low affinity transport system, completely comparable to that of rat hepatocytes.

Under equilibrium conditions it can be argued that the intracellular free hormone concentration is only dependent on the extracellular free hormone concentration, the activity of the uptake system transporting hormone into the cells, and the efflux processes transporting hormone out of the cells, regardless the amount of intracellular binding proteins. As it was shown in chapter V that efflux of thyroid hormone out of the cell is a passive mechanism, probably driven by the free hormone concentration gradient, the intracellular free hormone concentration is therefore (apart from the rate

of the metabolic processes) probably only dependent on the activity of the uptake process. This implies that inhibition of this process will lead to a lower free hormone concentration inside the cells and thus to a lower nuclear receptor occupancy and lower substrate availability to the deiodinating and conjugating enzymes, and consequently to a lower metabolism. We could actually show this sequence of events, both in the case of the occupancy of the nuclear receptor by T_3 (chapter V) and in the case of diminished metabolism when uptake of thyroid hormone is inhibited, irrespective of the type of inhibition, either by ouabain, or a monoclonal antibody (chapter VI) or by chemical modification of the carrier protein (chapter VIII).

In a number of publications Pardridge et al. claimed that only hormone bound to albumin is available for transport into the liver. From this finding they conclude that cellular transport into the liver is dependent on the albumin concentration in plasma. With a recirculating liver perfusion technique we could demonstrate that <u>total</u> transport of T_3 to the liver is indeed dependent on the albumin concentration (chapter IX). However, it could also be shown that not all T_3 transported to the liver is being translocated to the cellular compartment and subsequently metabolized, but part is bound outside the cellular compartment. Only transport to this latter pool of T_3 is dependent on the albumin concentration in the medium. The amount of T_3 transported into the cells for further metabolism is solely determined by the free T_3 concentration and is independent of total T_3 or albumin concentration in the medium.

Chapter II

Plasma membrane transport of thyroid hormones

1. INTRODUCTION

Thyroxine (T_4), the principal secretory product of the thyroid gland, has little intrinsic biological activity (1). Production of the actual hormone 3,3',5-triiodothyronine (T_3) involves enzymatic deiodination of the phenolic ring of T_4 (1). It is also possible that the tyrosyl ring of T_4 is deiodinated, in which case 3,3',5'-triiodothyronine (rT_3) is formed with complete loss of thyromimetic activity (1). Deiodinases, which act not only on T_4 but also on its derivatives, are present in various tissues (2). Prominent among these is the liver deiodinase, or type I deiodinase (2), the liver being the major source of peripheral T_3 production from T_4 (3). This enzyme catalyzes both deiodination of the outer- or phenolic-ring, in which case T_4 is converted to T_3, or rT_3 to 3,3'T_2, as well as the deiodination of the inner- or tyrosyl-ring, producing rT_3 from T_4, or 3,3'T_2 from T_3 (fig. 1). Normally deiodination of T_4 accounts for 80% of total T_3 production and an even greater fraction of rT_3 production in humans and in rats (4,5). Because the deiodinases are located inside the cell in the endoplasmic reticulum, both in the liver (6) and in the pituitary (7), it is clear that transport of T_4 over the plasma membrane is a first and important step in its activation. Furthermore there exists abundant evidence suggesting that T_3 regulates gene expression by binding to nuclear receptors found in a wide variety of cells and tissues (8,9). The majority of cells predominantly relies on plasma T_3 for their supply of hormone (10,11), although there are some notable exceptions as for instance rat brain, in which > 50% of T_3 is obtained from local conversion of T_4 (10). In rat liver about 40% of T_3 is produced by local conversion (10), except for nuclear T_3 which is for 80% or more derived from plasma (11,12). It is therefore clear that T_3 has to be transported over the plasma membrane too, as a first step before it can exert its activity, or before it can be degraded. It has been postulated that thyroid hormones enter the cell by passive diffusion (13,14), based on the lipophilicity of the hormones, which would therefore easily pass the lipid bilayer of the plasma membrane.

However since 1976 a large body of evidence has been presented from different laboratories, that thyroid hormones are transported from the extracellular compartment through the plasma membrane into the cell by an energy-dependent, saturable

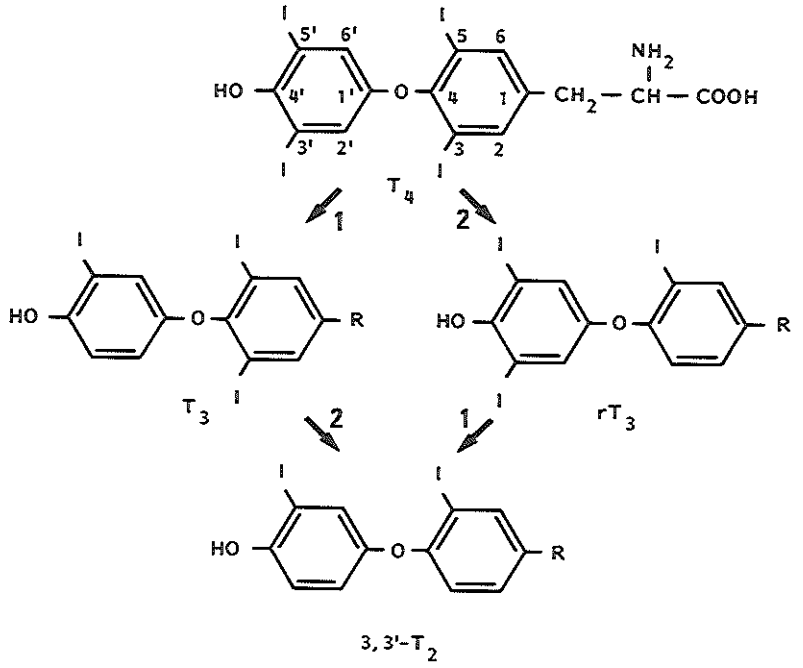


Figure 1.

Pathways of thyroxine (T_4) deiodination.

1 = outering deiodination, producing 3,3',5-triiodothyronine (T_3) from T_4 or 3,3'-diiodothyronine (3,3'-T_2) from 3,3',5'-triiodothyronine, or reverse triiodothyronine (rT_3). 2 = innering deiodination, producing rT_3 from T_4 or 3,3'T_2 from T_3. Both reactions are catalyzed by the type I deiodinase as present in liver and kidney. Type II deiodinase as present in pituitary, brain and brown adipose tissue, catalyzes only reaction I, while type III deiodinase present in brain, skin, and placenta only catalizes reaction II (2).

process (15-30).

2. INFLUENCE OF PLASMA BINDING ON MEMBRANE TRANSPORT

In euthyroid humans differences in the sizes of the extracellular pools of thyroid hormones are encountered, due to variations in serum binding proteins. Normally over 99% of the hormones are bound to plasma proteins. The three major carriers are thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) and albumin. In normal serum TBG binds 78%, TBPA 11.5% and albumin 10.3% of circulating T_4 (31[chapter III]). According to "the free hormone hypothesis" as originally proposed by Robbins and Rall (32), the free hormone concentration in

serum governs hormonal delivery to the cell and ultimately regulates hormonal action. It can be calculated that in the human liver the rate of dissociation of T_4 from TBG, TBPA and albumin together is more than one order of magnitude larger than the unidirectional removal of T_4 from the plasma during a single pass (31[chapter III],33-35; see Table 1). Because in equilibrium the efflux from the cells is almost as large as the influx, the net removal of T_4 is three orders of magnitude smaller than the combined dissociation rates of T_4 from its binding proteins. Thus in this model the intracapillary free hormone concentration is maintained at a constant level. Similar calculations show that TBPA, the major carrier protein in rats (36,37), liberates T_4 at a faster rate than required for tissue entry during a single capillary

Table 1

Bound hormone		Free hormone		Liver	disposal
T_4-TBG $t_{1/2}$ = 39 sec	→ 1588 ←				
T_4-TBPA	→	FT_4	542 →	Pool T_4	> 3.0 →
$t_{1/2}$ = 7.4 sec	1230 ←	2.5 pmol	← 534.8	539 nmol	
T_4-Alb $t_{1/2}$ < 1 sec	→ > 7910 ←			↓ T_4 → T_3 conversion < 4.3	
T_3-TBG $t_{1/2}$ = 4.2 sec	→ 266 ←			↓	
T_3-TBPA	→	FT_3	53.6 →	Pool T_3	2.7 →
$t_{1/2}$ = 1 sec	30 ←	0.4 pmol	← < 55.2	6.8 nmol	
T_3-Alb $t_{1/2}$ < 1 sec	→ > 285 ←				

Hormone fluxes (pmol/10 sec) in the euthyroid human liver during one capillary transit (10 sec, 123 ml plasma (33)). $t_{1/2}$: halflife of dissociation of the hormone from the protein at 37 C (34). Distribution of T_4 over its binding proteins from (31), and of T_3 (TBG: 77 ± 4.5%, TBPA: 2.1 ± 1.1%, Albumin: 20.2 ± 4.2% (mean ± SD, n=20) estimated with the method described in (31)). All other data are taken from (35). FT_4 and FT_3: extracellular amount of free hormone in pmol/123 ml. Values between the first and second column indicate dissociation of hormone in pmol/10 sec at 37 C based on normal total hormone concentrations. Values between the second and third column indicate transport of hormone from the free hormone pool into the liver and vice versa.

pass. However, the actual situation may be more complicated by molecular events occuring in the microcirculation such as intracapillary diffusion and capillary wall perfusion (38,39).

In contrast with Robbins and Rall, Tait and Burstein (40), in their studies on transport of steroid hormones into the liver put forward the concept that only the free hormone pool is available for transport into the liver during a single pass of blood through the organ. However, from Table 1 it is clear that this cannot be the case in transport of thyroid hormones, because the unidirectional removal of hormone greatly exceeds the free hormone pool. Therefore, Pardridge and coworkers have extended this concept in a number of presentations (see ref. (41) for a review) in such a way that besides the free hormone pool also the hormone bound to albumin is available for transport. To elaborate this model these authors have used a single pass injection technique (42). In this technique tracer hormone is injected together with tritiated water, a freely diffusable substance, in various media into the portal vein. After one pass through the liver, cellular uptake of thyroid hormone is calculated from the difference between the amount of tritiated water and thyroid hormone retained by the liver. However, besides large methodological problems, which are discussed elswhere (43,44), it is clear that this method measures the unidirectional flux to the liver, which is two orders of magnitude greater than the net removal of hormone in an equilibrium situation (Table 1). Because it is clear from the data presented in Table 1 that albumin is able to deliver the largest amount of hormone by dissociation, the authors conclude that only hormone bound to albumin can be transported into the liver. To explain the large difference between the size of the intravascular free hormone pool and the amount of hormone transported into the liver, they postulate that T_4 in plasma enters tissues by protein-mediated transport or enhanced dissociation from plasma-binding proteins (45). This leads to the conclusion that all T_4 uptake in the rat occurs via the pool of albumin bound T_4 (45), because in the same article the authors conclude that T_4 bound to TBPA in rat serum is not available for transport. But recently it was shown (46) that transport of T_4 into the rapidly exchangable pool (REP), which is mainly composed of liver and kidney (47,48), and the size of the REP in analbuminemic rats is indistinguishable from normal rats. This finding makes a special role for albumin in T_4 transport in

the rat very unlikely.

According to Pardridge et al. (45) T_4 bound to human TBPA is highly transportable in liver as is T_4 bound to human albumin (42). Therefore one would expect that T_4 bound to albumin present in subjects with familial disalbuminemic hyperthyroxinemia (FDH) is also transportable in liver, because the affinity constant of this modified albumin (K_a 0.7 - 1.0 x 10^7 l/mol) (31,49[chapter III]) is in between that of TBPA ($K_a \approx 10^8$ l/mol) (31[chapter III]), and albumin ($K_a \approx 10^5$ l/mol) (31[chapter III]). FDH is an autosomal dominant biochemical syndrome, which is characterized by elevated total T_4 concentrations, normal free T_4 levels, normal total T_3 concentrations and an euthyroid clinical status (50[chapter III],51,52). Originally it was claimed by Pardridge and coworkers (53) that T_4 bound to FDH albumin was freely transportable in rat liver, leading to a hyperthyroid liver with increased plasma concentrations of sex hormone binding globulin (SHBG) in many subjects with FDH, similar to the increased concentrations of SHBG in patients with hyperthyroidism (54). These findings were presented in extended form in a later study by a number of the same authors (55). In the original description of the FDH syndrome (50[chapter III]) it was found that T_4 turnover was normal in affected subjects, and subsequently, Mendel and Cavalieri (56) showed that the absolute rate of T_4 flux into the rapidly exchangeable cellular compartment, the intracellular T_4 pool size, and the T_4 disposal rate are all normal in FDH, consistent with the normal serum concentrations of free T_4 and the eumetabolic state of these individuals. In a following paper of Pardridge and coworkers (57) it was shown that the bioavailability of T_4 bound to FDH albumin was low, in contrast to their original study (53) and a later study of a number of the same authors (55), but in accordance with the findings of Hennemann et al. (50[chapter III]), Mendel and Cavalieri (56) and Sarne and Weinberg (58). The issue was reopened by Bianchi et al. (59), who showed, using a three compartmental model of hormone distribution and metabolism (47,48), that T_4 disposal rate was significantly increased (by 42%) in subjects with FDH as compared to controls, while there was no change in subjects with TBG excess, a condition with comparable serum T_4 elevations as FDH. T_4 and T_3 distribution volumes and T_3 clearance rate were unchanged. T_3 peripheral production was increased by 24% in FDH subjects. From these data the authors conclude that T_4 bound to FDH albumin is more

available to tissues than T_4 carried by TBG, thus suggesting an important role of albumin in T_4 availability to peripheral tissues. However, close inspection of the data presented (59) revealed that the mean free T_4 concentration in the FDH group was 40% higher than in the normal group or the group with TBG elevation. Therefore, the raised T_4 disposal in the FDH group could be easily explained by this higher free T_4 concentration on the basis of the free hormone hypothesis of Robbins and Rall (32).

Finally, it was shown in a recent study (60) that cellular uptake of T_4 by human hepatoma (Hep G2) cells from media containing serum from normals, subjects with FDH, or subjects with TBG excess, reflects the free T_4 concentration and is not influenced by the fraction or amount of T_4 bound to TBG or to the variant albumin of FDH. Therefore, it can be concluded from the data presented that most probably there is no special role for albumin in the transport of thyroid hormones into the intracellular compartment, and that transport of thyroid hormones is governed by the free hormone concentration, if the membrane carrier processes are unchanged, which is clearly not always the case (vide infra).

3. IN VIVO STUDIES

Clinical conditions such as nonthyroidal illness, starvation, surgery, or use of compounds such as x-ray contrast agents, dexamethasone, propranolol or propylthiouracil (PTU) induce a "low T_3 syndrome" (61), which is characterized by decreased serum T_3 concentrations and elevated rT_3 levels, while serum total T_4 concentrations are usually normal. Kinetic studies of the turnover of the hormones have revealed that the production of T_3 is decreased, but its clearance is unchanged, whereas production of rT_3 is unchanged, but its clearance is diminished (Fig. 2) (1,61). One explanation could be a reduced 5'-deiodinase tissue activity, resulting in a decreased T_3 production from T_4 and a reduced breakdown of rT_3 (61). Another explanation, however, could be a change in transport of thyroid hormone across the plasma membrane leading to a reduced substrate availability for the type I deiodinase in the tissues, which will also lead to a reduced production of T_3 from T_4 and breakdown of rT_3.

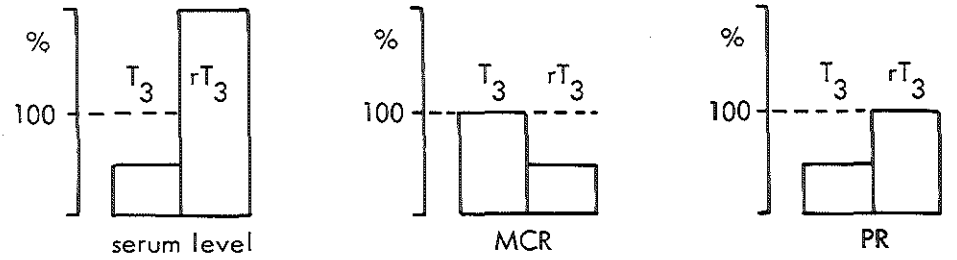


Figure 2.

Effects of various clinical conditions such as nonthyroidal illness, starvation, surgery, or use of compounds such as x-ray contrast agents, dexamethasone, propranolol or propylthiouracil (PTU) on serum levels, metabolic clearance rate (MCR), and production rate (PR) of T_3 and rT_3 (expressed as % of control).

3.1 T_4 kinetics

Most of the kinetic studies mentioned above have been performed with the non-compartmental analysis as first applied to thyroid hormones by Oppenheimer et al. (62). This calculation technique yields values for total body hormone pool sizes, fractional clearance rates and total body production rates. However, it is not possible to estimate tissue pool sizes or fluxes into tissues (63). These data can be obtained using a three compartmental model of thyroid hormone distribution and metabolism. This model consists of a central plasma pool in equilibrium with a rapidly equilibrating tissue pool (REP) and a slowly equilibrating tissue pool (SEP). It is assumed that disposal of hormone only occurs in the tissue pools. Since the original publications of DiStefano et al. (47,48) on the use of this three compartmental kinetic model of T_3 and T_4 metabolism, a large number of reports has appeared using this model. It can be argued that the REP in this model is mainly composed of liver and kidney (47). Because the size of the liver pool is quantitatively the most important, mass transport to this REP can be equated to hormone transport to the liver. With this method we showed that transport of T_4 to the REP was diminished during caloric restriction (35), a condition leading to a low T_3 syndrome as indicated by low serum T_3 and raised serum rT_3 concentrations, and to a decrease in size of the rapidly

equilibrating T_4 pool, i.e. liver compartment (35) (Table 2). This finding could indicate a lower substrate availability for the type I deiodinase, the enzyme responsible for T_3 production (64,65) and rT_3 breakdown (33,66) in the liver, leading to a lower production rate and serum concentration of T_3 with an unaltered metabolic clearance rate of T_3 (35). On the other hand, induction of a low T_3 syndrome with d-propranolol (67) is not associated with an altered transport of T_4 into the liver, nor to a smaller REP (Table 2). Therefore, it seems that in this case the lower T_3 production and rT_3 degradation has to be explained on the basis of an inhibition of the type I deiodinase.

In the normal and low-T_4 states of nonthyroidal illnesses the fractional rate of T_4 transport from serum to tissues is reduced to one-half of the normal value (Table 2). There are indications that a similar alteration is present in the high-T_4 state induced by illness (68) and with acute amiodarone administration (69). This decrement in fractional rate of T_4 transport is not related to the serum levels of total or free T_4 (70-75; Table 2). On the other hand the fractional rate of T_4 transport is reduced in high TBG states (76) or FDH (56), and increased in low TBG states (71,75,76) in healthy subjects. This results in a normal mass transport of T_4 to the tissues. Because in illness the reduction in the fractional rate of T_4 transport from serum to tissues cannot be attributed to alterations in serum T_4 binding, other causes such as an impairment of transport into tissues must be assumed. However, this is not always the explanation for the occurrence of a low T_3 syndrome, as was shown in subjects after ingestion of d-propranolol (67), suggesting that the changes in T_4 handling were restricted to the type I deiodinase. This is probably also the case in patients with a low T_3 syndrome caused by chronic renal failure (CRF). In two studies by Kaptein et al. (73,74) a diminished fractional rate of T_4 transport in CRF was found, while in a later study the same authors did not find any change in transport of T_4 as compared to normals (77) (Table 2). This latter finding is in accordance with Faber et al. (78) who reported a strongly reduced serum T_4, a normal FT_4 with an elevated metabolic clearance rate in CRF, leading to a normal T_4 transport and disposal. These findings can be explained on the basis of normal T_4 production with diminished serum binding of T_4. However, T_3 production is diminished (78), which indicates a diminished T_4 to T_3 conversion, and be cause transport of T_4 seems not

Table 2
Kinetic data of thyroxine transport, and tissue concentrations in nonthyroidal illnesses, and other conditions causing a low T_3 syndrome.

Diagnosis	T_4	FT_4	fractional transfer rate	mass transport rate	T_4 content: All tissues	T_4 content: REP	reference
	(% of normal mean values)						
Liver disease	105	-	45	48	-	-	(70)
Liver disease	99	174	41	46	42[a]	-	(71)
Cirrhosis	-	-	38		-	54	(72)
Chronic renal failure	112	119	48	54	84	75	(73,74)
Critical illness	25	56	54	29	24	21	(75,74)
Chronic renal failure	99	93	99	116	128	149	(77)
Caloric deprivation	98	125	68	69	72	80	(35)
D-propranolol treatment	114	113	98	112	111	123	(67)

[a]) 78% present in the liver (as estimated by liver biopsy)

to be changed, this can be explained by an altered type I deiodinase activity.

3.2 T_3 kinetics

In normal subjects 20% of the T_3 production stems from thyroidal secretion and 80 % from peripheral deiodination of T_4. In nonthyroidal illness thyroidal production of T_3 is normal (79), but the peripheral production of T_3 is decreased, which diminution is responsible for the low serum T_3 levels. In fact, a direct correlation between the serum T_3 concentrations and the production rates of T_3 can be found in patients with nonthyroidal illnesses (80), because usually the metabolic clearance rate of T_3 is not different from normal (35,67,80). Thus, a decreased serum T_3 concentration is indicative for a diminished production of T_3, because production rate is equal to the product of metabolic clearance rate and serum concentration (62). The fractional rate of transport of T_3 to tissues is not significantly altered in patients with liver cirrhosis (72), critically ill patients (75) or during caloric deprivation (35). Since these patients have a marked reduction in their serum T_3 binding, one would expect

Table 3

Thyroid hormone binding to plasma membranes of various tissues

Source of membranes	Dissociation constants (K_d) (nM)						reference
	T_4		T_3		rT_3		
	K_{d1}	K_{d2}	K_{d1}	K_{d2}	K_{d1}	K_{d2}	
Rat liver	-		3.2	220	-		(85)
	0.4	23	6.0	300	-		(86)
	0.6	23	9.7	237	-		(87)
	0.6	1)	200	1)	4	1)	(88)
	6.6	1)	-		-		(89)
	9.9	-	-		-		(92)
Rat spleen	NM		NM		-		(87)
Rat kidney	4.5	127	16	270	-		(87)
Rat testis	28	286	2.7		-		(87)
Rat thymocytes	-		1.0	25	-		(90)
Human placenta	-		2.0	18500	-		(91)

K_{d1} = high affinity; K_{d2} = low affinity;
1) = second class of binding sites present; - = not tested;
NM = not measurable

an increase in the fractional rate of transport of T_3 to tissues as is seen in patients with low TBG (81). The finding of a normal fractional rate of transport of T_3 to tissues in these patients is suggestive of the presence of a tissue transport defect of T_3 too.

3.3 rT_3 kinetics

rT_3 serum concentrations are increased and metabolic clearance rates are decreased in patients with nonthyroidal illness (67,75,82-84), and both are about normal in those with renal disease (73,78). This results in normal production rates in both groups of patients. Data on the fractional rate of transport of rT_3 to tissues are scarce. We have shown (67) that during d-propranolol treatment rT_3 transport to the tissues is normal, as was also found for T_4. This is in accordance with the finding that in rat hepatocytes rT_3 and T_4 are transported by the same mechanism (19-21),

which would imply that inhibition of transport of T_4 to the liver, as in caloric deprivation (35), will be accompanied by a diminished transport of rT_3 to the liver. Because the liver is the main site of disposal of rT_3 (33), this will lead to a diminished metabolic clearance rate of rT_3. Production however is not altered (75,82-84), and therefore serum concentration of rT_3 is elevated. This has been actually found (35), but whether the sequence of events as outlined here is the cause of this elevation remains to be proven experimentally.

4. IN VITRO STUDIES

4.1 Thyroid hormone binding to purified plasma membranes

Plasma membrane preparations of rat liver, kidney, testis and thymocytes contain two sets of binding sites for T_4, T_3, and rT_3 (85-92; see Table 3 for an overview), although not all authors tested all three compounds. In purified plasma membranes of rat spleen no binding sites were detectable (87). Because spleen response to thyroid hormone is limited (93), there may be a relationship between the presence of membrane binding sites and the sensitivity to thyroid hormone in different tissues (87). High-affinity binding of T_4 and T_3 occurs to different sites (87), as deduced from mutual displacement experiments. Furthermore, it has been suggested that these binding sites are involved in transport of thyroid hormone over the plasma membrane (85,87).

4.2 Thyroid hormone uptake into isolated cells

4.2.1 *Triiodothyronine*

Kinetic studies of the initial uptake of T_3 into rat liver parenchymal cells in primary culture have shown the presence of two saturable sites, one with a low K_m or high-affinity site (HAS), and the second with a high K_m or low-affinity site (LAS), respectively (see table 4). Similar findings were reported by other authors for a number of different cell types, such as rat hepatocytes (15,27,94), human cultured fibroblasts (22,26[chapter VII],95), cultured GH_3 cells (96), human Hep G2 hepato-

carcinoma cells (28), human cultured lymphocytes (18), mouse thymocytes (30), and mouse neuroblastoma cells (97).

The HAS is (partially) blocked when cells are preincubated with metabolic inhibitors such as KCN, dinitrophenol (DNP) or oligomycin, indicating the energy-dependency of the system (Table 4). This was also found in other cell types (97). The HAS is also strongly influenced by changes in temperature. An increase in incubation temperature leads to an increase in the apparent Michaelis constant (K_m) (Table 4) and maximal velocity (V_{max}) of this high-affinity system (19). This dependence on both temperature and energy strongly suggests that the HAS of T_3 represents an active transport process. Uptake of T_3 by the HAS at 21 C can be inhibited competitively by high concentrations of T_4, with a K_i of 46 nM (19), a much higher value than the K_m for the HAS of T_4 (1.2 nM; Table 5). Furthermore, the HAS is inhibited by ouabain, a specific inhibitor of Na^+,K^+-ATPase, which could imply that a sodium gradient over the plasma membrane is of importance in this transport process. This sodium-dependence of the T_3 uptake was also found in rat skeletal muscle (98). It is however clear that the HAS and the Na^+,K^+-ATPase are not identical, because a recently developed monoclonal antibody, which inhibits uptake of T_3 by rat hepatocytes, does not influence the transport of Rb^+ into these cells (99), in contrast to ouabain which strongly inhibits this transport (99). On the

Table 4

Characteristics of initial uptake of 3,3',5-triiodothyronine by different cell types.

Cell type	temp. °C	K_{m1} nM	K_{m2}	Inhibitors of low K_m system	reference
Rat hepatocytes	0	NM	1500	KCN 2 mM	(17,19,20,99)
	21	21	1800	DNP 2 mM	
	37	61	2800	Oligomycin .1 mM	
				Ouabain .5 mM	
				Monoclonal antibody	
Human fibroblasts	37	29	646	KCN .1 mM	(26)
				Ouabain 2 mM	
Human erythrocytes	37	128	NM	not energy dependent	(103)

K_{m1} - low K_m (high affinity) system; K_{m2} - high K_m (low affinity) system; NM - not measurable

other hand, the LAS of T_3 does not show an important temperature- or energy-dependence (19; Table 4). On this and other evidence, like the fact that the V_{max} of the LAS of T_3 but not of the HAS is lowered by washing the monolayers with medium containing a T_3-binding protein after the 1 min incubations with tracer T_3, it has been suggested that this LAS represents binding of T_3 to the outer surface of the cell (17,100).

It has been shown that transport of T_3 into rat skeletal muscle is saturable, stereospecific and energy dependent (25,98,101). Similar findings have been reported for rat skeletal myoblasts in culture (102). This transport is dependent on an intact sodium gradient over the plasma membrane, because diminution of this gradient either by replacement of extracellular Na^+ by choline or lithium, or by inhition of Na^+/K^+-ATPase with ouabain, reduced the specific (saturable) uptake component (98). However, T_4 uptake was not affected by changes in extracellular Na^+ (98). Insulin stimulated specific T_3 uptake more than two-fold (101). The effect could be blocked by addition of 10 μM T_3 to the medium or by substitution of extracellular sodium with lithium. In contrast, T_4 uptake is insensitive to insulin (101).

Uptake of T_3 by human erythrocytes on the other hand involves only one saturable process, which is not energy-dependent and cannot be inhibited by ouabain (103[chapter IV]). This has also been found for tadpole, frog (104), and rat erythrocytes (105,104), and recently it has been shown that transport of T_3 into rat erythrocytes follows a so-called simple pore model (106).

4.2.2 *Thyroxine*

Similar to T_3 it has been shown with T_4 tracer kinetic studies that initial uptake of T_4 into cultured rat hepatocytes (19,20,99,107) and human cultured fibroblasts (26[chapter VII]) consists of two saturable uptake systems: a high-affinity system (HAS) and a low-affinity system (LAS) (see Table 5). In hepatocytes the V_{max} of the HAS is dependent on temperature; i.e., an increase in temperature leads to an increase in the V_{max} (107). Analogous to T_3 the HAS of T_4 is energy-dependent and ouabain-sensitive, which findings suggest a transport function, while the LAS of T_4 is neither dependent on energy nor on ouabain, both in hepatocytes (107) and in fibroblasts (26[chapter VII]). Uptake of T_4 by the HAS at 21 C is inhibited competi-

Table 5

Characteristics of initial uptake of thyroxine and 3,3',5'-triiodothyronine by different cell types.

Cell type	temp. °C	K_{m1} nM	K_{m2}	Inhibitors of low K_m system	reference
T_4					
Rat hepatocytes	21 37	1.2 1.4	1000 1)	KCN 2 mM Oligomycin .1 mM Ouabain .5 mM Monoclonal antibody	(19,20,99,107)
Human fibroblasts	37	1.9	141	KCN .1 mM Ouabain 2 mM	(26)
rT_3					
rat hepatocytes	37	6.0	1)	Ouabain .5 mM Monoclonal antibody	(20,99)

K_{m1} = low K_m (high affinity) system; K_{m2} = high K_m (low affinity) system;
1) = second class of binding sites present

tively (by high concentrations of) T_3 with a K_i of 90 nM (19), a much higher value than the K_m for the HAS of T_3 (21 nM; Table 4). Because a similar difference was found between the K_i of inhibition of T_3 uptake by T_4 and the K_m of the HAS of T_4 (vide supra), it was concluded that T_3 and T_4 uptake proceed via different systems.

T_4 uptake by freshly isolated rat hepatocytes in suspension has been reported to take place by a saturable, sodium-independent, low-affinity system (27), comparable to the LAS mentioned above, or by simple diffusion (108). Simple diffusion as the mechanism of T_4 uptake has also been reported for rat skeletal muscle (98,101) and rat liver slices (109). This may be explained by the fact that in freshly isolated hepatocytes the intracellular ATP concentration is only 25 nmol ATP/35 μg DNA, which figure corresponds well with published data (< 40 nmol ATP/35 μg DNA) (110), while in cultured hepatocytes ATP concentration is much higher (64 nmol ATP/35 μg DNA (20); see legend to Fig. 3). Active uptake of T_4 by the HAS is strongly dependent on this intracellular ATP concentration (Fig. 3). It is therefore possible that mainly the LAS has been measured in the hepatocyte suspensions, which system is not energy-dependent or inhibited by ouabain (19).

In the studies with rat skeletal muscle (98,101), isolated intact muscles were

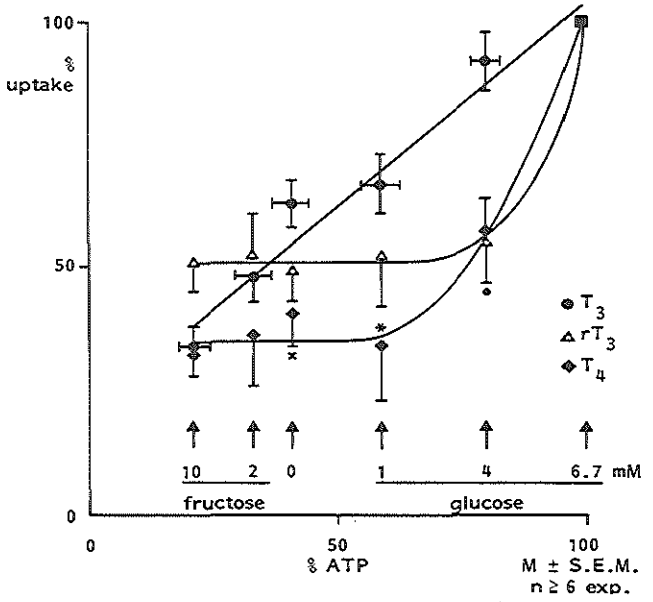


Figure 3.

*Active transport (in % of control) of iodothyronines into rat hepatocytes in primary culture (4 hours after isolation) as a 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, 3.8 rT_3 pmol/35 µg DNA/min and 64 nmol ATP/35 µg DNA. ATP content was varied by preexposure of the cells (during 30 min) to the indicated concentrations of glucose or fructose. Thereafter, the monolayers were exposed to the following free hormone concentrations for 1 min at 37 C: 0.1 nM T_4, 9 nM T_3 and 1.2 nM rT_3. Each uptake value represents the mean ± SEM of ≥6 experiments (in quadruplicate) and ATP values are from ≥ 16 experiments (at least in duplicate). Statistical evaluation on T_3 and T_4 transport with Student's t-test results in ·) $p<0.005$; *) $p<0.025$; x) $p=0.025$. Reproduced from (20).*

incubated in medium, and it is conceivable that O_2 supply is less than optimal in these circumstances compared to the in vivo situation, leading to a less than optimal ATP concentration in the incubated tissues. In the studies with rat liver slices (109) tissue was incubated with micromolar concentrations of T_4, orders of magnitude above the K_m of the high-affinity (energy-dependent) uptake system, in an atmosphere of nitrogen, which will result in low cellular ATP concentrations. For these two reasons it is clear that in this case the uptake of T_4 will follow diffusion kinetics. More studies under optimal conditions are necessary before firm conclusions can be drawn concerning T_4 uptake in different cell systems.

4.2.3 *Reverse triiodothyronine*

Initial uptake of rT_3 into rat hepatocytes in primary culture occurs by an energy-dependent, high-affinity system, similar to T_3 and T_4 (Table 5). This uptake can be inhibited (at least partially) by a monoclonal antibody and ouabain (19,99). On the basis of a similar dependence of the rT_3 and the T_4 uptake into cultured rat hepatocytes on intracellular ATP (Fig. 3), it was postulated that these two iodothyronines share a common pathway of transport into the cell (19). No studies have been performed to show the existence of a low-affinity system or a temperature effect on rT_3 transport.

4.2.4 *Influence of albumin on transport*

With 1 g/l or less albumin in the medium no active uptake of T_3 can be measured into rat hepatocytes in monolayer culture (100). This finding can be explained by assuming that in this case the diffusion over the unstirred waterlayer around the cell is rate limiting in uptake. With increasing albumin in the medium the T_3 concentration around the cell in this unstirred waterlayer will be stabilized. Measurement of initial uptake velocities of T_3 into rat hepatocytes in media containing in creasing concentrations of albumin (5-20 g/l) results in a linear increase of the V_{max} of both the HAS and the LAS of T_3, without a change in the respective K_m's (100). It has been shown that $\approx$ 0.3% of added albumin remains associated with the cells after washing the monolayers with medium without albumin (100). This indicates that with increasing amounts of albumin in the incubation medium, more albumin remains at the outer cellular surface, or in the unstirred waterlayer, which could be an explanation for the increase in the V_{max} of the LAS of T_3 (100). The finding of an increase in the V_{max} of the HAS of T_3 suggests a positive function of albumin in the transport mechanism of T_3 (100). Albumin has been shown to bind specifically to saturable binding sites on rat liver cells (111). The putative receptor appeared to mediate uptake of albumin-bound fatty acids (112), by binding the albumin-ligand complexes and catalyzing their dissociation. Albumin-mediated uptake has also been described for rose bengal (113,114), sulfobromophthalein (115), bilirubin (115) and iopanoic acid (116; for a review see (117)). However, it is unclear how albumin stimulates uptake, the more because the presence of a specific albumin-binding site

on the plasma membrane of hepatocytes has been challenged (118,119). Albumin-bound ligands are rapidly transferred to affinity chromatography columns composed of albumin-agarose beads (120), indicating that direct exchange of bound ligand between albumin in solution and albumin bound to the column takes place. Thus, it is possible that the albumin receptor effect may derive, not from an albumin receptor, but from the direct exchange of ligands between albumin in the plasma and membrane proteins with high affinities for these ligands on the sinusoidal liver cell membrane. Such high-affinity binding proteins, with the function of transport proteins, have been shown to exist for fatty acids (121), for bilirubin and sulfobromophthalein (122) and for iodothyronines (19). Transport of ligand by these proteins into the cells can be inhibited by specific poly- or monoclonal antibodies directed against the respective transport proteins (121-123[chapter VI]). Furthermore, a model has been proposed, which explains the rapid uptake of albumin-bound substances by hepatocytes without the inferrence of a specific albumin receptor (124). Another argument against the existence of a special role for albumin in the uptake of these compounds into the liver is the finding that in analbuminemic humans and rats mass transport of these compounds to the liver is normal despite a considerable larger volume of distribution and plasma clearance rate due to the diminished serum binding (125), in agreement with the normal transport of T_4 to the REP in analbuminemic rats (46). Furthermore, it has been shown in rat liver perfusion studies that the presence of albumin is necessary to obtain a uniform distribution of T_4 over the liver cells, while in the case of perfusion without any binding protein in the medium virtually all of the T_4 is taken up by the periportal cells (126). It was also shown that the same effect could be obtained by the addition of TBG or TBPA to the perfusion medium. Therefore it appears that in these liver perfusion studies the stabilisation of the free hormone concentration in the perfusate is the common denominator on which the effect depends.

Finally, we have recently shown (127[chapter IX]) that in perfused rat livers transport of thyroid hormone to the intracellular compartment is only dependent on the free hormone concentration in the medium, and not on the albumin concentration. This makes a special role for albumin in the uptake of thyroid hormone into the liver unlikely.

4.2.5 *Importance of intracellular ATP in the uptake of thyroid hormone*

Preincubation of monolayers of rat hepatocytes with increasing amounts of T_3 during 4 hours leads to a progressive decrease in intracellular ATP (128). In a subsequent incubation, initial T_3 uptake was found to be progressively decreased, and a positive and linear correlation was found between T_3 transport and intracellular ATP content (128). Similar results were obtained, when the decrease of intracellular ATP was induced by incubation of the monolayers with varying concentrations of glucose or fructose (20,128; Fig. 3). On the other hand, similar experiments with T_4 and rT_3 reveals not a linear but a curved relationship between uptake and ATP concentrations (20; Fig. 3). A decrease in intracellular ATP content of 50% results in a statistically significant greater inhibition of T_4 or rT_3 transport than of T_3 transport (Fig. 3). Furthermore, it seems that T_4 and rT_3 transport are in a similar way dependent on ATP, which is different from that of T_3 transport (Fig. 3). This is compatible with different pathways for T_4 and rT_3 on one hand and for T_3 on the other hand.

4.3 Thyroid hormone release from isolated cells

The intracellular bioavailability of thyroid hormone is the net result of uptake, intracellular production, intracellular breakdown and efflux from the cells. Particularly in the liver, this bioavailability is mainly governed by uptake and efflux rates, because intracellular production and degradation rates are small in comparison with influx and efflux rates (35,67; Fig 2). Only limited data are available on the mode of efflux of thyroid hormones from cells. It has been shown that cell- associated T_3 was released from rat liver cells into the medium with an half-life of about 100 sec (129). However, no evidence has been provided that this rapid process represented transmembrane movement of T_3. In fact evidence has been presented (130[chapter V]) that this process represents efflux of hormone bound to the outside of the cells. Efflux of both T_4 as T_3 from the intracellular compartment of rat hepatocytes proceeds with a half-life of about 7 to 8 min (130[chapter V]). Diminishing the cellular ATP content with 40%, or loading the cells using very high free T_3 concentrations (0.5 or 54 nM) did not affect this efflux, indicating the absence of energy dependency and saturation of the system with unlabeled hormone at these concentra-

tions (130[chapter V]). However loading the cells with 5 μM free T_3 leads to a partial saturation ($\approx$ 40%) of the efflux (RD, EPK unpublished observations), which could indicate that the efflux process is carrier mediated too, although it is clear that under physiological conditions efflux will follow diffusion kinetics. Variations in albumin concentrations in the efflux medium have an influence T_3 efflux. The half-life values decreased with increasing albumin concentrations from 0 to 1 g/l and remained constant thereafter. This shows that albumin has a permissive effect on the efflux of thyroid hormone, probably by facilitating the diffusion of thyroid hormone through the unstirred waterlayer around the cell (130[chapter V]).

It has been shown (106) that both influx and efflux of T_3 from rat erythrocytes is inhibited by high intracellular as well as high extracellular T_3 concentrations. Therefore, it was concluded that the transport mechanism of erythrocytes was compatible with a carrier-mediated diffusion following a simple pore model (106).

Because the uptake process of T_3 into hepatocytes is an active energy-dependent process, while efflux seems to proceed passively, it is very likely that there exists a gradient of free hormone over the plasma membrane, as the driving force of this diffusion process. In fact the ratio of free cytosolic/free plasma concentrations for L-T_3 has been shown to be about 2.8 (131) in vivo, indicating that the free T_3 concentration in the cytosol is higher than in the plasma. Surprisingly enough, these authors also found a much greater gradient from cytosol to nucleus. A free nuclear/ free cytosolic ratio for L-T_3 of 58.2 was found for rat liver (131). This was unexpected, because it has been shown that there exists large pores in the nuclear envelope (5.6-5.9 nm), allowing the free diffusion of macromolecules as large as 19.5 kDa, and excluding dextrans with a mol wt of > 62 kDa (132). In another study (133) it was shown that the nuclear envelope is very permeable to inulin (mol wt $\approx$ 5.5 kDa), with a resistance to the passage of inulin similar to that of cytosol. Therefore, it seems that the nuclear envelope plays a negligible role in regulating the nucleoplasmic movement of solutes smaller then macromolecules (133). However, inulin concentrates in the nucleus to four times its cytoplasmic level, which is attributed to solute exclusion from cytoplasmic water (133), a purely physicochemical passive process. Thus, it is difficult to explain that such a large gradient as reported (131) exists for T_3 over the nuclear envelope, because the mol wt of T_3 is only 651 Da.

In contrast to the free T_3 gradient from plasma to cytosol found in vivo, the same group of authors found a lesser gradient of 1.65 in GH_1 cells (134) and could not find a free T_3 gradient from medium to cytosol after incubation of freshly isolated rat hepatocytes with ^{125}I-T_3 and various concentrations unlabeled T_3 (135). However, in view of the results discussed here this is not unexpected. Firstly, they performed the incubations without any protein in the incubation media, and it has been shown that in these circumstances uptake of T_3 into hepatocytes follows diffusion kinetics, because the diffusion through the unstirred waterlayer around the cell is rate limiting (100). Secondly, ATP concentrations in freshly isolated hepatocytes are low (vide supra) and are restored to normal after 4 h culture (20). Because it has been shown that T_3 uptake into hepatocytes is linearly related to ATP (Fig. 3), it can be argued that a possibly existent gradient is diminished with a similar factor as the ATP concentration, e.g. 2.5 times as compared to 4 h cultured hepatocytes. However, it seems that even in these cells active transport is present, because 2 mM KCN inhibited T_3 uptake significantly with a further 24% (135).

5. STUDIES WITH LIVER PERFUSION TECHNIQUES

No change in the rate of conversion of T_4 to T_3 was observed in perfused livers of fasted rats, as compared to controls. However fasting resulted in a progressive decrease in hepatic T_4 uptake to 42% of control levels by the third day of fasting, accompanied by a proportionate decrease in T_3 production (136). A similar decrease in hepatic uptake of T_4 (41%) after 2 days fasting was found in another study (167). When T_4 uptake in 2 days fasted rat livers was raised to levels found in fed rats by increasing the perfusate T_4 concentration, T_3 production returned to normal (136). However, restoration of serum T_4 levels in fasted rats, which are low during fasting, failed to correct the decrease in hepatic T_4 uptake or T_3 production during the subsequent perfusion (137). It was hypothesized that decreased hepatic uptake was caused by loss of intracellular T_4-binding proteins. Another explanation can be that the active uptake is decreased due to a diminished intracellular ATP concentration, which is shown to be decreased in livers from fasted rats (138,139). This role of ATP can also be concluded from the finding that early hepatic T_4 uptake is diminished

when rat livers are perfused with fructose, a compound known to elecit a transient depletion of liver ATP, while after 20 min of perfusion this inhibition has disappeared (167). On the other hand, in other studies no change in T_4 uptake from the medium could be demonstrated during perfusion of livers from diabetic rats (137), hypo- or hyperthyroid rats (140), dexamethasone treated rats (141), or when PTU was added to the perfusion medium (136). All changes in T_3 production from T_4 should therefore be explained in these cases by changes in 5'deiodinase activity.

Recent investigations have shown that uptake of T_3 by the perfused rat liver is inhibited in livers of rats fasted for 2 days (168,169). This conclusion was drawn from the finding that the relative amounts of T_3 glucuronide, T_3 sulfate and iodide produced from T_3 was not changed, only the total amount of T_3 metabolized (168), indicating that intracellular metabolism was not affected by fasting. The inhibition could be completely normalized by a preperfusion of the livers with a combination of insulin, cortisol and glucose (168). Transport into livers of hypothyroid animals was not altered as compared to euthyroid rats, while uptake by perfused livers of hyperthyroid rats was significantly lower (169). However due to the fact that intracellular metabolism was raised in livers from hyperthyroid rats, the total efficiency of T_3 metabolism was not different from euthyroid rats (169).

In two studies with perfused livers (42,136) no saturability of thyroid hormone uptake could be demonstrated, which is in contrast to the findings with isolated cells in a large number of papers from different laboratories (15-30). However, in the first study (136), the maximal free T_4 concentration used to saturate the uptake system was < 0.4 nM (60 μg/dl in 3% bovine serum albumin (dialysable fraction < 0.05%) with 15% human erythrocytes), far below the apparent K_m of T_4 transport into rat hepatocytes (table 5), and therefore not sufficient to detect saturation of uptake. Lack of saturation in the second study (42) is probably inherent to the technique used. As discussed previously, the authors have used a single pass injection technique, which method measures the unidirectional flux to the liver, and allows only a very short period of time for hormone-tissue interactions. This time interval is probably too short for hormone transport over the cell membrane, and therefore only reflects binding to the outer cell surface, which has a very high K_m (17,100 and table 4 & 5). We have recently shown that these extracellular pools are also present

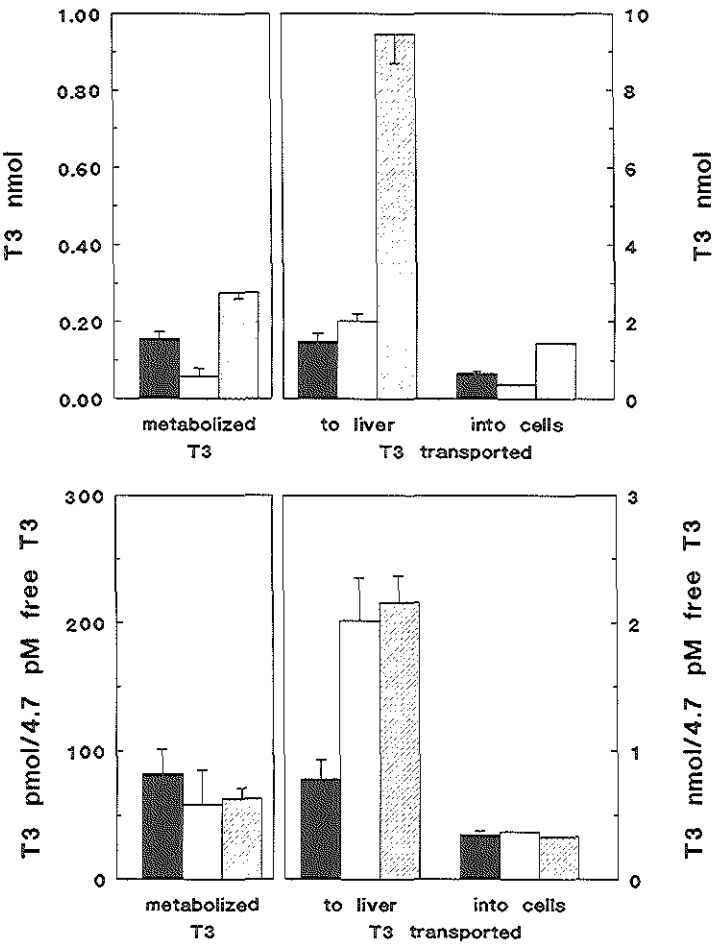
in the perfused rat liver (127[chapter IX]). It was concluded that not all T_3 is transported to metabolic active pools in the liver, but that part is bound outside the cellular compartment. This latter pool of T_3 is dependent on the albumin concentration in the medium. The amount of T_3 metabolized is solely determined by the free T_3 concentration, rather than by the total T_3 or albumin concentration in the medium (127[chapter IX] and Fig. 4). Due to the presence of these large extracellular hormone binding pools, it is clear that it is not possible to measure uptake of hormone into liver cells with this single pass injection technique (42). Injected hormone will for the greater part spread over these extracellular pools, and not enter the cells. Therefore uptake seems to follow diffusion kinetics in this system, because binding of hormone to these pools is linearly related to the free hormone concentration (127[chapter IX]).It is thus impossible to distinguish between hormone bound to the cell surface and intracellularly located hormone. In addition, it has been shown (100) that it is impossible with the concentration of BSA (0.1%) used in this liver perfusion study (42) to measure saturable uptake in rat hepatocytes in primary culture.

6. EFFECTS OF ALTERED MEMBRANE TRANSPORT ON METABOLISM

If the active transport system for thyroid hormones has physiological significance, then it is to be expected that blockade of this uptake system would result in decreased intracellular concentration and metabolism of T_3 and notably attenuated occupancy of the nuclear receptor by hormone. Indeed, it has been found that ATP depleted rat hepatocytes in monolayer show a diminished content of T_4 (130[chapter V]; Table 6). Inhibition of uptake by ouabain, a substance which does not affect intracellular ATP concentration, nuclear receptor affinity or maximal binding capacity, effects a decrease in total uptake of T_3 of $\approx$ 30% after 1 and 2 hours incubation with tracer T_3. Moreover, a more substantial decrease with regard to nuclear binding is seen, amounting to 50 and 41% after 1 and 2 hours, respectively (Table 6). The decrease in total uptake is less than for nuclear uptake and may be explained by the fact that ouabain does not affect the binding of thyroid hormone to the outer cell surface (19). Another observation also indicated that inhibition of

Figure 4.

Influence of various concentrations of albumin and free T_3 on T_3 transport and metabolism during 60 min in a recirculating rat liver perfusion system. Closed bars: 1% albumin, mean FT_3 8.9 ± 1.16 pmol/l during the perfusion. Open bars: 4% albumin, mean FT_3 4.7 ± 0.97 pmol/l. Hatched bars: 4% albumin, mean FT_3 20.6 ± 2.12 pmol/l. Upper left panel: total amount of T_3 metabolized. Upper right panel: total amount of hormone transported to the liver, and into the cells, respectively. Lower panels: the same parameters, but corrected for differences in FT_3: values were adjusted to a mean FT_3 of 4.7 pmol/l. Raising the albumin concentration from 1 to 4%, without a change in total added T_3 leads to a fall in mean FT_3 from 8.9 to 4.7 pmol/l, with a concomitant diminution of T_3 metabolized (upper left panel: closed bars versus open bars; $p < 0.001$). However, <u>total</u> transport to the liver increases significantly ($p < 0.001$) despite the lower FT_3 concentration, while transport <u>into</u> the cells diminishes in parallel with the free hormone concentration. After correction for the differences in FT_3 (lower panel) it appears that the amount of T_3 metabolised and transported <u>into</u> the cells is not significantly different in the experiments with 1% and 4% albumin in the medium and therefore not dependent on the albumin concentration in the medium, but <u>total</u> transport to the liver is raised more than two-fold, when the albumin concentration in the medium is increased from 1 to 4%. This indicates that the major part of the hormone transported to the liver does not enter the cell, but is sequestered in a metabolically inert pool, probably located outside the cell. Transport to this pool is dependent on both the free hormone and the albumin concentration in the medium, in contrast to transport of hormone into the cells, which is only dependent on the FT_3 concentration in the medium. Similar conclusions can be drawn from the results with 4% albumin and a high FT_3 concentration in the medium (hatched bars). Redrawn from data presented in (127).



uptake of T_3 will lead to lower occupancy of the nuclear T_3 receptor. Rats on a

nutritionally deficient diet with normal iodine intake displayed markedly elevated serum free T_3 levels but showed no increase in oxygen consumption. This was associated with greatly reduced hepatic cellular and nuclear ^{125}I-T_3 to serum ^{125}I-T_3 ratios following administration of the labeled hormone. Kinetic data showed that cellular uptake of T_3 was decreased. The lack of metabolic effect of the elevated serum T_3 levels was attributed to the reduced availability of serum T_3 to tissue nuclear receptor sites (142)2. Finally, it has been shown in GH_3 cells that monodansylcadavarine, an endocytosis inhibitor, which also inhibits T_3 uptake in fibroblasts (22), blocked virtually all of the cellular uptake of T_3 and the accumulation of T_3 in the nucleus (23), while it was shown that this compound does not inhibit T_3 binding to the nuclear receptor itself. Similar findings have also been reported by other authors, for cytochalasin B (24), dansylcadavarine (24), chloroquine (24), and 5,5'-diphenylhydantoin (143). The latter compound also inhibits T_3 binding to isolated nuclei (144), although to a much lesser extent than the overall effect on whole cells, both in vivo in rat anterior pituitary cells and in vitro in cultured GC cells (143).

The decrease in T_3 medium clearance caused by inhibition of uptake by ouabain or by monoclonal antibody ER-22 (99) will also lead to a diminished metabolism of the hormone (123[chapter VI],145[chapter VIII]; Fig. 5) to I^- and conjugates. This indicates that intracellular metabolism is altered if transport of T_3 over the plasma

Table 6

Percent inhibition of thyroid hormone uptake in rat hepatocytes by 0.5 mM ouabain or 45% ATP depletion (mean ± SEM)

Condition	incubation time (h)	hormone	% inhibition	
			total uptake n = 4	nuclear uptake n = 4
ATP depletion	0.5	T_4	20 ± 7[b]	-
Ouabain	1	T_3	29 ± 3.5[b]	50 ± 4.4[b]
Ouabain	2	T_3	30 ± 7.4[a]	41 ± 5.2[b]

Difference versus control: [a]) $p < 0.05$, [b]) $p < 0.005$

Data from (130)

membrane is inhibited. If 6-propyl-thiouracil (PTU), a known inhibitor of liver deiodinase both in homogenates (146) and in cultured hepatocytes (147), is included in the incubation medium, no significant change in the disappearance of T_3 is observed, but now mainly conjugates are formed (Fig. 5) instead of I^-. This is in accordance with the recent finding that sulfation facilitates hepatic T_3 deiodination (147,148). It appears, therefore, that production of iodide from T_3 can be inhibited by diminution of uptake of T_3 by hepatocytes, for instance by ouabain or a monoclonal antibody, or by inhibition of deiodinase using PTU, and that both processes can be influenced independently. In a similar way it has been shown that inhibition of rT_3 and T_4 transport into rat hepatocytes will lead to a diminished breakdown of

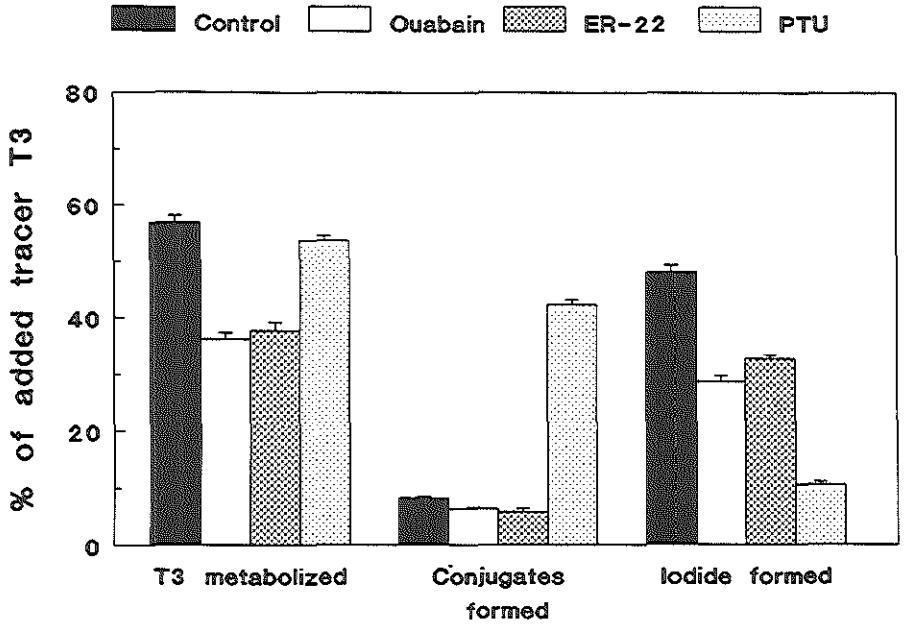


Figure 5.

Uptake and metabolism of T_3 in monolayers of rat liver cells, after incubation with ^{125}I-T_3 during 20 h at 37 C. Results are expressed as % T_3 metabolized. In control experiments 60 % of the T_3 was metabolized, with iodide as the main product (50%), while about 10% was conjugated. Production of iodide was diminished after inhibition of uptake by the monoclonal antibody ER-22 or by ouabain, without a rise in conjugate fraction. Inhibition of deiodination by 6-propyl-thiouracil (PTU) leads to an accumulation of T_3 conjugates in the medium, without a change in disappearance of T_3. Drawn from data reported in refs. (123[chapter VI],145[chapter VIII]).

rT_3 and production of T_3 from T_4 during longterm incubation (123[chapter VI]). It is therefore apparent that changes in the uptake of iodothyronines into cells have an important effect on their bioavalability and metabolism.

7. IMPLICATIONS OF ALTERED MEMBRANE TRANSPORT ON THYROID HORMONE METABOLISM IN PATHOPHYSIOLOGICAL SITUATIONS

Different diagnostic and therapeutic agents have been shown to interfere with the transport of T_3 and T_4 into hepatocytes (107) and other cell types. Among these are nonsteroidal antiinflammatory drugs (29), phloretin (28), cytochalasin B (24), dansylcadavarine (24), chloroquine (24), 5,5'-diphenylhydantoin (143), iodinated substances such as radio contrast agents (107) and amiodarone (107). The latter are structural analogues of thyroid hormone and probably compete for binding to the membrane transport protein (107). Inhibitory effects have also been observed with propranolol but this is thought to be due to a decrease in ATP content of the cultured hepatocytes which probably does not occur in vivo (107). It has been shown that transport of thyroid hormones into the tissues in vivo is not inhibited by d-propranolol (67), although T_3 production and rT_3 degradation are diminished, leading to the so-called "low T_3 syndrome". This indicates that in this case the most likely explanation is inhibition of type I deiodinase in the liver (67) (Fig. 6), which inhibition can only be reproduced in vitro with propranolol concentrations (149), which are three orders of magnitude higher than the therapeutic concentrations in humans (150).

Of special interest is the observation that plasma of patients with severe non-thyroidal illness (NTI) contains a factor that inhibits the binding of T_3 to plasma proteins (151,152) as well as its uptake by rat hepatocytes (151) and human hepatocarcinoma (Hep G2) cells (153), although it is not known from these studies if besides cellular binding, transport is inhibited too. Recent findings from our laboratory show that the latter is indeed the case as well (166). NTI and a number of the afore-mentioned compounds elicit a low T_3 syndrome, and it is tempting to suppose that inhibition of the uptake of T_4 and rT_3, which share the same uptake system (107), into the liver is causual in the diminished T_3 production and rT_3 breakdown

(Fig. 6), apart from any possible inhibition of the type I deiodinase in the liver as well. Indeed, it has been shown in T_4-substituted humans, that serum T_4 concentrations, which were elevated during starvation, decreased significantly during refeeding, suggesting normalization of T_4 transport into tissues when adequate caloric intake

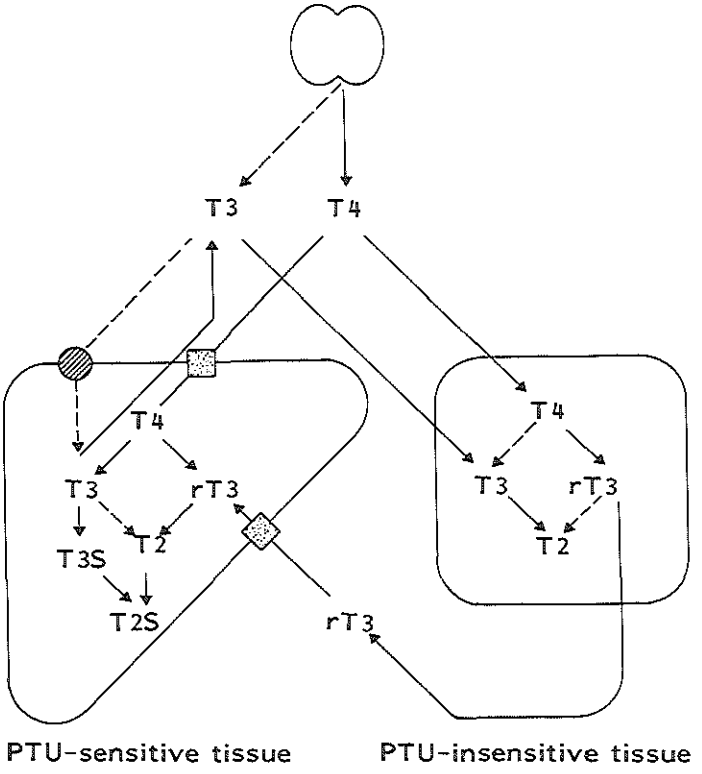


Figure 6.

Model of the peripheral metabolism of thyroid hormone in normal humans.

Production of plasma T_3 and clearance of rT_3 is placed predominantly in tissues with PTU-sensitive, type I deiodinase activity, e.g. the liver, although contribution of the kidneys is not excluded (33,162). Because T_3 is not a good substrate for the type I deiodinase, breakdown of T_3 in the liver is slow, unless T_3 is sulfated first (T_3S). In a similar way, deiodination of T_2 occurs only after sulfation, because T_2S is a much better substrate for the type I deiodinase than T_2 itself.Clearance of plasma T_3 and production of plasma rT_3 is located mainly in tissues such as brain and perhaps skin with PTU-insensitive, type III deiodinase activity. The model suggests that type II deiodination of T_4 does not contribute significantly to the production of plasma T_3 in euthyroid subjects. However, this does not negate the important function of the type II enzyme as a local source of intracellular T_3 in, for instance, brain and pituitary but also as a major producer of plasma T_3 in hypothyroidism (163). For a discussion of intracellular metabolism of thyroid hormones see (164). T_4 and rT_3 share the same uptake system (depicted as a square in the diagram), while T_3 is transported into the liver by a different system (19,20,21) (represented as a circle). Reproduced from (165).

was resumed (154). In another study of tracer T_4 and T_3 kinetics it has been found (35) that during caloric restriction mass transfer rates of T_4 to both the rapidly equilibrating pool (REP, composed mainly of liver and kidney (47)) and the slowly equilibrating pool (SEP) diminished significantly, despite an increase in free T_4. This led to significantly smaller tissue T_4 pools, lower T_3 production rates and serum total and free T_3 (35). The lower T_3 production rate was explained by the smaller T_4 pool in the REP, leading to a diminished substrate availability to the deiodinating enzymes. 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 due to liver cirrhosis have shown, however, that rT_3 production is normal (4). This would suggest that plasma rT_3 production takes place mainly outside the liver. Many arguments are in fact in favor of an extrahepatic origin of plasma rT_3 and that the liver is the site of degradation of plasma rT_3 (33,66).

There are other indications of alterations in thyroid hormone transport in vivo. In T_4-substituted rats, serum T_4 increased and plasma disappearance of labeled T_4 decreased during fasting (155). In other reports however, no effects of dietary manipulation on serum T_4 levels in T_4-substituted rats are observed (156,157). In humans, it has been shown that prednisone slows the acute disappearance of injected labeled T_4 (158) from plasma and decreases the hepatic T_4 pool. A similar effect on the rapid phase of plasma disappearance of T_4 (table 2) and rT_3 (75) has been reported in patients with severe NTI. In a number of situations, such as fasting, diabetes, and dexamethasone treatment, liver deiodinase activity in rats is decreased as tested in tissue homogenates (159-161). But it is not clear to what extent this decrease contributes to the low T_3 syndrome, because it is unknown whether the deiodination reaction is the rate-limiting step in the tissue conversion of iodothyronines. Further investigations should learn us more about the precise role of inhibition of transport and of deiodination of thyroid hormones in the generation of the low T_3 syndrome. The study of mechanisms that lead to lowered T_3 production is considered to be important. T_3 is an energy-consuming hormone and a decrease of T_3 production is seen as an energy-saving mechanism in situations of stress, starvation or illness.

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Chapter III

Studies on thyroxine binding albumin

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

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Summary 2 people from different families had high levels of serum-thyroxine (T4) and a high free T4 (FT4) index but a normal serum triiodothyronine (T3) and serum-reverse-T3 (rT3). The abnormal serum thyroid hormone profile appeared to be inherited in an autosomal dominant manner. Serum-FT4 in affected relatives was normal. The increases in serum-T4 and FT4 index are explained on the basis of an observed increase in affinity of T4 for thyroxine-binding globulin, thyroxine-binding prealbumin, and albumin. The FT4 index did not reflect the true concentration of circulating free T4 in these cases. Thyroid function in the propositi was normal and the results of T4, T3, and rT3 kinetic studies accorded with increased binding of T4 by serum proteins and normal binding of the other iodo-thyronines. This "euthyroid high total T4, normal T3 syndrome" should be kept in mind during diagnostic evaluation of thyroid function.

Introduction

PATIENTS with a raised serum-total-thyroxine (T4) and free thyroxine index (FT4 index) but a normal serum-total-triiodothyronine (T3) and free triiodothyronine index (FT3 index) have been described several times. This unusual combination of findings has been ascribed to a special form of hyperthyroidism,[1-4] so called "T4 toxicosis", which may be common among euthyroid elderly people.[5] It was suggested that, in T4 toxicosis, serum-T3 was not increased because the peripheral conversion of T4 into T3 (normally the major source of T3 production[6]) was diminished by accompanying non-thyroidal illness.[1] Indeed it has been reported[7] that, in hyperthyroidism in conjunction with

non-thyroidal illness when serum-T4 alone is raised, serum-T3 increased only after recovery from the co-existing disease. The same authors, however, also described a group of subjects in whom T4 was raised and T3 was normal during acute non-thyroidal illness and in whom the serum hormone concentrations remained unaltered after recovery from the concurrent illness.

We report here serum estimations of total T4, T3, and reverse T3 (rT3) and the free index of these hormones in two healthy euthyroid people who had a raised serum-T4 and normal T3. We also investigated a possible genetic basis for the abnormalities. The serum concentration of FT4 and the equilibrium association constants (K_a) between T4 and its serum transport proteins were estimated in relatives of both subjects and turnover studies of T4, T3, and rT3 were done in the probands.

Patients

A woman aged 61 presented with mild fatigue. Serum-T4 was raised but she was clinically euthyroid. She took no drugs. Physical examination, and urine and blood were normal. Kidney function, electrolytes, glucose, serum proteins, calcium, phosphate, cholesterol, and triglycerides were also normal. Liver function tests were slightly abnormal: serum alkaline phosphatase 85 I.U./l (normal <45); S.G.O.T. 55 I.U./l (<30); S.G.P.T. 67 I.U./l (<30). Serum bilirubin was normal. A liver biopsy showed slight fibrosis and evidence of previous hepatitis.

A 47-year-old man was referred with an unexplained raised serum-T4 without clinical evidence of thyrotoxicosis. He had mild depression but was otherwise healthy and took no drugs. No physical abnormalities were found. Routine urine and blood analysis was normal. Kidney and liver function, electrolytes, glucose, serum proteins, calcium, phosphate, and cholesterol were normal. Serum triglycerides were moderately raised (2·48–3·40 mmol/l). X-ray of the thorax and cardiogram were normal.

Serum of relatives of both subjects was analysed to investigate the genetics of their disorder.

Thyroid hormone turnover studies were done in both subjects and in healthy controls after informed consent.

Methods

T4,[8] T3,[9] rT3,[10] and thyrotrophin (T.S.H.) (Calbiochem Zurich, standard M.R.C. 68/38) measurements in serum were done with specific radioimmunoassays. T3 resin uptake was measured with the 'Triosorb' kit (Abbott Laboratories, Chicago). The FT4 index was calculated as serum T4×T3 resin uptake(%)/100. FT3 and FrT3 indices were calculated similarly. Serum-FT4 was estimated with a commercial kit (Corning Medical, Medfield, Massachusetts). Serum thyroxine-bind-

ing globulin (T.B.G.) and thyroxine-binding prealbumin (T.B.P.A.) capacities were determined by agar gel electrophoresis.[11] Autoantibodies against thyroglobulin and cytoplasm were detected as described earlier.[12] Antibodies against T4, T3, and rT3 were detected by agar gel electrophoresis.[13] Estimates of the equilibrium association constants (K_a) for the interactions of T4 with T.B.G., T.B.P.A., and albumin were calculated from the measurements of FT4, T.B.G., T.B.P.A., and albumin (radial immunodiffusion technique, Behringwerke, Hoechst) and the percentage T4 tracer bound to these proteins (assessed from agar electrophoresis[11]). For calculation the general formulation of interaction between thyroid hormones and their binding proteins was used.[14]

Thyrotrophin-releasing hormone (T.R.H.) tests were done by rapid intravenous injection of 200 μg T.R.H. and taking blood for T.S.H. determination at 0, 20, 30, 60, and 120 min.

T4 and T3 turnover studies were done as described previously.[15,16] Turnover of rT3 was studied by injecting purified and sterilised ^{131}I-rT3 and by plasma collection at frequent intervals during 48 h. Plasma samples were processed as described for T3 turnover studies.[16] Labelled rT3 was obtained as described earlier.[10] Approximately 20 μCi ^{125}I-T4 and 15 μCi ^{131}I-T3 were injected simultaneously and 6 days later (after complete disappearance of ^{131}I-activity) approximately 15 μCi ^{131}I-rT3 was given. In this manner the turnover studies of the three hormones were completed within 10 days. Starting 1 day before the study until the end, three drops of saturated solution of potassium iodide were administered four times a day to prevent thyroidal reutilisation of liberated ^{131}I and ^{125}I. Calculations were done by the non-compartmental approach.[17,18]

Results

In both subjects serum-T4 and FT4 index were clearly raised while T3, rT3, and T.B.G. capacity were normal (table I).

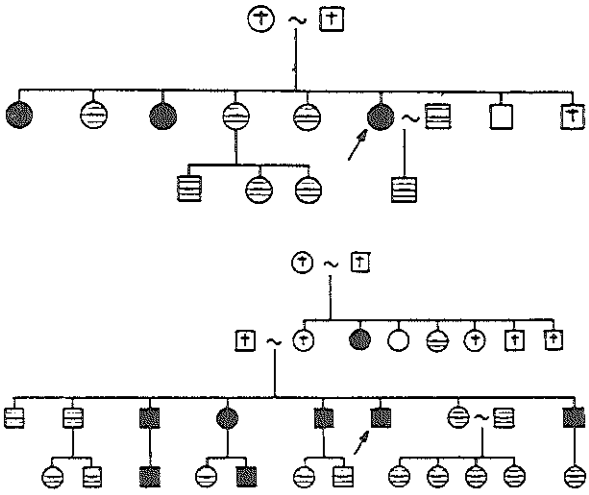
Both subjects were clinically euthyroid and the T.S.H. response to T.R.H. was normal; patient 1: T.S.H. <1, 7·2, 8·5, 5·9, and 1·7 mU/l at 0, 20, 30, 60, and 120 min, respectively; patient 2: T.S.H. <1, 7·8, 8·8, 4·9, and 2·6 mU/l. Thyroid uptake studies were normal (patient 1: uptake of ^{99m}Tc after 3 and 15 min 2·1 and 4·1% of dose, respectively; patient 2: uptake of ^{131}I after 4, 24, and 48 h 38, 50, and 50% of dose, respectively) as were the thyroid scans. Circulating antibodies against T4, T3, and rT3, which may result in falsely raised serum values of the corresponding thyroid hormones in the radioimmunoassays,[13] were not detected. Thyroid autoantibodies were also absent.

The figure shows that the abnormal thyroid hormone profile was inherited. FT4 index was much higher in relatives with the trait than without it but FT3 and

FrT3 indices, serum-T.S.H., and, most remarkably, serum-FT4 were normal in both groups (table II).

TABLE I—SERUM THYROID HORMONE LEVELS, FT4 INDEX AND T.B.G. CAPACITY IN PATIENTS

—	T4 nmol/l	T3 nnmol/l	rT3 nmol/l	T.B.G. nmol/l	FT4 index
Patient 1	240	1·59	0·35	395	56
Patient 2	216	1·44	0·31	301	68
Normal range	60–150	1·3–2·6	0·15–0·52	214–404	18–38



Family studies

Family 1 above, family 2 below. Arrow indicates the probands. Circles represent females; squares represent males. Filled symbols: positive for the trait; shaded symbols: negative; open symbols: not tested.

In affected members K_a for interactions of T4 with T.B.G., T.B.P.A., and albumin were significantly higher than in the non-affected members (table III).

In thyroid hormone kinetic studies (table IV) production (disposal, D), distribution volume (V), and total body pool (P) of T3 and rT3 were normal. Mean VT4 was lower but mean PT4 was higher than normal, while DT4 was normal.

Discussion

In both patients the serum-T4 and the FT4 index were raised while T3 and rT3 were normal. T.B.G. (the

most important thyroid hormone transport protein) was also normal. Clinically the patients were euthyroid and the serum-T.S.H. response to T.R.H. was normal, showing that the pituitary-thyroid axis was intact. Furthermore, thyroid uptake studies were consistent with normal function. The possibility of a genetic basis for this abnormal thyroid hormone profile was considered and investigations in relatives of the propositi revealed that the mode of inheritance was of the dominant autosomal type because the ratio of affected *vs.* unaffected in those members who had relatives in the first degree positive for the trait was not significantly different from 1 (3/4 in family 1 and 8/8 in family 2). The trait was not X-linked because it had been transmitted from male to male.

Despite the difference in FT4 index, serum-FT4, the other indices of iodothyronines, and serum-T.S.H. were normal in affected and non-affected members. The difference between FT4 index and serum-FT4 is quite unexpected and to our knowledge has not been reported before. Indeed, the FT4 index is generally considered to closely mirror the serum-FT4.[19]

TABLE IV—RESULTS OF THYROID HORMONE KINETIC STUDIES (PER 1·73 m^2 BODY SURFACE)

—	DrT3	DT3	DT4	VT4	PT4
Patient 1	18·1	26·7	128·7	6·76	1623
Patient 2	19·4	34·9	154·8	8·07	1748
Patients (mean±S.D.)	18·8 ±0·9	30·9 ±5·8	143·6 ±21	7·42* ±0·93	1685† ±88
Controls (mean±S.D.)	21·6 ±5·2 (n=5)	41·2 ±14·6 (n=10)	119·6 ±21·3 (n=16)	12·49 ±1·68 (n=16)	1341 ±222 (n=16)

D=production (disposal) (nmol/24h).
VT4=volume of distribution of T4(l).
PT4= total body pool of T4 (nmol).
*P<0·001
†P<0·05

A discrepancy between serum-FT4 and FT4 index could be explained on the basis of increased binding of T4 by serum proteins with unaltered binding of T3. When the affinity of binding proteins for T4 is increased, the serum total T4 will be increased. Because the subjects were euthyroid we suggest that serum-FT4 (T4×fraction unbound T4) remained unchanged because the fraction of total T4 circulating in the free form was decreased proportionally to the increase of total T4. This sequence of changes can be argued because there is an equilibrium between a hormone and

TABLE II—MEAN (S.D.) THYROID HORMONES IN RELATIVES POSITIVE OR NEGATIVE FOR THE TRAIT AND IN CONTROLS

	Relatives of patient 1		Relatives of patient 2		
	Positive (n=3)	Negative (n=7)	Positive (n=8)	Negative (n=14)	Controls (n=71)
FT4 index	52·9 (8·5)	31·7 (6·5)	60·7 (5·4)	33·3 (4·1)	28·2 (5·1)
FT3 index	0·36 (0·08)	0·35 (0·03)	0·49 (0·08)	0·60 (0·13)	0·51 (0·10)
FrT3 index	0·064 (0·012)	0·055 (0·022)	0·066 (0·014)	0·055 (0·014)	0·052 (0·017)
FT4 (pmol/l)	22·3 (3·6)	16·9 (4·4)	23·7 (3·0)	18·5 (1·8)	18·9* (3·2)
T.S.H. (mU/l)	2·0	2·6	2·7	1·8	1·4
(range)	(<1–4·3)	(<1–5·0)	(<1–5·5)	(<1–3·3)	(<1–4·9)

*n 11

TABLE III—MEAN±S.D. APPARENT EQUILIBRIUM ASSOCIATION CONSTANTS (K_a) IN RELATIVES POSITIVE OR NEGATIVE FOR THE TRAIT

K_a	Relatives of patient 1		P	Relatives of patient 2		P
	Positive (n=3)	Negative (n=5)		Positive (n=8)	Negative (n=14)	
T.B.G.×10^{10}l/mol	3·3 (0·4)	2·2 (0·2)	<0·005	3·4 (1·0)	2·2 (0·4)	<0·001
T.B.P.A.×10^{8}l/mol	0·67 (0·08)	0·39 (0·14)	<0·025	0·84 (0·26)	0·39 (0·14)	<0·001
Albumin×10^{6}l/mol	3·8 (0·5)	1·9 (0·5)	<0·005	3·8 (0·5)	1·3 (0·3)	<0·001

its binding proteins.[14] The FT4 index is, in essence, T4×T3 resin uptake. T3 resin uptake is, for practical purposes, taken to represent the fraction of unbound T4, although T3 tracer is being used. There is no objection to this procedure and it is permissible to use the FT4 index (as an estimate of FT4) for comparison between patients, provided that the affinity characteristics of the binding proteins do not differ between the subjects tested. If serum binding of T4 is increased but serum binding of T3 is not, FT4 index will give (falsely) raised values because the unbound fraction of T4, as estimated with T3 tracer, will not be found to be decreased. Indeed we found that the affinity of T4 for the three thyroid-hormone-binding proteins in the subjects positive for the trait was higher than that in their unaffected relatives. Although the apparent K_a values of the affected and unaffected members are of the same order of magnitude as those reported by others,[20] the differences between the family subgroups are highly significant. The finding that the affinity of T4 for all three binding proteins is increased, not just for one, is remarkable and suggests either that a circulating substance interferes positively with the interaction between T4 and T.B.G., T.B.P.A., and albumin, or that there is a deficiency of a natural occurring inhibitor.

These possibilities, however, are speculative and further investigations are necessary. Because T.B.G. and T.B.P.A. capacity and albumin concentration was normal in all the members of both families who had the abnormal thyroid hormone profile, the increased affinity of T4 would have led to increased occupancy of binding sites by T4 and consequently a decreased number of free binding sites. Serum-T3 and serum-rT3 were normal and tracer T3 binding to serum was unaltered (as shown by the T3 resin uptake). If fewer sites for binding of these hormones were available, the affinity of these other iodothyronines for the three serum proteins was, presumably, proportionally increased relative to T4.

The results of the turnover studies in the propositi accorded with our in-vitro findings of increased serum binding of T4 with a normal FT4 and normal serum binding of T3 and rT3. The decreased VT4 and increased PT4 are fully explained by increased serum binding of T4 and are of the order described in subjects with idiopathic elevation of serum T.B.G.[21] The normal turnover of T4 is consequently expected when FT4 is normal.

The frequency in the population of the "euthyroid high total-T4, normal T3 syndrome" is unknown but,

from a practical point of view, its existence should be kept in mind in the diagnostic evaluation of thyroid function in patients.

We thank Dr C. v. d. Peyl, Juliana Hospital, Terneuzen, and Dr B. A. de Planque, Municipal Hospital, Dordrecht, for the opportunity to study their patients, and Miss B. Engelhard for secretarial assistance.

Requests for reprints should be addressed to G. H.

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Addendum

After we told doctors in our hospital about the syndrome, we were presented with 3, probably 4, additional similar patients, of whom 3 had been previously treated with antithyroid drugs for supposed hyperthyroidism.

If this syndrome turns out to be a common one, some thought should be given to abandoning the FT4 index as a routine screening test for thyroid function and using the measurement of the FT4 instead.

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INHERITED THYROXINE EXCESS: A SERUM ABNORMALITY DUE TO AN INCREASED AFFINITY FOR MODIFIED ALBUMIN

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SUMMARY

Further analysis of sera from euthyroid subjects with dominantly-inherited, elevated serum total thyroxine (T4) and free T4 index but with normal free T4 levels was performed as an extension of a previous study (Hennemann *et al.*, 1979a). Scatchard analysis and isoelectric focusing of whole sera and purified serum fractions suggest that this T4 excess is due to increased T4 binding by modified serum albumin.

Recognition of this syndrome and appreciation that the free T4 index does not reflect the free T4 levels is important to protect patients from the consequences of an incorrect diagnosis of thyrotoxicosis.

Recently we described two families with high serum thyroxine (T4) levels and a high free T4 index, but normal serum triiodothyronine (T3) and serum reverse T3 (rT3) levels in about half of the members (Hennemann *et al.*, 1979a). All subjects were clinically euthyroid. This abnormal serum thyroid hormone profile appeared to be inherited as an autosomal dominant. Serum free T4 levels in both affected and unaffected subjects were normal as measured by equilibrium dialysis (Hennemann *et al.*, 1979a; 1979b; 1979c). It was postulated that the increases in total serum T4 and free T4 index were due to an increase in the affinity of T4 for all three binding proteins.

Inherited T4 excess was subsequently reported by Lee *et al.* (1979) and Barlow *et al.* (1980). Lee *et al.* described a 9-year-old boy and his father with a high serum T4 and free T4 index, associated with an euthyroid clinical state. The T4 excess was due to the presence of an additional binding protein with a high capacity and an association constant (K_a) similar to that of thyroxine-binding prealbumin (TBPA).

Barlow *et al.* (1980) reported a euthyroid Australian family in which four members had high T4 and free T4 indices again with an autosomal dominant type of inheritance. They also reported that this family had normal free T4, T3, rT3 and basal TSH levels and a

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normal TSH response to TRH. They suggested that T4 binding by serum was altered in these subjects. We report further studies of our patients with inherited T4 excess using techniques designed to separate and characterize serum thyroid hormone binding proteins. The data indicate an alteration in the structure of albumin leading to increased binding of T4.

SUBJECTS

A full description of the two families had been published previously (Hennemann *et al.*, 1979a). Ten relatives were studied in family 1 over two generations. Three were positive for the trait, and seven negative. Family 2 spanned three generations; a total of twenty-two relatives were studied, of whom eight were positive and fourteen negative.

Sera from six members of the second family, who were positive for the trait, and five negative members, were studied. From the first family only serum of the proband was available for further analysis.

METHODS

Maximal binding capacities (MBC) of serum T4 binding globulin (TBG) and TBPA, were determined by agar gel electrophoresis (DiGiulio *et al.*, 1964; Wieme, 1965). Separation gels were poured on microscope slides (0·9% Agar Noble (Difco) in 0·2 M glycine, 0·13 M sodium acetate, pH 8·6). Electrophoresis was performed at 150 V 80 mA per slide for 60 min. Temperature was maintained at 15°C, by immersing the slides in petroleum spirit (boiling range 40–60°C). Excess heat was removed by forced evaporation.

Before application onto the gel the sera were mixed with bromophenol blue, which comigrates with albumin, labelled T4 and saturating concentrations of unlabelled T4 (1·9 μmol T4/l for TBG, and 8·4 μmol T4/l for TBPA). These concentrations were chosen because preliminary studies had shown that saturation of TBG and TBPA were achieved at 1·3 μmol T4/l and 6·5 μmol T4/l respectively. After electrophoresis gels were sliced into three parts for counting; the region containing the albumin stained with bromophenol blue, the region between albumin and the origin containing TBG, and the region anodal to albumin containing TBPA. After determination of the fraction of the total radioactivity present in each region, MBC was calculated by multiplying this fraction with the concentration of T4 added plus the endogenous T4 level.

Albumin concentration was measured by radial immunodiffusion (Behringwerke, Hoechst) and total protein according to Lowry *et al.* (1951).

The K_a of TBG for T4 was measured using isolated TBG. TBG was separated from serum with immobilized anti-TBG antiserum (Corning Medical, Medfield, Mass.). The immobilized anti-TBG antiserum was washed once with phosphate buffer (0·05 M, 0·15 M NaCl, pH 7·4) and resuspended to the same concentration. 25 μl 1:20 diluted serum and 0·5 ml anti-TBG suspension were mixed in appropriately labelled tubes. After incubation for 30 min at 25°C the suspension was centrifuged (10 min, 1200 *g*) and washed once with 1 ml phosphate buffer. The TBG-antibody complex was incubated with tracer T4 and unlabelled T4 (from 0·01 to 125 ng T4/tube) in 1·0 ml phosphate buffer. After 30 min incubation at 37°C, the suspension was centrifuged and the sediment counted. All incubations were performed in duplicate. Data were analysed according to Scatchard (1949).

Binding parameters of TBPA and the additional T4 binding protein present in the sera of the positive subjects were measured as described by Sutherland & Simpson-Morgan (1974; 1975). Data were analysed according to Rosenthal (1967).

Preparative electrophoresis was performed in a thin layer of Sephadex G-75 Superfine, with equipment from LKB-produkterAB, Bromma, Sweden, using glycine-acetate (0·2 M glycine, 0·13 M sodium acetate, pH 8·6) buffer. Albumin was stained with bromophenol blue and after 20 h of electrophoresis, 5 mm bands of the TBPA and albumin regions were transferred to small columns and the proteins eluted with buffer. These fractions were used in subsequent assays.

Immuno-, and disc gel-electrophoresis was performed as described by Clausen (1969) and Maurer (1968) respectively. Isoelectric focusing was done with the materials and apparatus from LKB produkterAB, Bromma, Sweden.

RESULTS

The distribution of T4 between its binding proteins was similar in the normals and subjects negative for the trait (Table 1). However, in sera of members of the family positive for the trait, there was significantly more T4 bound in the albumin region than in normal sera, whereas the binding in the TBG region was significantly less. There were no differences in the binding of T4 to TBPA between the positive and negative members of the family, nor between normals and the negative members.

In contrast to our previous study (Hennemann *et al.*, 1979a), which was performed utilizing whole sera, we could not find a significant difference in K_a of TBG after isolation from sera (Fig. 1) from the various groups. MBC's as estimated with this technique were similar to those estimated with agar gel electrophoresis (Fig. 1).

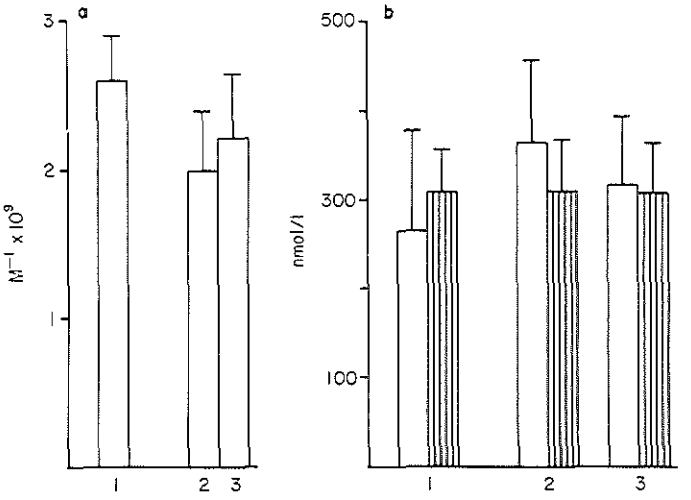

Fig. 1. (a) Association constants and (b) maximal binding capacities of TBG. □ Values estimated after separation of TBG from serum; ▩ values estimated by agar gel electrophoresis. 1 = normals ($n = 9$); 2 = subjects negative for the trait ($n = 5$); 3 = subjects positive for the trait ($n = 6$). Depicted are means ± SD.

Table 1. Distribution of T4 with the binding proteins (mean ± SD) as estimated by agar gel electrophoresis after labelling serum T4 with only a tracer dose of radioactive T4

Group	*n*	TBG %	Albumin %	TBPA %
Normals	20	75±6·6	10·3±1·3	11·5±3·3
Positives	8	55·5±4·0	25·3±1·8	15·6±2·2
Negatives	9	72±6·8	10·8±2·4	13·6±3·9

Measurement of the K_a of the binding of T4 to TBPA after preparative electrophoresis showed no significant differences between members of the family positive and those negative for the trait (Fig. 2). Estimation of MBC's with this method is not possible because of the unknown recovery of the preparative electrophoresis. There were no differences in MBC's estimated with agar gel electrophoresis between the members of the family positive and those negative for the trait (Fig. 2).

Binding analysis in whole sera (Sutherland & Simpson-Morgan, 1974; 1975) disclosed an additional binding protein with a K_a of $1{\cdot}0 \pm 0{\cdot}1 \times 10^7\ M^{-1}$ and a MBC of 280±30 μmol/l (mean ± SD, $n=6$) only in the sera of the family members positive for the trait (Fig. 3).

Preparative electrophoresis of serum yielded pure albumin fractions as judged by the immunoelectrophoresis with the anti total human antiserum (Fig. 4) as well as the anti-prealbumin antiserum, which gave clean precipitation arcs with whole sera and the TBPA fractions, but no visible arcs with the albumin fractions. This purity was further

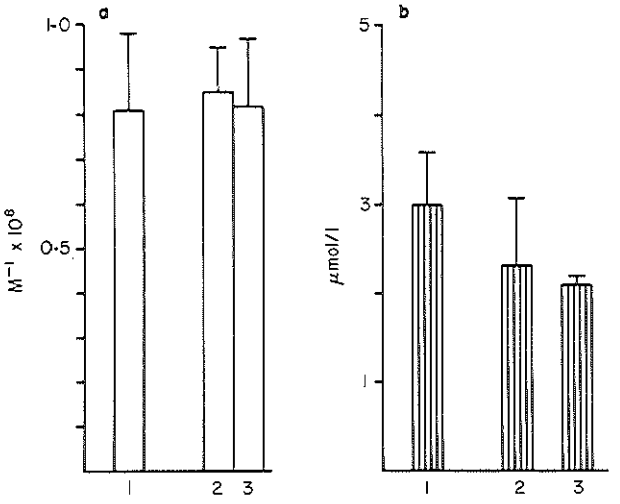

Fig. 2. (a) Association constants of TBPA after separation from serum. (b) Maximal binding capacities estimated by agar gel electrophoresis. 1 = normals ($n=9$); 2 = subjects negative for the trait ($n=3$, a; $n=5$, b); 3 = subjects positive for the trait ($n=4$, a; $n=6$, b). Depicted are means ± SD.

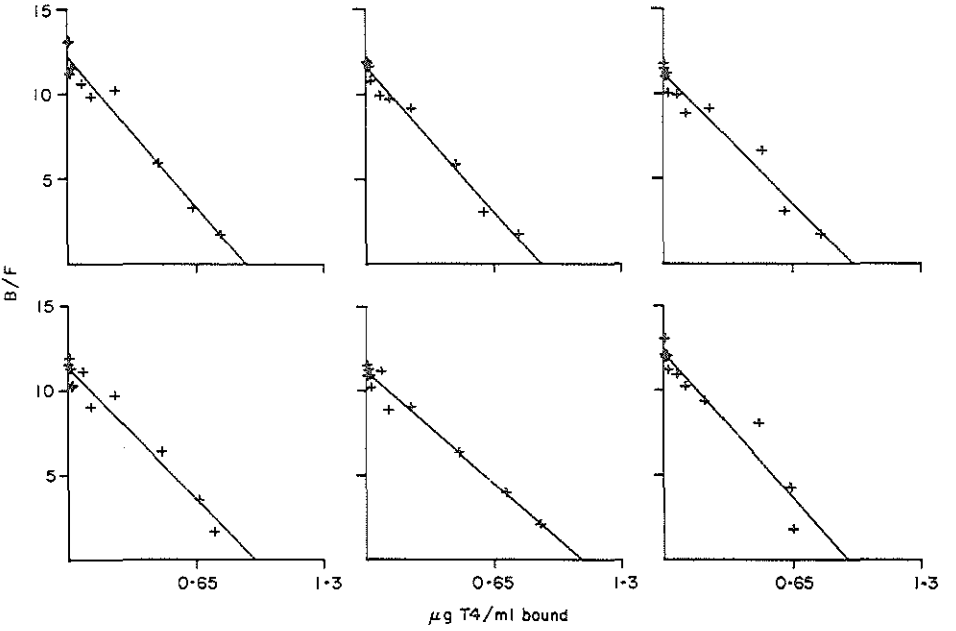

Fig. 3. Scatchard plots of whole sera from the positive family members. Mean (±SD) K_a is 1·0 (±0·1) × 10^7 M^{-1} with a maximal binding capacity of 280±30 μmol/l. Serum was diluted 200-fold. Data were obtained with the method of Sutherland & Simpson-Morgan (1974; 1975).

Table 2. Composition of the peak albumin fraction obtained from preparative electrophoresis and the band at pH 5·5 obtained from preparative isoelectric focusing

Subject	Protein (mg/fract.)	Albumin (mg/fract.)	K_a × 10^7 M^{-1}	MBC (nmol/fract.)	mol MBC / mol alb.
1 After preparative electrophoresis					
Normal	21·0	22·2	—	—	
Family 2					
neg.	17·1	17·8	—	—	
neg.	22·6	24·0	—	—	
pos.	24·8	24·0	1·0	116	0·29
pos.	22·8	24·0	0·9	130	0·33
pos.	14·8	13·6	0·9	59	0·26
Family 1					
pos.	20·3	20·0	1·1	73	0·22
2 After preparative isoelectric focusing (fraction at pH 5·5)					
Family 2					
pos.	7·4	7·6	1·2	78	0·62
pos.	7·6	8·0	1·3	74	0·59

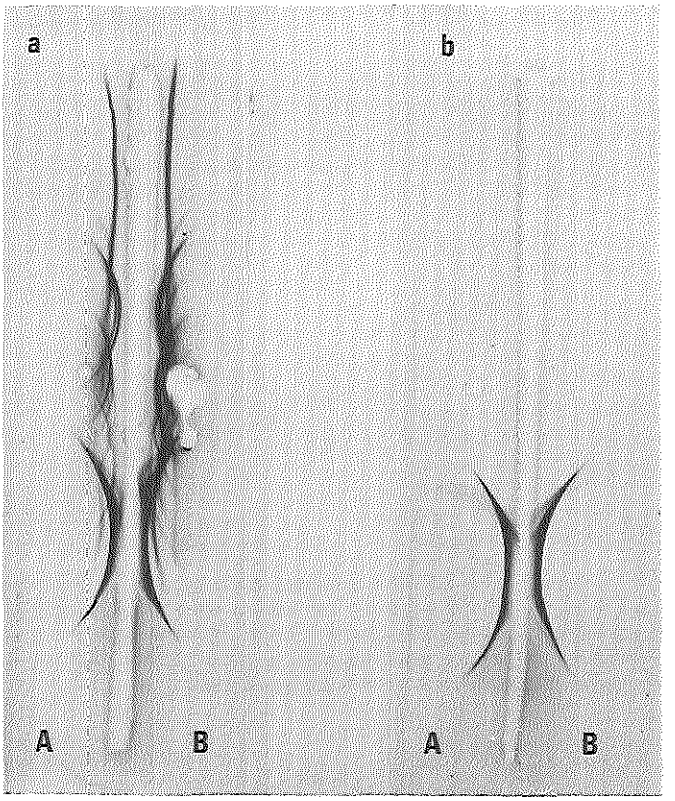

Fig. 4. (a) Immunoelectrophoresis patterns of total serum and (b) albumin fractions. Centre well contained anti-total human anti-serum. A = negative family member; B = positive family member.

substantiated by disc polyacrylamide gel electrophoresis (data not shown), and comparison of albumin (by immunodiffusion) and total protein (Lowry *et al.*, 1951) in these fractions (Table 2). Binding of T4 to albumin fractions of positive family members was characterized by K_a values of 1×10^7 M^{-1}. This binding protein could only be measured in the albumin fractions prepared from sera of subjects positive for the trait.

Isoelectric focusing of these fractions and of whole sera showed a prominent band in the albumin region at pH 5·5 in the family members positive for the trait (Fig. 5). This band is absent or only just visible in the negative members. It could be demonstrated that at pH 5·5 the fractions from the positive family members contained a protein immunologically indistinguishable from albumin which bound T4 with an association constant of 1×10^7 M^{-1} (Table 2). Although we have not performed autoradiographic studies, the prominent band in panel f of Fig. 5 did not bind T4 specifically; a different preparation (d) from the same family member showed a negligible band in this area. Whether or not the presence—but not the ability to bind T4—of this band is a consistently variable finding in normal sera remains to be explored.

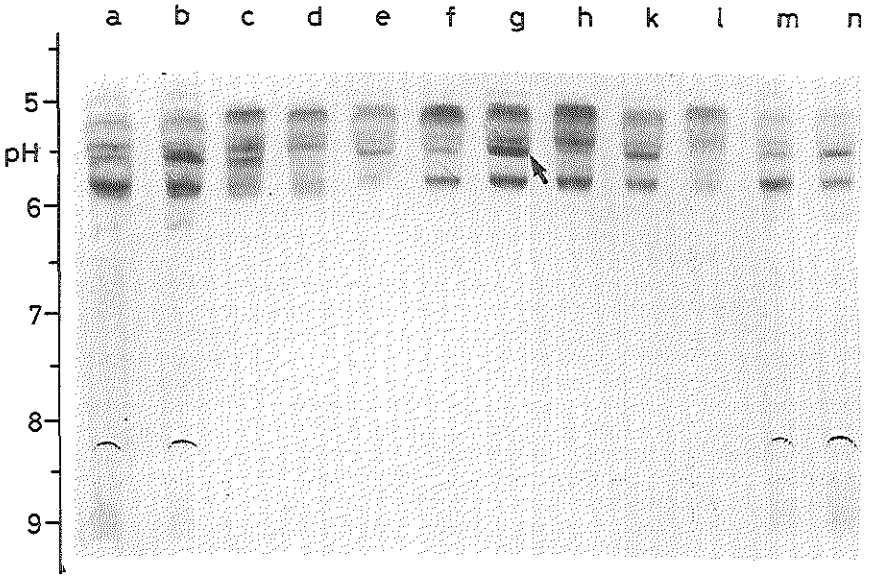

Fig. 5. Results of electrofocusing. pH scale is depicted on the left. The following samples were applied: a and b, whole serum of a negative and positive subject respectively; c–l, albumin fractions; c, e, g and k, different positive subjects; d, h and l, different negative subjects; d and f, different preparations of the same negative subject; m and n, 2·5-fold dilutions of a and b respectively. Arrow indicates the location of the additional band.

DISCUSSION

The combination of raised total serum T4 and normal free T4 concentrations can only be due to increased binding of T4 to serum proteins. From the data presented in Table 1 the most likely explanation for this augmented binding of T4 is an increased affinity to a modified form of albumin, or a protein comigrating with albumin on agar gel.

Estimation of the K_a's of TBG and TBPA after separation yielded values which did not show any difference between normals and members of the family. The K_a we report here for TBG ($2{\cdot}5 \times 10^9$ l/mol) is consistent with other recent reports ($1{\cdot}6 \times 10^9$ l/mol, Cheng *et al.*, 1979; $2{\cdot}5 \times 10^9$ l/mol, Snyder *et al.*, 1976), although higher values have also been reported ($1{\cdot}6 \times 10^{10}$ l/mol, Korcek & Tabachnick, 1976; $2{\cdot}4 \times 10^{10}$ l/mol, Marshall *et al.*, 1972). To isolate TBG from serum immobilized TBG antibody was chosen. Because TBG migrates more slowly than albumin at pH 8·6, preparative electrophoresis will yield TBG preparations which are still contaminated with albumin.

In contrast to our previous findings (Hennemann *et al.*, 1979a) we could not find increased K_a's for TBG and TBPA in the positive members of the family. This discrepancy could occur because, for calculation of the K_a's of these binding proteins in previous studies, measurements were performed in whole sera and the presence of an additional binding protein could alter the results. Measurement of the K_a's using purified binding proteins would overcome this problem. Another explanation could be that the procedure employed to isolate the binding proteins removes a circulating factor which promotes the

interaction between T4 and carrier proteins. This explanation seems less likely since our observations can be adequately explained by the identification of an extra T4-binding protein. Scatchard analysis of whole sera revealed a binding protein in the positive subjects with a K_a of 1×10^7 M^{-1} and a MBC of 280 μmol/l.

From the data in Fig. 1 it appears that there are no differences in concentrations of TBG between the groups. Although levels of free T4 were within the normal range, it appears that occupancy of TBG is elevated in the positive subjects (Table 1), possibly due to a redistribution of T4 during electrophoresis at pH 8·6, compared with the distribution in charcoal binding studies at pH 7·4.

From these data we calculate that the modified albumin (K_a 10^7 M^{-1}; MBC 280 μmol/l) should bind 9·3 times as much T4 as TBPA (K_a 10^8 M^{-1}; MBC 3 μmol/l). Mean T4 in the negative members of the family is 105 nmol/l, of which 13·6% is bound to TBPA (Table 1). Modified albumin therefore should bind $105 \times 0{\cdot}136 \times 9{\cdot}3 \sim 133$ nmol T4/l and because there are no changes in free T4, the other proteins will still bind 105 nmol T4/l. The mean T4 in the positive members should be 238 nmol T4/l, but the observed value was 236 (range 180–282) nmol/l. This would imply that the carriage of T4 by modified albumin is almost 56% of the bound T4, and not 25% as shown in Table 1. This difference can be explained by redistribution of T4 during electrophoresis as mentioned above.

Preparation of albumin by electrophoresis yields fractions considered pure when the albumin content was measured by an immunological method, specific for albumin, and compared with chemical protein determination (Table 2). This purity is confirmed by immuno- and polyacrylamide gel disc-electrophoresis. In the albumin fractions of the family members with raised T4 a binding protein with a K_a of 1×10^7 M^{-1} could be detected (Sutherland & Simpson-Morgan, 1974; 1975), which was not measurable in normals and family members with normal T4. Because these fractions contain only albumin it is most likely that this binding protein is a modified form of albumin for which T4 has a higher affinity. Albumin contains one major binding site and a set of secondary binding sites (Princé & Ramsden, 1977). If we assume that only the principal binding site is modified, it can be calculated from the MBC and the albumin content of the fractions that on a molar basis about 25% of the albumin is changed, without alteration to its immunological properties (Table 2).

Isoelectric focusing of the albumin fractions from the preparative electrophoresis (Fig. 5) showed that although immunologically homogenous, these fractions consist of a number of proteins with isoelectric points ranging from pH 4·8–5·9. This is consistent with previous reports (Malamud & Drysdale, 1978). Both in the albumin fractions and in whole sera of patients with a raised T4 at pH 5·5 a protein band is observed which binds T4 (Table 2) and which is absent or only just detected in subjects with normal T4 levels.

It is possible that the binding protein abnormality observed in our subjects with inherited T4 excess is the cause of inherited T4 elevation in two other reports (Lee *et al.*, 1979; Barlow *et al.*, 1980). In both studies free T4 was normal and the subjects were clinically euthyroid with a normal pituitary–thyroid axis (Hennemann *et al.*, 1979a; Barlow *et al.*, 1980). Barlow *et al.* (1980) report that the binding characteristics of the abnormal protein are similar to prealbumin but that the electrophoretic properties are similar to albumin.

Recognition of this syndrome and appreciation that the free T4 index does not reflect actual free T4 concentrations is important in order to protect patients from inappropriate treatment.

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SPECIFIC THYROXINE BINDING ALBUMIN IS A CONSTITUENT OF NORMAL HUMAN SERUM

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SIR,—In 1979 we described two families showing autosomal dominantly inherited hyperthyroxinaemia due to increased binding of T_4 by serum protein.[1] Free thyroxine (FT_4) serum levels in those affected were normal but the FT_4 index was falsely raised. We subsequently showed that the serum protein responsible was in the albumin fraction.[2] The existence of this syndrome has been repeatedly confirmed,[3-9] and it has been assumed that the T_4 binding albumin (TBA) component was present only in patients with this hyperthyroxinaemia syndrome—hence the suggested term "familial dysalbuminaemic hyperthyroxinaemia". We report here the possible occurrence of this TBA in normal serum.

Serum from twelve unrelated normal persons (laboratory personnel, three females) and three patients with familial hyperthyroxinaemia were investigated. Albumin was separated from serum by preparative gel electrophoresis.[2] Affinity association constant (K_a) and maximal binding capacity (MBC) for T_4 were estimated[10] by incubating the albumin samples with increasing amounts of T_4 followed by separation of bound and unbound T_4 on dextran-coated charcoal.

The results are summarised in the table. Normal sera also contain an albumin component which binds T_4 specifically with a mean K_a of $0{\cdot}9 \times 10^7$/mol, not different from the K_a of the albumin fraction of subjects with hyperthyroxinaemia. However, the MBC differs greatly between subjects with hyperthyroxinaemia and controls: serum albumin fractions of controls contain only one-tenth of the albumin specifically binding T_4 found in patients with the syndrome (12 versus 157 mmol T_4/mol total albumin). These data suggest that the TBA component, which is present in considerable amounts in serum of patients with familial hyperthyroxinaemia, is also detectable in serum of normal controls, albeit in limited quantity.

AFFINITY ASSOCIATION CONSTANT (K_a) AND MAXIMAL BINDING CAPACITY (MBC) OF THYROXINE BINDING ALBUMIN (TBA) IN SUBJECTS WITH HYPERTHYROXINAEMIA AND NORMAL PERSONS: MEAN±SD (RANGE)

Group	K_a ($\times 10^7$/mol)	MBC (mmol T_4/mol total albumin)
Hyperthyroxinaemia (n = 3)	0·7±0·11 (0·5–0·8)	157±7 (150–164)
Normal (n = 12)	0·9±0·37 (0·5–1·9)	12±7 (5–33)

We have studied only twelve subjects but our finding of this protein in all twelve suggests that it is a normal constituent of human serum. We suggest that the term "familial dysalbuminaemic hyperthyroxinaemia" be abandoned in favour of "congenital thyroxine binding albumin elevation" in familial cases where hyperthyroxinaemia is caused by increased levels of TBA. This in analogy to congenital TBG elevation, which is an X-linked dominant condition.

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Chapter IV

Evidence that the uptake of triiodothyronine by human erythrocytes is carrier-mediated but not energy-dependent

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Evidence that the uptake of tri-iodo-L-thyronine by human erythrocytes is carrier-mediated but not energy-dependent

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We investigated 3,3',5-tri-iodo-L-thyronine transport by human erythrocytes and by 'ghosts' prepared from these cells. Uptake of tri-iodothyronine by erythrocytes at 37°C was time-dependent with a maximum reached after 60 min. Tracer analysis after incubation for 1 min revealed only one saturable binding site, with K_m 128 ± 19 nM (mean ± S.E.M.; $n = 7$) and $V_{max.}$ 4.6 ± 0.7 pmol of tri-iodothyronine/min per 6×10^7 cells. After 10 min incubation K_m 100 ± 16 nM ($n = 10$) was found with $V_{max.}$ 7.7 ± 1.2 pmol of tri-iodothyronine/10 min per 6×10^7 cells. At 0°C the uptake system is still active, with K_m 132 ± 26 nM and $V_{max.}$ 1.8 ± 0.3 pmol of tri-iodothyronine/10 min per 6×10^7 cells. The $V_{max.}$ with intact cells is 5-fold greater than the $V_{max.}$ with membranes derived from the same amount of cells when uptake studies are performed in media with similar free tri-iodothyronine concentrations. This indicates that at least 80% of tri-iodothyronine taken up by the intact erythrocytes enters the cell. This saturable uptake system can be inhibited by X-ray-contrast agents in a dose-dependent fashion. (±)-Propranolol, but not atenolol, has the same effect, indicating that the membrane-stabilizing properties of (±)-propranolol are involved. Furthermore, there is no inhibition by ouabain or vanadate, which indicates that tri-iodothyronine uptake is not dependent on the activity of $Na^+ + K^+$-dependent adenosine triphosphatase. We have prepared erythrocyte 'ghosts', resealed after 2.5 min with 0 mM-, 2 mM- or 4 mM-ATP inside. Inclusion of ATP and integrity of the membrane of the erythrocyte 'ghosts' were verified on the basis of an ATP-concentration-dependent functioning of the Ca^{2+} pump. No difference was found in the uptake of tri-iodothyronine by erythrocyte 'ghosts' with or without ATP included, indicating that uptake of tri-iodothyronine is not ATP-dependent. The following conclusions are drawn. (1) Tri-iodothyronine enters human erythrocytes. (2) There is only one saturable uptake system present for tri-iodothyronine, which is neither energy (i.e. ATP)-dependent nor influenced by the absence of an Na^+ gradient across the plasma membrane. This mode of uptake of tri-iodothyronine by human erythrocytes is in sharp contrast with that of rat hepatocytes, which uptake system is energy-dependent and ouabain-sensitive [Krenning, Docter, Bernard, Visser & Hennemann (1978) *FEBS Lett.* **91**, 113–116; Krenning, Docter, Bernard, Visser & Hennemann (1980) *FEBS Lett.* **119**, 279–282]. (3) X-ray-contrast agents inhibit tri-iodothyronine uptake by erythrocytes in a similar fashion to that by which they inhibit the uptake of tri-iodothyronine by rat hepatocytes [Krenning, Docter, Bernard, Visser & Hennemann (1982) *FEBS Lett.* **140**, 229–233].

The biological action of 3,3',5-tri-iodo-L-thyronine (referred to below simply as tri-iodothyronine) is initiated by the binding of the hormone to receptors in the target cell (Samuels, 1978). To reach the cellular compartment the hormone has to be translocated through the plasma membrane. We have shown with transport studies with rat hepatocytes in primary culture (Krenning *et al.*, 1978) that this uptake of tri-iodothyronine is carrier-mediated and energy-dependent and can be inhibited by KCN, 2,6-dinitrophenol or oligomycin. Further investigations have shown that this active

transport of tri-iodothyronine is regulated by the ATP content of the hepatocytes (Krenning *et al.*, 1980). It was found that a highly significant positive correlation existed between the intracellular ATP concentration and the transport of tri-iodothyronine. The energy-dependency of tri-iodothyronine uptake is in harmony with the finding by Cheng *et al.* (1980) that the mechanism of tri-iodothyronine uptake is by the so-called 'receptor-mediated endocytosis'.

In the present paper we describe studies concerning tri-iodothyronine uptake by human erythrocytes. It has been reported that erythrocyte membranes bind tri-iodothyronine and thyroxine competitively (Singh *et al.*, 1976). Holm & Jacquemin (1979) have shown that human erythrocyte membranes contain two separate saturable uptake systems for tri-iodothyronine, with K_m values almost identical with those found with rat liver cells (Krenning *et al.* 1978).

We decided to investigate further the characteristics of human erythrocytes with regard to transmembranal transport of tri-iodothyronine. Our reasoning was that, if we could confirm that transport of tri-iodothyronine into erythrocytes is based on similar principles to those that apply for hepatocytes, erythrocytes could possibly be used as a model system for tissue uptake of tri-iodothyronine (and possibly other iodothyronines) in studies performed *in vivo*.

Part of this work was presented at the 11th Annual Meeting of the European Thyroid Association, Pisa, Italy, on 7–11 September 1981.

Materials and methods

Materials

3,3',5-Tri-iodo-L-thyronine labelled at the 3'-position ([^{125}I]tri-iodothyronine, 1200 μCi/μg) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). The iodide content of this material, as determined by high-pressure liquid chromatography [C_{18} column; methanol/aq. 7.35 mM-KH_2PO_4 (57:43, v/v) as solvent] did not exceed 3%. Unlabelled tri-iodothyronine, X-ray-contrast agents, inhibitors of the cytoskeleton, ouabain and oligomycin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). (±)-Propranolol and atenolol were a generous gift from I.C.I. (Rotterdam, The Netherlands). All other chemicals were of reagent grade and were obtained from BDH Chemicals (Poole, Dorset, U.K.). Polypropylene reaction vessels from Eppendorf (Marburg, West Germany) were used throughout.

Preparation of the erythrocytes

Blood from healthy volunteers was collected in heparinized tubes and centrifuged at room temperature. The plasma and the buffy coat were removed and the erythrocytes were washed three times with 3 vol. of ice-cold phosphate-buffered saline (150 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.4). Finally, the erythrocytes were suspended in phosphate-buffered saline and diluted to obtain a relative packed-cell volume of 0.25, as estimated with a haematocrit centrifuge. This suspension will contain 3×10^5 cells/10 μl (Dacie & Lewis, 1968). Erythrocytes were kept at 4°C until use.

Preparation of erythrocyte lysate

Erythrocytes were mixed with 5 mM-sodium phosphate buffer, pH 7.4, containing 4 mM-$MgCl_2$. After 10 min membranes were separated by centrifugation at 9500 *g* for 1 min. Then 10 μl of 2 M-KCl and 65 μl of 2 M-NaCl per ml were added to the supernatant to obtain iso-osmoticity (130 mM-NaCl and 20 mM-KCl).

Uptake studies with erythrocytes

Incubation mixtures (0.8 ml) were made in Eppendorf reaction vessels containing 6×10^7 cells in phosphate buffer (130 mM-NaCl/20 mM-KCl/4 mM-$MgCl_2$/5 mM-sodium phosphate buffer, pH 7.4). After temperature equilibration at 37°C during 15 min, the reaction was started by the addition of [^{125}I]tri-iodothyronine with various concentrations of unlabelled tri-iodothyronine in 0.2 ml of phosphate buffer at 37°C. The reaction vessels were closed and the contents were mixed and incubated for 1 min or 10 min. The cells were then separated from the medium by centrifugation at 9500 *g* for 1 min. Cells were pelleted within the first 10 s of centrifugation. The supernatant was discarded and the radioactivity in the pellet was counted after an additional wash with ice-cold phosphate buffer. Blanks for all hormone concentrations used were prepared in the same way, but the centrifugation was omitted, to correct for non-specific binding to the reaction vessels.

Binding studies with erythrocyte membranes were performed similarly, except that NaCl and KCl were omitted from the phosphate buffer, which caused lysis of the cells. To correct for non-specific binding to the membrane and/or diffusion the fractional uptake at the highest tri-iodothyronine concentration used (7.7 μM) was subtracted from the fractional uptake values obtained at lower concentrations of tri-iodothyronine. Then a double-reciprocal plot was constructed to assess the kinetic parameters.

Measurement of the inhibition of tri-iodothyronine uptake by various compounds

Incubation mixtures (0.8 ml) were made in phosphate buffer containing 6×10^7 cells, with or without

the compound to be tested (added as 0.08 ml of a 10-fold concentrated solution in phosphate buffer). After 30 min preincubation at 37°C, the reactions were started by the addition of tri-iodothyronine and tracer tri-iodothyronine in 0.2 ml of phosphate buffer. Incubation for 10 min and separation of cells and medium were performed as described above. Specific uptake of tri-iodothyronine was calculated as the difference in percentage uptake between a low dose of hormone (1.5 nM-tri-iodothyronine), far below the K_m of the transport process, and a very high concentration of tri-iodothyronine (7.7 μM). Percentage inhibition was calculated as the percentage fall in specific uptake in erythrocytes in the presence of the compound tested versus control incubations of mixtures not containing the inhibitor.

Preparation of pink erythrocyte 'ghosts'

Erythrocyte 'ghosts' were prepared as previously described (Larsen *et al.*, 1978), with some modifications. Erythrocytes were haemolysed by mixing 1 vol. of packed cells with 10 vol. of hypo-osmotic buffer containing 10 mM-Tris/HCl buffer, pH 7.4, 4 mM-$MgCl_2$, 1 mM-$CaCl_2$ and various concentrations of ATP (0–4 mM). After 2.5 min the erythrocyte 'ghosts' were resealed by addition of 2 M-KCl and 2 M-NaCl solutions to adjust the concentration of KCl to 20 mM and that of NaCl to 130 mM. The whole procedure was performed at 0–4°C, and the erythrocyte 'ghosts' were kept on ice until use. When the erythrocyte 'ghosts' were used to study the function of Ca^{2+}-stimulated ATPase, $^{45}Ca^{2+}$ was included in the hypo-osmotic buffer (final specific radioactivity 10^5 d.p.m./μmol of Ca^{2+}) and the sealed erythrocyte 'ghosts' were washed twice with Tris buffer (4 mM-$MgCl_2$ / 1 mM-$CaCl_2$ / 130 mM-NaCl / 20 mM-KCl / 10 mM-Tris/HCl buffer, pH 7.4) to remove radioactive Ca^{2+} not incorporated into the erythrocyte 'ghosts'.

Preparation of white erythrocyte 'ghosts'

For comparison purposes erythrocyte 'ghosts' were also prepared as described by Holm & Jacquemin (1979). Erythrocytes were haemolysed by mixing 1 vol. of packed cells with 40 vol. of 5 mM-sodium phosphate buffer, pH 8.0. After 10 min the membranes were collected by centrifugation at 22000 *g* for 10 min at 4°C. They were washed five times with the same buffer to remove all haemoglobin. Then the membranes were suspended in the hypo-osmotic buffer used to prepare pink erythrocyte 'ghosts' and treated further as described in the preceding paragraph.

Measurement of the Ca^{2+} pump in erythrocyte 'ghosts'

Transport experiments were started by warming the $^{45}Ca^{2+}$- and ATP-loaded erythrocyte 'ghosts' suspended in Tris buffer at 37°C. Packed 'ghosts' were diluted 4-fold. After various time intervals, 0.5 ml samples were chilled on ice and centrifuged at 9500 *g* for 1 min. The packed 'ghosts' were washed once with ice-cold Tris buffer, treated with 6% (w/v) $HClO_4$ and centrifuged, and the radioactivity of samples of supernatant were counted.

Statistical analysis

Statistical analysis of the data was performed by using Student's *t* test for unpaired groups (Snedecor & Cochran, 1967).

Results

Uptake of tri-iodothyronine at 37°C by human erythrocytes is time-dependent (Fig. 1). Binding of the hormone is maximal and constant after 60 min of incubation; 60% of the maximal value is obtained within 10 min. There is a linear relationship between the number of erythrocytes in the incubation mixture and the uptake of tri-iodothyronine by the cells, both at a low concentration of hormone and at a much higher concentration (Fig. 2). From the fact that the fraction of the hormone bound by the cells is lower at high concentrations of tri-iodothyronine, it can be concluded that the process is saturable.

To extend this finding further, cells were incubated with various concentrations of tri-iodothyronine, and the fraction associated with the cells was plotted against the logarithm of the con-

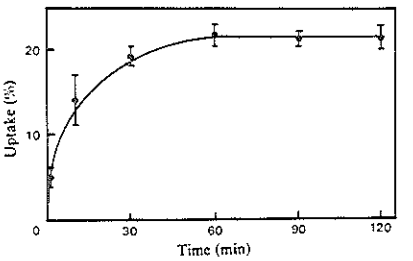

Fig. 1. *Tri-iodothyronine uptake by erythrocytes plotted versus time*

Erythrocytes (6×10^7) were incubated with 15 pmol of tri-iodothyronine in 1 ml of phosphate buffer for various time intervals. For full experimental details see the text. Time in minutes is plotted on the abscissa. On the ordinate the amount of tri-iodothyronine associated with the erythrocytes is shown as percentage of total tri-iodothyronine added. Bars indicate S.D. Each data point is the mean for five different experiments performed in duplicate.

From the difference in maximal uptake velocity between membranes and intact erythrocytes (Table 1) it can be concluded that after 10 min only 16% of the hormone is associated with the membranes of erythrocytes, the remainder (i.e. 84%) being transported into the interior of the cell.

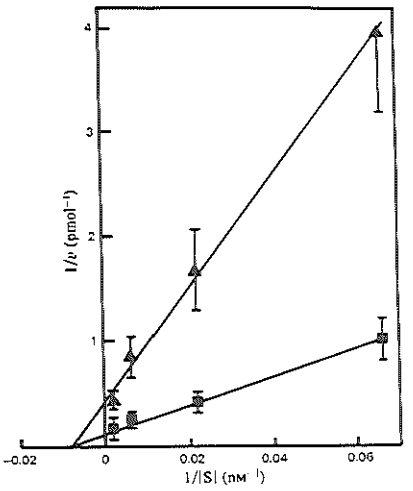

Fig. 4. *Double-reciprocal plot of tri-iodothyronine uptake by erythrocytes*

Erythrocytes (6×10^7) were incubated with various concentrations of tri-iodothyronine for 1 min (▲) or 10 min (■). Means ± S.D. are plotted of the reciprocals of values for tri-iodothyronine uptake for seven (▲) and ten (■) experiments respectively. Fur further experimental detail see the text and the legend to Fig. 3.

In order to determine whether the uptake of tri-iodothyronine by erythrocytes is dependent on ATP, we prepared pink erythrocyte 'ghosts' without or with 4 mM-ATP included after resealing. With these 'ghosts' we performed tri-iodothyronine-uptake studies during 10 min at 37°C. There is no significant difference between the kinetic parameters of the erythrocyte 'ghosts' with ATP included and those without ATP (Table 1). This indicates that the presence of ATP is not necessary for the uptake process.

To assess the integrity of the membranes of the erythrocyte 'ghosts' used in the tri-iodothyronine-uptake studies and to verify the inclusion of the ATP inside these 'ghosts', we performed studies with $^{45}Ca^{2+}$ to measure the activity of the Ca^{2+} pump. Normally the erythrocyte membrane maintains a steep Ca^{2+} gradient, with concentrations of Ca^{2+} higher outside than inside the cells. The gradient is maintained by a low permeability to Ca^{2+} in the inward direction and an ATPase that is stimulated by Ca^{2+} and requires Mg^{2+}. This ATPase is directly responsible for Ca^{2+} efflux (Schatzmann, 1973). The enzyme sites for ATP and Ca^{2+} are located on the inner face of the membrane.

Fig. 5 shows that the efflux of Ca^{2+} from the interior of pink erythrocyte 'ghosts' is dependent on the concentration of ATP inside. This indicates that ATP is indeed included inside these erythrocyte 'ghosts'. Since only a small amount of Ca^{2+} (about 15%) diffuses out of the erythrocyte 'ghosts' when no ATP is present inside, it can be concluded that the membranes of pink erythrocyte 'ghosts' are still impermeable for small ions.

Finally, we performed studies with various compounds to test interference with tri-iodothyronine uptake. A summary of the results is given in Table 2.

Table 1. *Kinetic parameters of tri-iodothyronine uptake in erythrocytes (6×10^7), erythrocyte membranes (of 6×10^7 cells) or erythrocyte 'ghosts' (6×10^7)*

For full experimental details see the text. Key to medium used: A, phosphate buffer; B, phosphate buffer with lysate of 6×10^7 cells; C, phosphate buffer without NaCl and KCl, which causes lysis of the added cells. All experiments were performed in triplicate. Kinetic parameters of each individual experiment were calculated and means ± S.E.M. are reported.

Incubation conditions						
Material	Medium	Time (min)	Temp. (°C)	No. of observations	K_m (nM)	$V_{max.}$ (pmol per 6×10^7 cells)
Erythrocytes	A	10	37	10	100 ± 16	7.7 ± 1.2
Erythrocytes	A	1	37	7	128 ± 19	4.6 ± 0.7
Erythrocytes	A	10	0	3	132 ± 26	1.8 ± 0.3
Erythrocytes	B	10	37	6	127 ± 12	9.6 ± 1.1
Membranes	C	10	37	5	75 ± 10	1.6 ± 0.3
'Ghosts' (0 mM-ATP)	A	10	37	4	118 ± 13	3.5 ± 0.3
'Ghosts' (4 mM-ATP)	A	10	37	4	128 ± 8	3.9 ± 0.4

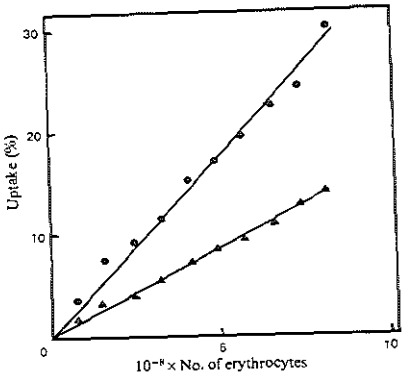

Fig. 2. *Tri-iodothyronine uptake (1 min) by erythrocytes plotted versus the number of cells*
Various amounts of erythrocytes were incubated with 15 pmol (●) or 3 nmol (▲) of tri-iodothyronine in 1 ml of phosphate buffer. For full experimental details see the text. Number of erythrocytes is plotted on the abscissa, and the amount of tri-iodothyronine bound to the erythrocytes after 1 min of incubation is given on the ordinate. Each data point is the mean of two experiments, performed in duplicate.

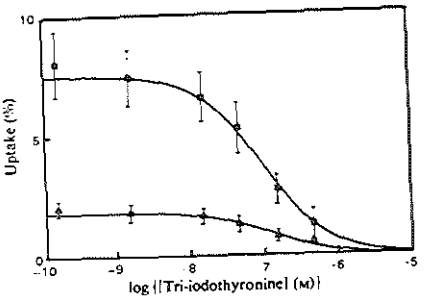

Fig. 3. *Tri-iodothyronine uptake by 6 × 10⁷ erythrocytes plotted versus the concentration of tri-iodothyronine*
For full experimental details see the text. On the ordinate the fractional uptake of hormone is plotted, and on the abscissa the logarithm of the concentration of tri-iodothyronine in the incubation mixtures. Data points are means ± S.D. for ten (■: 10 min incubation time) or seven (▲: 1 min incubation time) experiments. Each experiment was performed in triplicate and the data points were corrected for non-saturable binding by subtraction of the percentage uptake of hormone at a tri-iodothyronine concentration of 7.7 μM before further calculations were performed. Non-saturable binding was the same at 1.5 μM- and 7.7 μM-tri-iodothyronine added to the cells and amounted to 2.5 ± 0.8% (mean ± S.D., $n = 7$) after 1 min incubation and 6.5 ± 2.1% (mean ± S.D., $n = 10$) after 10 min incubation.

centration of the hormone (Fig. 3). A sigmoidal pattern, typical for a saturable process, is observed, both after 1 min and after 10 min incubation.

Extension of the concentration range down to 0.5 pM-tri-iodothyronine did not reveal a second saturable binding site; extension to higher concentrations was not possible owing to the limited solubility of the hormone. From these findings it can be concluded that only one saturable uptake system for tri-iodothyronine exists in erythrocytes. Plotting these data in a double-reciprocal plot (Fig. 4) revealed that both curves have the same intercept with the abscissa. This indicates that the K_m of the uptake process is not dependent on incubation time. The intercepts with the ordinate are different, showing a higher maximal uptake after 10 min incubation than after 1 min. A summary of the uptake parameters is listed in Table 1.

If maximal uptake velocities are calculated it appears that upake during the first minute is 6-fold greater than the mean uptake during the first 10 min. This finding is in accordance with the curved relationship between uptake of hormone and time (Fig. 1). At 0°C the uptake system is still active, although the maximal velocity is significantly lower than at 37°C (Table 1).

In order to answer the question whether tri-iodothyronine enters human erythrocytes, we measured the binding of tri-iodothyronine to erythrocyte membranes, prepared by lysis of cells, and compared the results obtained with the uptake parameters for intact cells. Because the incubation mixture of the membranes still contained the cell cytosol liberated during lysis, we have incorporated a similar amount of cytosol in the incubation mixtures of the intact erythrocytes. Although it is known that erythrocyte cytosol contains tri-iodothyronine-binding proteins (Yoshida & Davis, 1977; Davis *et al.*, 1980), it has also been shown that association of tri-iodothyronine with these proteins proceeds only slowly, about 5% of the added tri-iodothyronine being bound during the first 30 min. Because we started the incubations by the addition of hormone, it is therefore assumed, for the calculations of the kinetic parameters, that all tri-iodothyronine in the incubation mixtures was unbound.

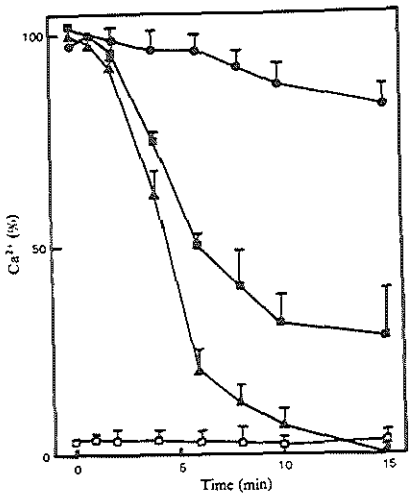

Fig. 5. *ATP-dependent efflux of Ca^{2+} from erythrocyte 'ghosts'*

Pink erythrocyte 'ghosts' were prepared with 0mM- (●), 1mM- (■) and 4mM- (▲) ATP and $^{45}Ca^{2+}$ included. For full experimental details see the text. The amount of radioactivity present in the erythrocyte 'ghosts' at the start of the incubation at 37°C was taken as 100%. Depicted are means ± S.E.M. for four experiments performed in duplicate. A similar amount of white erythrocyte 'ghosts' prepared with 1mM-ATP and $^{45}Ca^{2+}$ (□) included only 5% of the radioactivity compared with the pink erythrocyte 'ghosts', and no transport could be measured.

Table 2. *Percentage inhibition of tri-iodothyronine uptake in erythrocytes by various compounds*

For full experimental details see the text. Each experiment was performed in triplicate. N.S., No significant inhibition.

Compound	Concentration (μM)	Inhibition (%)	No. of observations
Tyropanoic acid	100	22	6
	10	12	5
Iopanoic acid	100	78	6
	10	37	6
Ipodic acid	100	86	4
	10	32	5
(±)-Propranolol	100	30	6
	10	N.S.	4
Atenolol	100	N.S.	6
	10	N.S.	2
Ouabain	1000	N.S.	9
Vanadate	10	N.S.	3
Colchicine	125	16	7
	25	N.S.	7
Vinblastine	60	57	3
	12	22	3
Cytochalasin	100	N.S.	2
	20	N.S.	2
Oligomycin	110	73	2
	22	43	2

Discussion

The present study demonstrates that uptake of tri-iodothyronine by human erythrocytes involves only one saturable process. Careful examination of tri-iodothyronine uptake over a wide range of hormone concentrations (from 1pM to 7.7μM) did not reveal a second system. This is in contrast with the existence of two saturable uptake systems in rat hepatocytes (Krenning *et al.*, 1978, 1980; Eckel *et al.*, 1979) and with the presence of two different saturable binding sites on rat liver membranes (Pliam & Goldfine, 1977). We have shown (Krenning *et al.*, 1978, 1980) that the hepatic transport system with the highest affinity is energy (i.e. ATP)-dependent and can be inhibited by metabolic inhibitors such as KCN, 2,6-dinitrophenol and oligomycin, or by ouabain (Krenning *et al.*, 1981*a*), which last-mentioned finding indicates that the Na^+ gradient over the membrane may be of importance. However, the presence or absence of ATP in erythrocyte 'ghosts' seems not to be important for the uptake of tri-iodothyronine by these 'ghosts' (Table 1), which finding argues against a role of ATP in the uptake process.

Nevertheless the possibility remains that an Na^+ gradient generated by the presence of ATP via $Na^+ + K^+$-dependent ATPase is important for the uptake process. Because the method used for the preparation of erythrocyte 'ghosts' may partially restore the Na^+ gradient across the membrane, independent of the presence of ATP, it is therefore possible that the effect of the depletion of the erythrocyte 'ghosts' of ATP is masked. However, the fact that ouabain or vanadate does not inhibit the uptake of tri-iodothyronine by erythrocytes (Table 2) makes this hypothesis unlikely. This implies that the tri-iodothyronine-transport system present in erythrocytes is not energy-dependent. This conclusion is further substantiated by the finding that this tri-iodothyronine-transport system is still active at 0°C (Table 1), in contrast with the energy-dependent uptake system of rat hepatocytes, which is not measurable at 0°C (Krenning *et al.*, 1978). That oligomycin strongly inhibits tri-iodothyronine uptake by erythrocytes (Table 2) cannot be attributed to its inhibition of the $Na^+ + K^+$ pump (Hoffman et al., 1980), because neither ouabain nor vanadate, which also block $Na^+ + K^+$-dependent

ATPase (Hoffman *et al.*, 1980), inhibits the uptake of tri-iodothyronine by erythrocytes (Table 2). Furthermore, it is known that oligomycin blocks the production of ATP (Cantarow & Schepartz, 1967) by inhibition of the phosphorylation of ADP, but this mechanism also cannot be involved, because erythrocytes do not contain mitochondria, the usual site of action of oligomycin. The mechanism of action of this antibiotic therefore remains unclear, although it is possible that this compound has membrane-stabilizing properties too.

That membrane stabilization causes an inhibition of tri-iodothyronine uptake by erythrocytes can be concluded from the finding that (±)-propranolol in high concentrations inhibits tri-iodothyronine transport by erythrocytes, whereas atenolol does not (Table 2), although both are β-blocking agents. This discrepancy can be explained by the membrane-stabilizing properties of (±)-propranolol (Pritchard, 1978), in contrast with atenolol. The mechanism by which colchicine and vinblastine interfere with tri-iodothyronine uptake by erythrocytes is unclear. It is known that these two compounds inhibit the assembly of microtubules (Sternlicht & Ringel, 1979; Beck, 1980). However, these structures are not present in erythrocytes (Nicolson, 1976). On the other hand, erythrocytes do contain microfilaments and their associated proteins (Goldman *et al.*, 1979; Nicolson, 1976), but cytochalasin, which inhibits these structures (Weihing, 1976), does not impair the uptake of tri-iodothyronine by erythrocytes.

More than 80% of the tri-iodothyronine associated with erythrocytes after 10 min exposure to the hormone is transported into the interior of the cell. This can be concluded from the difference between the maximal uptake velocity of intact erythrocytes and the maximal binding of the hormone by a membrane preparation derived from the same amount of erythrocytes (Table 1). It is rather likely that the affinity of the carrier in the membrane will be different when binding is measured in the membrane preparation as compared with intact cells, because in the former case both sides of this carrier are exposed to the uptake buffer, whereas this is not the case when intact erythrocytes are used. Therefore a difference in K_m can be expected.

We prepared white (i.e. haemoglobin-free) erythrocyte 'ghosts' by washing the lysed erythrocytes extensively before resealing them. In our hands it was not possible to show any saturable uptake process in this type of 'ghosts', in contrast with pink erythrocyte 'ghosts', which are prepared by opening the erythrocytes for only a short time.

The absence of a saturable uptake process in white erythrocyte 'ghosts' may be due to damage of the membranes. Electron microscopy revealed that the membranes of this type of erythrocyte 'ghosts' show many blebs, representing small protrusions at the edge of the 'ghosts' (Ting-Beall *et al.*, 1981). Furthermore, it appears not possible to confine $^{45}Ca^{2+}$ inside the white erythrocyte 'ghosts', which indicates that these 'ghosts' are leaky for small ions, and no active transport of Ca^{2+} could be measured (Fig. 5). These findings shed some doubt on the structural integrity of these white erythrocyte 'ghosts'.

Our results are in sharp contrast with a previous report (Holm & Jacquemin, 1979) in which the existence is described of two saturable tri-iodothyronine-uptake processes in white erythrocyte 'ghosts'. The uptake process with the highest affinity could be inhibited by ouabain (Holm & Jacquemin, 1979). In our study only one saturable transport system for tri-iodothyronine in intact erythrocytes or pink erythrocyte 'ghosts' (Table 1) could be demonstrated. This system could not be inhibited by ouabain (Table 2). The reason for this discrepancy is not clear, although Holm & Jacquemin (1979) did not describe any test to assess the structural integrity of the erythrocyte 'ghost' preparations that they used.

From the data in Table 2 it can be concluded that X-ray-contrast agents such as ipodic acid, tyropanoic acid and iopanoic acid are strong inhibitors of tri-iodothyronine uptake by erythrocytes. A similar influence was found on the uptake of tri-iodothyronine and thyroxine by the high-affinity system of rat hepatocytes (Krenning *et al.*, 1981*b*) and *in vivo* on the uptake of thyroxine by human liver (Felicetta *et al.*, 1980). It is also known that these compounds strongly inhibit the conversion of thyroxine (3,3′,5,5′-tetraiodo-L-thyronine) into tri-iodothyronine (Suzuki *et al.*, 1979) *in vivo* as well as *in vitro* (Kaplan *et al.*, 1979). However, it is possible that at least part of the described inhibition of conversion *in vivo* is due to the inhibition of the transport of the thyroid hormones across the cell membrane.

The K_m of the tri-iodothyronine-transport system in erythrocytes (100/130 nM) that we describe in the present paper compares well with the K_m reported for the tri-iodothyronine-transport system present in lymphocytes (100 nM) (Holm *et al.*, 1980). This uptake system appeared to be energy-dependent. It is possible that the uptake systems of erythrocytes and lymphocytes are similar, because both cells come from the same stem cell (Quesenbery & Levitt, 1979*a*,*b*,*c*), but that the erythrocyte system lost its energy-dependency at the time that its nucleus disappeared, a target of the tri-iodothyronine inside the cell.

Finally, we can conclude on the basis of the results presented above that the human erythrocyte cannot be used as a model system for tissue uptake of tri-iodothyronine in studies performed *in vivo*.

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Chapter V

Regulation of influx and efflux of thyroid hormones in rat hepatocytes: Possible physiologic significance of the plasma membrane in the regulation of thyroid hormone activity.

Regulation of Influx and Efflux of Thyroid Hormones in Rat Hepatocytes: Possible Physiologic Significance of the Plasma Membrane in the Regulation of Thyroid Hormone Activity

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Introduction

One if not the most important reason to study thyroid hormone metabolism is the notion that most (in humans about 80%) of the overall 3,3',5-triiodothyronine (T_3) production is generated in peripheral tissues by conversion of thyroxine (T_4) into T_3 (Chopra 1976). The liver plays an important role in this process but other tissues like kidney and brain are also of significance. The process of T_4-deiodination into T_3 is subject to regulation, e.g. in conditions of caloric deprivation or starvation or in chronic or acute disease extra-thyroidal T_3 production is diminished (Vagenakis 1981).

It has been shown that the greater part of T_3 that occupies the nuclear receptor of rat liver, which is the main site for the initiation of thyroid hormone action, is not derived from local intracellular deiodination of T_4 but is derived from circulating plasma T_3 (Larsen 1982). In order to reach the nucleus, T_3 has to be translocated from the extracellular fluid into the cell via the plasma membrane. The concentration of plasma T_3 is, among other things, dependent on the amount of T_4 which enters the tissue cells where it is converted into T_3. In the pituitary and especially in the cerebrum, most of the T_3 bound to the nucleus is derived from intracellular deiodination of T_4, less being derived from the plasma (Larsen 1982).

If transport of T_4 and T_3 over the plasma membrane of target cells are controlled processes, then they may determine (apart from processes which influence intracellular thyroid hormone metabolism) the ultimate production of T_3 and the exertion of thyromimetic activity. Once, having entered tissue cells, thyroid hormones may be further metabolised or leave the cell unaltered. What has been mentioned with respect to the regulatory role of thyroid hormone influx may also apply to the efflux of these substances. In other words, if the efflux of thyroid hormones is subject to regulation then this process may also play a part in the control of thyroid hormone activity.

We will discuss here studies concerning influx and efflux of thyroid hormones with special attention to possible regulation of these processes. Also the significance of these transport processes as studied by in vitro techniques will be discussed in the light of a possible physiological mechanism.

Uptake of Thyroid Hormones into Rat Hepatocytes

Initial uptake rates of tracer T_3 and T_4 by rat hepatocytes in primary culture, i.e. uptake after 1 min of incubation, were studied in the presence of increasing doses of unlabeled hormone (Krenning et al. 1978). These studies were done at different temperatures and for T_3 in the presence of T_4 and for T_4 in the presence of T_3. Also the influence of preincubation with metabolic blockers like KCN, DNP and oligomycin on initial uptake rates was studied. Table 1 depicts the results after analysis of one min uptake of T_3 under the different circumstances. It can be seen that 2 uptake systems, one with relatively high K_m (low affinity system: LAS) and one with relatively low K_m (high affinity system: HAS)

Table 1 Characteristic 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/35 μg 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(2.0-3.1)	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

Table 2 Kinetics parameters of thyorid hormone transport

T_4 :	HAS	K_m = 1.2 nM
	inhibition T_3 transport	K_i = 21 nM
T_3 :	HAS	K_m = 21 nM
	inhibition T_4 transport	K_i = 90 nM

were detected. Also the corresponding V_{max} is given in Table 1. The LAS appears to be unaffected when studied under different temperatures and does not change when T_4 is present during the studies or when the cells were preincubated with metabolic blockers. On the contrary, the HAS depends very much on temperature, is suppressed by T_4, and is not detectable anymore after preincubation with metabolic blockers. From these and other studies (Krenning et al. 1979), it was argued that the LAS represents binding of T_3 to the outer cell surface possibly attached to albumin, whereas the HAS represents the mechanism by which T_3 is transported into the cellular compartment. Similar findings were obtained with regard to the uptake of T_4. Also here two saturable uptake systems were found; one with low affinity (K_m 1.0 μM) that is not influenced by temperature, the presence of T_3, or preincubation with metabolic blockers, and one with high affinity (K_m 1.2 nM at 21 °C) that is dependent on temperature, suppressible by T_3 or after preincubation with metabolic blockers.

Of interest was the question whether T_3 and T_4 were transported via the same transport route or by different pathways. This problem was studied (Krenning et al. 1981) by measuring the K_i of T_4 for the inhibition of T_3 transport and comparing this value with the K_m of T_4 for its own transport system. Also the inhibitory activity of T_3 on T_4 transport was studied and the K_i value of T_3 was compared with the K_m for its own transport system (Table 2). From the differences of the K_i values with the respective K_m values it is concluded that T_4 and T_3 are transported via different uptake systems although they do inhibit each others transport.

Since metabolic inhibitors abolished the detection of the HAS for T_3 and T_4, the relationship between intracellular ATP concentration and initial uptake rates or T_3 and T_4 was studied. This relationship was also studied with regard to reverse T_3 (rT_3) which also appeared to be translocated via a high affinity system with a K_m value similar to that of T_4. The relationship of initial uptake of the 3 thyroid hormones and the intracellular ATP concentration is depicted in Fig. 1. It can be seen that the relationship between ATP concentration and uptake is linear with regard to T_3 and curvilinear with regard to the other iodothyronines. The different types of relationship between ATP and T_3 on one hand and T_4 and rT_3 on the other hand underline the possibility of a different uptake route for these iodothyronines. The similarity in ATP-uptake relationship for reverse T_3 and T_4 is in concert with a shared uptake system as suggested from the similarity of the K_m values of the respective HAS.

The above mentioned results were all concerned with initial uptake rates. However, in biologic systems especially in physiological situations at least semi-

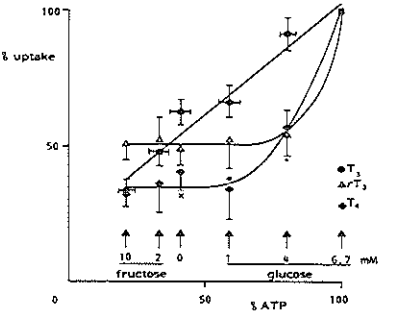


Fig. 1 Active transport (in % of control) of iodothyronines into rat hepatocytes in primary culture as function of cellular ATP content (in % of control). Values from incubations with 6.7 mM glucose are expressed as 100%. The absolute control values amounted to 0.67 T_4, 36 T_3 and 3.8 rT_3 pmol .35 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 °C: 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; xp=0.025

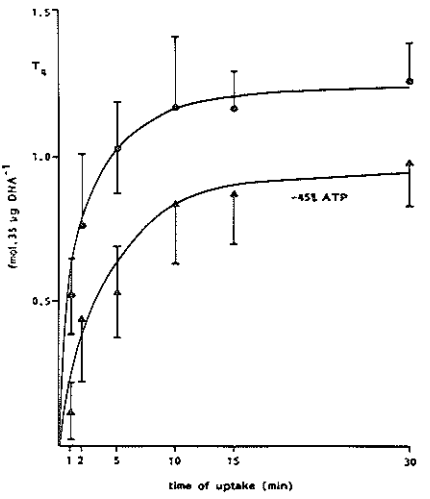


Fig. 2 Effect of intracellular ATP on total T_4 uptake (FT_4=0.1 pM) by cultured rat hepatocytes at 37 °C (mean ± SEM). At equilibrium, difference in uptake (20% versus control) remains significant (p<0.005)

equilibrium conditions prevail. It was therefore important to study the effect of intracellular ATP on the magnitude of uptake of thyroid hormone in equilibrium conditions. In Fig. 2 this relationship is shown with regard to the uptake of T_4 showing that a decrease of ATP concentration by 45% results in a permanent decrease of total cellular uptake.

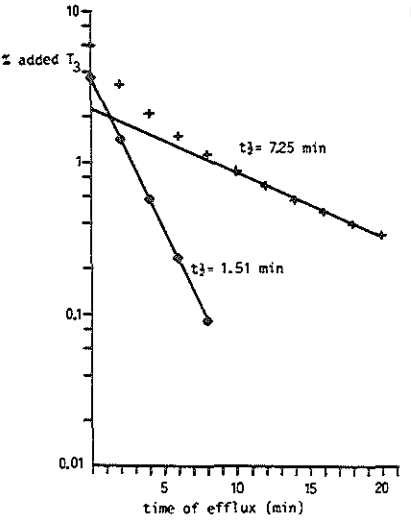
Efflux of Thyroid Hormones from Rat Liver Cells

Methods

Rat hepatocytes were obtained by the collagenase perfusion technique and about $2 \cdot 10^6$ cells ($\approx 35\,\mu g$ DNA) were cultured for at least 4 h, as described previously (Krenning et al. 1981). To study the release of cell-associated thyroid hormone, monolayers were incubated with [^{125}I]T_3 or [^{125}I]T_4 in concentrations of 0.5 and 54 nM free T_3 or 0.07 nM free T_4 in 4 ml incubation medium including 6.7 mM glucose and 0.5% bovine serum albumin (BSA) (Krenning et al. 1981). Incubation at 37 °C was carried out for 30 min, at which time equilibrium was reached. Cells were then washed twice with incubation medium without BSA. Efflux was measured in incubation medium containing BSA in concentrations ranging from 0 to 0.5%. To avoid appreciable re-uptake of hormone once entered the medium, efflux medium was replaced by fresh medium every 2 min. In order to characterize the first efflux component (vide infra) renewal of medium was performed in some experiments every 1/2 min during the first 2 min. The efflux curve was obtained by cumulative measurement of ^{125}I activity in the media in relation to time. High performance liquid chromatography of extracellular medium showed that under all conditions tested metabolism of [^{125}I]T_3 and [^{125}I]T_4 was negligible (data not shown). To study the potential role of intracellular ATP on efflux, cells were exposed in a preincubation for 30 min at 37 °C as well as in subsequent incubations to medium without glucose. This resulted in a diminution of ATP concentration by 40%. The data were analysed in semi-logarithmic diagrams. Best fit was obtained by inferring three linear, i.e. first order components. The half time of the faster phases were corrected for the contribution of the slower phases. Monolayers were also preincubated with [^{125}I]BSA for 30 min at 37 °C and release was measured similary as for T_3 and T_4. Statistical analysis was performed with Student's t-test. Data are the mean ± SD of n experiments, each with at least 3 monolayers per data point.

Results and Discussion

A typical efflux curve obtained is depicted in Fig. 3 for T_4 and T_4. Analysis of these uptake curves resulted in 3 components, the half times of 2 of these being illustrated in the figure. The component with the shortest half life (t½ 0.36 min for both T_3 and T_4, not depicted in the figure) probably



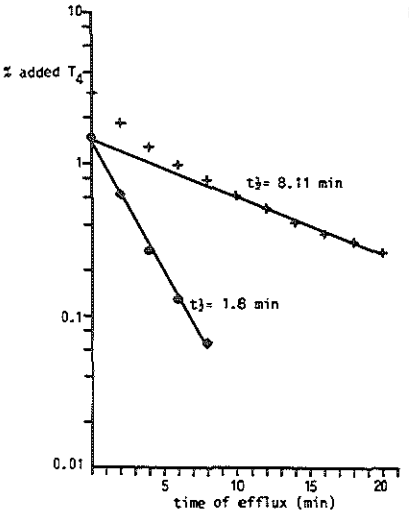


Fig. 3 T_3 (left panel) and T_4 (right panel) efflux from cultured rat hepatocytes: Percentage of added T_3 and T_4 released as function of time. Analysis revealed the presence of 3 efflux components. First component with t½ of 0.36 min (for both T_3 and T_4) is not shown

represents hormone bound to albumin in the water layer around the cell since it is similar to the t½ of the rapid efflux phase of albumin (Fig. 4). The component with a half time of 1.51 min for T_3 and 1.6 min for T_4 (fast phase) probably represents thyroid hormone bound to the outer cell surface. The component with the longest half times (slow phase) (T_3 7.25 min and T_4 8.11 min) represents

T_3 or T_4 efflux from the intracellular compartment. It appeared (not shown) that of the T_3 initially bound to the nucleus, 95% disappeared during an efflux period of 20 min.

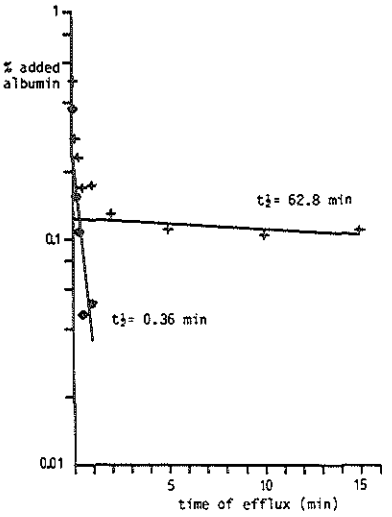


Fig. 4 Albumin release from cultured hepatocytes. Analysis disclosed 2 efflux components

In Table 3 results are given of studies where the effect of intracellular ATP decrease is measured on the fast and the slow phase with regard to T_3. As can be seen a decrease in intracellular ATP by 40% did not significantly affect the half times of both components. In Table 4 the results are shown of studies related to the question whether efflux of T_3 is a saturable process. Cells were loaded with T_3 at concentrations of 0.5 nM and 54 nM free T_3. No saturability of efflux for these concentrations of thyroid hormone was seen since the t½ of the slow phase and the rapid phase were not different under these circumstances. The effect of different albumin concentrations in the efflux medium on T_3 efflux is given in Fig. 5. The t½ values decreased with increasing BSA concentration from 0 to 0.1% and remained constant with higher BSA concentrations. The effect of the BSA concentration was particularly pronounced for the slow phase. These findings show that albumin has a permissive function on the efflux of thyroid hormone probably by facilitating the diffusion of thyroid hormone through the water layer around the cells.

Table 3 Effect of intracellular ATP(-40%) on efflux of T_3 from cultured rat hepatocytes (mean ± SEM)

	control n = 9	-40% ATP n = 2	
t½ fast phase (min)	1.3 ± 0.17	1.8 ± 0.007	NS
t½ slow phase (min)	7.6 ± 0.41	6.3 ± 0.25	NS

Table 4 Effect of T_3 loading on T_3 efflux from cultured rat hepatocytes. Loading concentrations 0.5 nM and 54 nM free T_3 (mean ± SEM)

	0.5 nM free T_3 n = 9	54 nM free T_3 n = 2	
t½ fast phase (min)	1.3 ± 0.17	1.3 ± 0.35	NS
t½ slow phase (min)	7.6 ± 0.41	5.8 ± 0.65	NS

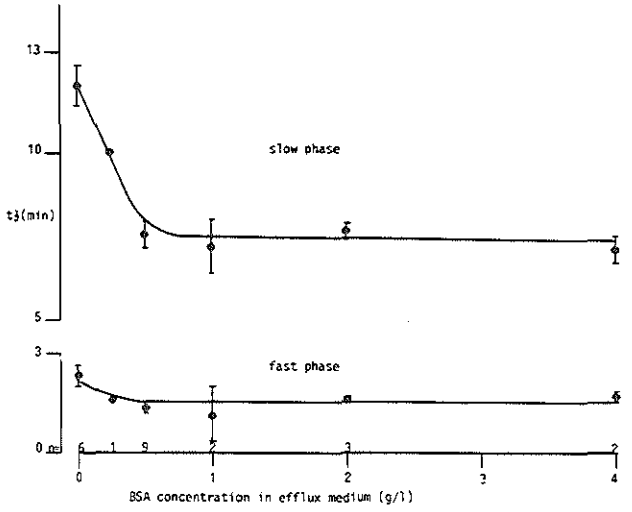


Fig. 5 Effect of bovine serum albumin (BSA) in efflux medium on efflux of T_3 from cultured rat hepatocytes (mean ± SEM)

Studies with Regard to the Possible Physiologic Significance of the Active Transport System

If the active transport system for thyroid hormones has physiological significance then it is expected that blockade of this uptake system would result in decreased intracellular concentration and notably, attenuated occupancy of nuclear bound thyroid hormone. Rat hepatocytes in primary culture were preincubated for 30 min with 0.5 mM ouabain and total and nuclear bound thyroid hormone (1.8 M Sucrose and Triton X-100 treated nuclei) were measured after 1 and 2 hrs of uptake of T_3 in the presence of the same concentration of ouabain. Previous studies (Krenning et al. 1981) showed that ouabain inhibits uptake of T_3 and T_4 probably because the uptake system needs a sodium gradient over the plasma membrane. The results are shown in Table 5. It can be seen that ouabain effects a decrease of total uptake of ≈ 30% after 1 and 2 h. Moreover a more substantial decrease with regard to nuclear binding is seen amounting to 50 and 41% respectively (ouabain does not influence nuclear receptor affinity or maximal binding capacity). The decrease in total uptake, although substantial, is less than for nuclear uptake and may be explained by the fact that ouabain does not affect the binding of thyroid hormone to the outer cell surface (Krenning et al. 1981).

Table 5 Percent inhibition of T_3 uptake in rat hepatocytes by 0.5 mM ouabain (mean ± SEM)

	total uptake n = 4	nuclear uptake n = 4
1 hr	29 ± 3.5**	50 ± 4.4**
2 hrs	30 ± 7.4*	41 ± 5.2**

p value versus control: *<0.05, **<0.005

Conclusions

From our experiments concerning the cellular uptake of thyroid hormones and from other studies (Eckel et al. 1979; Cheng et al. 1980) it is concluded that there exists an energy-dependent, carrier mediated uptake system for thyroid hormones. T_4 and reverse T_3 are probably translocated over the plasma membrane by the same route which is different from that of T_3. The K_m values of the transport systems are too high to be of any limiting value in the entry of thyroid hormone in physiological and patho-physiological situations. The studies on the effect of ATP suggest that also in equilibrium states the intracellular ATP concentration may have a regulatory function on the amount of hormone taken up by the cells. The investigations of the efflux of thyroid hormone do not point to ATP dependency or saturability (within the physiological concentration of free hormones) of the system. Efflux of thyroid hormone is therefore probably a passive process in which the intracellular free hormone concentration is the driving force. It is suggested that the efflux process of thyroid hormones from rat hepatocytes is not a site for the regulation of intracellular thyroid hormone metabolism and the ultimate bioavailability of thyroid hormones. The in vitro studies with regard to the effect of ouabain on the uptake of thyroid hormones suggest that the energy dependent carrier mediated uptake process may be of influence in the intracellular bioavailability of hormone and therefore may be a factor in the regulation of overall thyroid hormone activity. Other studies on the physiological importance of the carrier-mediated transport process are in line with our findings (Halpern and Hinkle 1982; Horiuchi et al. 1982; Cheng 1983). It should be realised that the information with regard to the regulation of influx of thyroid hormone into cells is obtained from in vitro studies. In vivo studies related to this aspect are of prime importance before any definite conclusions can be drawn.

In the light of the possible regulatory activity of intracellular ATP on uptake of thyroid hormones, it is noteworthy that under circumstances of starvation, disease states like anaemia, diabetes and circulatory shock and even thyrotoxicosis, lowered intracellular ATP concentrations have been found (Krenning et al. 1983). It is therefore feasible that intracellular ATP may appear to be of importance in the regulation of extrathyroidal T_3 production and may, apart from decreased $T_4 \rightarrow T_3$ conversion, play a role in the generation of the so-called low T_3 syndrome. In this way a decrease of intracellular ATP acts as a self-regulatory defence mechanism with regard to energy expenditure under the influence of thyromimetic substances.

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Chapter VI

Carrier-mediated transport of thyroid hormones into rat hepatocytes is rate-limiting in total cellular uptake and metabolism

CARRIER-MEDIATED TRANSPORT OF THYROID HORMONE INTO RAT HEPATOCYTES IS RATE-LIMITING IN TOTAL CELLULAR UPTAKE AND METABOLISM

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ABSTRACT: We investigated if carrier-mediated transport into rat hepatocytes is rate limiting in total cellular uptake and metabolism of thyroid hormone. Rat hepatocytes in primary monolayer culture were incubated under equilibrium conditions with tracer T_4, T_3 or rT_3 in the absence or presence of inhibitors of thyroid hormone uptake, i.e. ouabain and ER-22, a monoclonal antibody against the rat hepatocyte plasma membrane. The results for all three iodothyronines show that inhibition of clearance from the medium during incubation is paralleled by a similar decrease in iodide production. This indicates that the decrease in metabolism of thyroid hormone is directly related to the inhibition of cellular uptake. These findings underline the potential importance of the plasma membrane in the regulation of thyroid hormone metabolism and, therefore, determination of expression of thyroid hormone activity.

It has been thought that thyroid hormone enters target cells solely by passive diffusion (1,2). However, in recent years reports from several laboratories strongly suggest that carrier-mediated transport is involved in uptake into tissue cells. Thus, Rao et al. and we independently showed that 3,3',5-triiodothyronine (T_3) (3-5), T_4 (6,7) and rT_3 (6,7) are taken up by rat hepatocytes in vitro by a carrier-mediated, energy-dependent process. Similar transport processes for thyroid hormones have been subsequently reported to exsist for other cell types as well. It was found in mouse fibroblasts, that T_3 was bound to a saturable plasma membrane protein and subsequently internalized (8). Further studies demonstrated that uptake into these cells was also energy-dependent (9). Specific uptake of T_3 was also present in human epitheloid carcinoma cells, Chinese hamster ovary cells (9) and rat pituitary tumor cells (10,11). Affinity-labeling studies revealed similar characteristics of the plasma membrane carrier protein involved in T_3 transport in human, rat and mouse cultured cells (12). Similar properties with regard to affinity were found for rat hepatocytes

and human fibroblasts (13).

Evidence that in vivo transport of thyroid hormones into tissues is not a passive phenomenon either, has been obtained in several studies. In rats subjected to a nutritionally deficient diet, diminished cellular entry of thyroid hormone was found (14), possibly induced by altered membrane fluidity (15). In humans, prednisone slowed the acute disappearance of injected T_4 (16). We showed that during caloric deprivation in obese subjects transport of thyroid hormone into tissues, especially that of T_4, is inhibited (17). The results of this last study indicate that diminution of T_4 transport could at least in part, if not fully, explain low T_3 production in these circumstances. These studies suggest that inhibition of transport of thyroid hormones into cells has ultimate repercussions on thyroid hormone metabolism and bioavailability. One might speculate that if this inhibition of uptake is due to regulation of transport at the level of the plasma membrane, the specific transport mechanism involved should be rate-limiting in total cellular uptake and metabolism. To further investigate the significance of transmembranal transport, rat hepatocytes in primary monolayer culture were incubated in the absence or presence of inhibitors of thyroid hormone uptake, i.e., ouabain and a monoclonal antibody directed at the cell membrane of the rat hepatocyte (18). Under these conditions, inhibition of clearance of T_4, T_3 and rT_3 from the incubation medium could be measured, as well as the effect of inhibition of clearance on the production of metabolites of these iodothyronines.

MATERIALS AND METHODS

Reagents. [3',5'-^{125}I]T_4 and [3'-^{125}I]T_3 were purchased from The Radiochemical Centre, Amersham, UK. [3',5'-^{125}I]rT_3 was prepared by radioiodination of 3,3'-T_2 with the chloramine-T method and subsequently purified on Sephadex LH-20 (19). [3'-^{125}I]T_3-sulphate (T_3S) was prepared from [3'-^{125}I]T_3 as previously described (20).

Monoclonal antibody ER-22 was prepared against the rat hepatocyte plasma membrane in our laboratory. In other experiments (18) it inhibited T_4 and T_3 uptake into rat hepatocytes in a dose-dependent fashion (>90% uptake inhibition of both iodothyronines at a dilution of 1:100). The antibody was prepared in a partially purified form by ammoniumsulphate precipitation. ER-22 recognized a membrane

protein with a mol wt of 52,000.

In vitro measurement of deiodination. Possible inhibition of deiodination by ER-22 was measured as previously described (21). Production of $^{125}I^-$ from [^{125}I]rT_3 by rat liver microsomes in the presence of ER-22 was compared with that of microsomes alone, and expressed as % of added rT_3.

Animals. Adult male Wistar rats (150-300 g bodyweight) were used for all experiments.

Isolation and culture of hepatocytes. These techniques have been previously published by us (5,6).

Incubations. Monolayers of $1x10^6$ cells were incubated in 2 ml culture medium containing 0.5% BSA and tracer; approximately 3.0 pM of free [^{125}I]T_4, [^{125}I]T_3, [^{125}I]rT_3 or [^{125}I]T_3S. Incubations were done in the absence (control) or presence of ER-22 (dilution 1:1000) or 5 mM ouabain. Incubations using T_4, T_3 and T_3S tracer were performed at 37 C for 20 h and with rT_3 tracer for 90 min. All incubations were performed in the dark to minimize spontaneous deiodination. Dishes without cells but otherwise similarly processed served as blanks to correct for spontaneous deiodination.

Analysis of medium. After incubation, medium was isolated and analyzed for iodide, iodothyronines and iodothyronine conjugates with small Sephadex LH-20 (1 ml bed volume) columns, equilibrated with 0.1 N HCl. To 0.5 ml of medium, 0.5 ml 1 N HCl was added, and this mixture was applied to the column. Elution was effected with 0.1 N HCl, water and 1% ammonia in ethanol as solutions. After elution, collected fractions were counted. Good separation was achieved between iodide, conjugates (glucuronides plus sulphates) and iodothyronines. It appeared by HPLC that more than 94% of the iodothyronine fraction consisted of the tracer used in the experiment. Analysis of medium was not carried out when more than 20 % of hepatocytes did not exclude tryphan blue at the end of incubation (22), which happened only occasionally. Another criterion of viability was that at the end of incubation with or without ER-22 and ouabain cells still kept their capability to conjugate, a process which is sensitive to cell damage, as it is ATP

Table 1. Distribution of residual iodothyronine, derived iodide and iodothyronine conjugates in medium after incubation of labeled T_4, T_3 and rT_3 with rat hepatocytes in monolayer culture in the absence (control) or presence of uptake inhibitors ER-22 and ouabain.

	percentage mean ± SEM		
	Iodothyronine	Iodide	Conjugates
T_4			
control	82.9±0.8	12.7±0.4	1.9±0.1
ER-22	90.7±1.2*	6.9±0.6*	1.3±0.3**
ouabain	92.2±1.2*	7.0±0.6*	2.3±0.3
T_3			
control	32.0±1.4	51.5±0.6	10.3±0.6
ER-22	64.8±1.4*	24.6±0.6*	9.8±0.6
ouabain	66.2±1.4*	21.5±0.6*	10.6±0.6
rT_3			
control	45.8±0.9	54.1±0.5	1.8±0.2
ER-22	62.8±1.1*	36.9±0.7*	1.9±0.2
ouabain	56.8±1.6*	41.0±0.9*	1.7±0.3

* Significantly diffent from control, $P<0.001$, ** $P<0.005$.
Results are means of 2-6 experiments carried out at least in duplicate. SEM derived from analysis of variance.

dependent. Finally, attachment to the dish only occurs if the cells are in good condition. Radioactivity of the 3 fractions was expressed as percentage radioactivity applied to the column. As in the experiments cell-associated radioactivity was below 5 % of total activity added to the dish, the percentage distribution of iodothyronine and metabolites in the medium, in fact, approximates percentage of total radioactivity added.

Table 2. Distribution of residual T_3S, derived iodide and T_3 in medium after incubation of labeled T_3S with rat hepatocytes in monolayer culture in the absence (control) or presence of ouabain.

	percentage mean ± SEM		
T_3S	Iodothyronine	Iodide	Conjugates
control	0.4±1.2	49.1±0.5	50.2±2.7
ouabain	1.7±0.3	15.8±1.0*	81.6±3.1*

* Significantly diffent from control, $P<0.001$, ** $P<0.005$.
Results are means of 3 experiments carried out at least in duplicate. SEM derived from analysis of variance.

Free hormone concentrations in the incubation media were measured by equilibrium dialysis (23).
Statistical analysis of the results was performed with one way analysis of variance (24).

RESULTS

The distribution of residual iodothyronine and derived iodide and iodothyronine conjugates after incubation of rat hepatocytes with labeled T_4, T_3 and rT_3 in the absence (control) or presence of ER-22 or ouabain is shown in Table 1. It can be seen that for all 3 iodothyronines both ER-22 and ouabain inhibited clearance of iodothyronine and caused a decrease in iodide production of similar magnitude as the effect on clearance. That conjugate concentration was only slightly diminished (T_4) or unchanged (T_3 and rT_3) is caused by the fact that after release from the cells, uptake of conjugates by the hepatocytes is also inhibited by ouabain or ER-22. This phenomenon is examplified in Table 2, in which inhibition of clearance of T_3S from the medium by ouabain is

Table 3. Equilibrium dialysis of T_4, T_3 and rT_3 in culture medium with 0.5 % BSA and in the presence of ER-22 or ouabain.

Medium	% free hormone		
	T_4	T_3	rT_3
culture medium	0.24±0.03	2.45±0.27	0.79±0.05
" + ER-22 1:25	0.21±0.01	2.43±0.39	0.80±0.05
" + 5 mM ouabain	0.22±0.01	2.79±0.58	0.87±0.06
expts	4	10	4

Mean ± SD (4-10 experiments in duplicate)

shown, with concomitant decrease in I^- release. Deconjugation of T_3S to T_3 hardly takes place under these circumstances.

In Table 3 results of the free hormone determinations in the media are summarized. It appeared that addition of ER-22 or ouabain to the medium did not cause a significant change in free T_4, T_3 or rT_3.

From the data presented in Table 4 it can be concluded that ER-22 does not affect deiodination of rT_3 by rat livermicrosomes in vitro.

DISCUSSION

T_4 has little if any intrinsic biologic activity, and most if not all thyroid hormone activity is attributed to T_3 (25). About 80 % of total T_3 production in rats and in humans is from peripheral T_4 monodeiodination (26,27). It is obvious that T_4 has to be transported into tissues to serve as a substrate for T_3 production. It has been shown that in the liver the larger proportion of nuclear T_3 is derived from plasma (28,29), whereas in the pituitary and central nervous system 50 and 80 % of nuclear T_3 is derived from local T_4 deiodination, respectively (28). From these notions it follows that if regulation of T_4 and T_3 transport into tissue cells occurs, this process may be of great significance in the ultimate determination of availability of bioactive thyroid hormone. In this light it seemed important to study if carrier-mediated transport has a rate-limiting role in total cellular iodothyronine uptake (see also introduction). Our studies reported here indeed strongly support this concept. Thus, we found (Table 1 and 2), for all iodothyronines studied and

Table 4. I^- production from rT_3 by rat livermicrosomes.
Microsomes (5 ug protein) were preincubated with buffer, ER-22 (0.5 μg IgG) or normal mouse IgG (0.5 μg). Then I^- production from rT_3 (0.4 uM; total reaction mixture 300 μL) was measured after 20 min incubation at 37 C. Mean I^- production in controls was taken as 100 % and results with ER-22 and normal IgG are expressed as % of control.

Test condition	expt 1 n	expt 1 % I^-	expt 2 n	expt 2 % I^-
Control	16	100±9.0	7	100±1.2
ER-22	6	104±7.7	4	99±0.7
Normal IgG	4	94±8.5	4	103±1.6

n = number of incubates; Mean±SD

T_3S, that if uptake, i.e., clearance by hepatocytes, was inhibited, metabolite production, i.e., iodide release decreased to the same extent. The decrease in iodothyronine clearance could not be explained by a decrease in free hormone concentration by ouabain or ER-22 (Table 3), neither could the decrease in iodide production (i.e., deiodination) be explained on the basis of a direct effect on the deiodinase per se by ouabain or ER-22, as tested on rat liver microsomes (Table 4). Finally, the decrease in uptake and metabolism of thyroid hormone during incubation with ER-22 or ouabain was not due to loss of cell viability (see Methods).

In conclusion, our studies with inhibitors of uptake of thyroid hormones into rat hepatocytes demonstrate clearly that carrier-mediated uptake is rate limiting on total cellular uptake and metabolism. These findings underline the potential importance of this transport process in the regulation of expression of thyroid hormone activity in tissues.

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Chapter VII

Active transport of iodothyronines into human cultured fibroblasts

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Active Transport of Iodothyronines into Human Cultured Fibroblasts*

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ABSTRACT. Thyroid hormone uptake into human cultured fibroblasts was studied using 2-min incubations with labeled iodothyronines. The results indicate the presence of an active T_4 uptake process with two saturable sites with apparent K_m values of 1.9 and 141 nM, respectively, and an active T_3 uptake process with two saturable sites with K_m values of 29 and 650 nM. The uptake of both hormones was energy dependent, *i.e.* inhibited by KCN or by incubation of the cells in the absence of glucose. By analogy with similar findings in rat hepatocytes we postulate that the high affinity systems represent active transport of thyroid hormone into the cell. Preincubation of the cells with 2 mM ouabain resulted in a decrease in the uptake of both T_3 and T_4, suggesting that a sodium gradient is necessary for transport. Similar to that in rat hepatocytes, uptake of T_3 was inhibited by high concentrations of T_4, and uptake of T_4 was inhibited by high concentrations of T_3. These data indicate that regulation of thyroid hormone uptake at the level of the plasma membrane may be operative in humans. (*J Clin Endocrinol Metab* 65: 624, 1987)

ALTHOUGH T_4, the main secretory product of the thyroid gland, may have some intrinsic biological activity, it is considered predominantly as a prohormone, which becomes activated by intracellular 5′-deiodination (1, 2). This deiodination of T_4 occurs in peripheral tissues and results in the formation of T_3, the principal bioactive thyroid hormone (1, 2). Only about 20% of the total daily T_3 production directly originates from the thyroid; the remainder is generated by peripheral conversion of T_4 to T_3 (1–3).

To reach the nuclear receptors (4) and deiodinating enzymes, which are located in the endoplasmic reticulum (5), thyroid hormones have to be transferred from the extracellular compartment through the plasma membrane into the cell. This translocation has been thought to be a passive diffusion-controlled process (6). However, in recent years increasing evidence has been compiled by us and others that thyroid hormone accumulation in rat hepatocytes is an energy-dependent and saturable process (7, 8), which is inhibited by a monoclonal antibody directed against a plasma membrane protein (9). A similar active transport mechanism has been found in cultured GH_3 cells (10, 11) and cultured mouse fibroblasts (12). Data concerning iodothyronine transport processes into human cells are scarce, although Holm and coworkers (13) reported the existence of an energy-dependent saturable uptake system in cultured human lymphocytes. It is important to know if transmembrane transport of thyroid hormones is a more general process. If active iodothyronine transport is a common characteristic of human cells and organ systems, such transport may have an important influence on the regulation of thyroid hormone metabolism and, therefore, on the intracellular availability of hormone. We report here on the kinetic parameters of T_4 and T_3 uptake in human cultured fibroblasts. In addition, the influence of different circumstances and various compounds known to inhibit thyroid hormone uptake in hepatocytes (7) were tested. A preliminary account of this work has been published (14).

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Materials and Methods

Materials

Piperazine-*N,N′*-bis-[2-ethane sulfonic acid] (PIPES), HEPES, and *N,N*-bis-[2-hydroxy-ethyl]2-amino ethane sulfonic acid (BES) were purchased from Sigma Chemical Co. (St. Louis, MO). 3,5-[3′-^{125}I]triiodothyronine and [3′-^{125}I]T_4 (both SA, >1200 μCi/μg) were obtained from the Radiochemical Centre (Amersham, United Kingdom) and their purity was verified by high pressure liquid chromatography (15). *d*-Propranolol was a generous gift of I.C.I. Holland (Rotterdam, The

Netherlands). The sources of all other materials were described previously (16, 17).

Methods

Cell culture. Normal human fibroblasts were obtained from the Department of Cell Biology and Genetics, Medical Faculty, Erasmus University (Rotterdam, The Netherlands). Cell lines from eight normal subjects were used between passages 9–16. The data are reported as the mean of results obtained with different cell lines, unless stated otherwise. Fibroblasts were grown in Ham's F-10 culture medium, containing 10.6 mM PIPES, 11.2 mM BES, 8.9 mM HEPES, 12 mU/ml insulin, 2 mM $CaCl_2$, 10 U/mL penicillin/streptomycin, and 10% fetal calf serum, pH 7.4, at 37 C in air.

Incubation procedure. At confluence the cells were harvested by trypsinization (10-min incubation at 37 C with 1 mL/75 cm^2 0.25% trypsin in phosphate-buffered saline, pH 7.4). After one wash with culture medium, the cells were diluted to 2.5×10^5 cells/mL in culture medium and plated in six-well cluster dishes (2 mL/well). After 48-h incubation at 37 C in air, when the wells contained about 10^6 fibroblasts, the cells were washed with 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, 0.5% BSA, pH 7.4) and incubated at 37 C in triplicate for the indicated period of time with tracer iodothyronine, (total [^{125}I]T_3 concentration used, 150 pM, resulting in a free concentration in 0.5% BSA of 2.1 pM; total [^{125}I]T_4 concentration used, 90 pM, resulting in a free concentration in 0.5% BSA of 0.2 pM) with or without unlabeled iodothyronine or other compounds to be tested in 1 mL of the same medium. Uptake experiments were terminated by removing the supernatant and washing the monolayer with 2 mL incubation medium without albumin.

Diffusion was determined by measuring uptake at a very high concentration of free T_3 (18 μM). At this high concentration the contributions of the saturable processes were negligible. Since diffusion is linearly related to the free hormone concentration, it could be calculated at each hormone concentration used. To correct total uptake for diffusion, which is necessary for the calculation of specific uptake kinetics (17), diffusion was subtracted from total cell-associated radioactivity.

Cell-associated radioactivity and DNA content (18) were measured after lysis with 0.1 M NaOH. Free hormone concentrations were estimated by equilibrium dialysis (19). Statistical analysis of the data was performed with a one-way analysis of variance (20).

Results

As illustrated in Fig. 1, uptake of T_3 at 37 C was almost linearly related with time up to 2 min. To measure initial uptake kinetics we calculated K_m and maximum velocity (V_{max}) after 2-min incubation of 10^6 cells with various T_3 concentrations, as outlined previously for rat hepatocytes (17). Figure 2 shows the double reciprocal plot of T_3 uptake. Two saturable uptake processes appeared to be present, and the kinetic parameters of these two processes are summarized in Table 1. From Fig. 1 it is clear that uptake was temperature dependent, being lower at 22 C than at 37 C, and that preincubation of the cells with medium devoid of glucose lowered T_3 uptake. Furthermore, it appears that uptake of hormone by diffusion contributed only minimally to total uptake (Fig. 1).

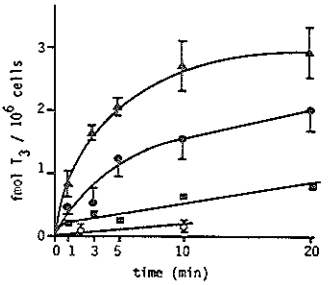

FIG. 1. Uptake of T_3 (free T_3 2.1 pM) by human cultured fibroblasts as a function of time at 37 C. Cells (10^6) were incubated with tracer T_3 in 1 mL incubation medium with 0.5% BSA. For details see *Materials and Methods*. ▲, Preincubation for 30 min with incubation medium including 0.5% BSA and 6.7 mM glucose (mean ±SEM; n = 4; two different cell lines). ●, Preincubation for 30 min with 0.5% BSA without glucose (mean ±SEM; n = 3; two different cell lines). ■, Uptake studies performed at 22 C (mean of two experiments with different cell lines). ○, Diffusion at 37 C at this free T_3 concentration (mean ±SEM; n = 6 different cell lines).

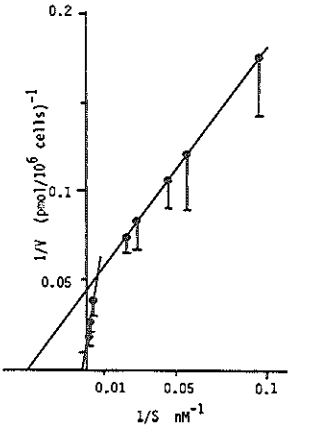

FIG. 2. Double reciprocal plot of 2-min T_3 uptake by human fibroblasts (V), after correction for diffusion, *vs.* the free T_3 concentration (S). Diffusion was measured at a free T_3 concentration of 18 μM, calculated for each free T_3 concentration and subtracted from the total cell-associated radioactivity. The results are the mean ±SEM from six different cell lines.

TABLE 1. Kinetic parameters of 2-min T_3 and T_4 uptake by cultured human fibroblasts at 37 C

	K_{m1} (nM)	V_{max1} (pmol/well)	K_{m2} (nM)	V_{max2} (pmol/well)
T_4	1.9 ± 0.6	10.9 ± 3.0	141 ± 33	306 ± 79
T_3	29 ± 7.3	22 ± 4.6	646 ± 150	156 ± 31
T_3[a]	Not measurable		429 ± 90	163 ± 50

Values are the mean ± SEM (six different cell lines; 10^6 fibroblasts/well; 8.5 ± 0.6 μg DNA/well).

[a] Measured in the presence of unlabeled T_4 (4.5 μM).

TABLE 2. Inhibition of 5-min thyroid hormone uptake into human cultured fibroblasts by various compounds or conditions

Compound or condition	Conc. (μM)	T_3 n	T_3 % Inhibition	T_4 n	T_4 % Inhibition
d-Propranolol	1	5	31 ± 5.5	3	37 ± 5.8
Ouabain	2000	4	39 ± 2.3	4	21 ± 3.8
KCN	100	3	76 ± 3.0	3	41 ± 8.8
Without glucose	0	4	26 ± 4.7	4	21 ± 7.9
Iodoacetic acid	10	4	34 ± 3.6	3	4 ± 5.0[a]
T_4 (free)	2.5	6	56 ± 2.6		
T_3 (free)	14			3	36 ± 6.5
22 C		3	76 ± 2.1	3	51 ± 6.1

Cells were preincubated for 30 min with the compound tested before uptake of tracer hormone was measured in the presence of the same compound. Addition of the compounds to the incubation medium in the given concentrations had no influence on the free hormone concentration. The results (percent inhibition of total uptake ± SEM) are the mean of triplicate experiments with cells of the indicated number (n) of cell lines. Inhibitions were significantly different ($P < 0.001$) unless indicated otherwise.

[a] P = NS.

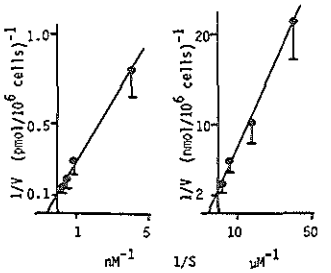

FIG. 3. *Left panel*, Double reciprocal plot of high affinity T_4 uptake (measured at 2 min) in human fibroblasts (V) *vs.* the free T_4 concentration (S). Correction for diffusion was performed as outlined for T_3 (free T_4, 4.5 μM). The results are the mean ±SEM from four different cell lines. *Right panel*, Magnification of the area near the origin of the *left panel*, showing the low affinity uptake system.

Uptake of T_4 by human fibroblasts was similarly dependent on time (curve not shown), except that equilibrium was reached between 5 and 10 min. Dependence of T_3 and T_4 uptake on temperature was also comparable (Table 2). Figure 3 shows the double reciprocal plot of T_4 uptake at various concentrations of free T_4. Here also, two saturable uptake systems were found, of which the kinetic parameters are summarized in Table 1.

Table 2 summarizes the studies with various compounds known to inhibit uptake of thyroid hormones into rat hepatocytes (16, 21). All of these compounds inhibited thyroid hormone uptake by human fibroblasts, except that T_4 uptake was not inhibited by iodoacetate. However, because the effect of this compound has not been studied in rat hepatocytes, we do not know whether this lack of inhibition is a real difference between human fibroblasts and rat hepatocytes.

Discussion

Our studies clearly demonstrate the existence of two saturable uptake processes for both T_3 and T_4 in human cultured fibroblasts. One system is characterized by high affinity and low capacity, and the other by low affinity and high capacity. The K_m and V_{max} values of the high affinity systems of T_4 and T_3 in these cells are similar to those reported previously for rat hepatocytes (22) (K_m of T_4, 1.9 *vs.* 1.4 nM for rat hepatocytes; K_m of T_3, 29 *vs.* 61 nM, respectively). There was a 4-fold difference between the K_m of T_3 uptake in human fibroblasts and that in human cultured lymphocytes (29 *vs.* 110 nM, respectively) (13), while the uptake systems of both cells can be inhibited by KCN (13). This difference could point to an intrinsic difference between the uptake mechanisms of thyroid hormones in these two cell types, but it also is possible that this difference is caused by methodological differences. The K_m in lymphocytes was measured under equilibrium conditions (after 60-min incubation with tracer and/or unlabeled hormone) (13), while we used initial velocity measurements.

The K_m and V_{max} of the low affinity T_3 system were about 4-fold lower than those in rat hepatocytes. Previously, we reported that for rat hepatocytes this low affinity system represented binding of thyroid hormone to the outside of the plasma membrane (17, 23). The low affinity systems for T_3 and T_4 in rat hepatocytes could not be influenced by metabolic inhibitors or coincubation with high concentrations of T_4 and T_3, respectively, while in these situations the high affinity systems were completely blocked (16, 17). Although we have not systematically tested the high and low affinity systems in fibroblasts, similar effects of T_4 on low and high affinity T_3 uptake in human fibroblasts were found. If we assume that in human fibroblasts low affinity uptake also represents binding of thyroid hormone to the cell surface, then from the difference in V_{max} of the low affinity system of T_3 between fibroblasts and hepatocytes, it seems that a much greater part of cell-associated hormone is located inside the fibroblasts compared to that in rat

hepatocytes. From the measured K_m and V_{max} values it can be calculated that at the tracer concentrations of iodothyronines used, 75% of the cell-associated radioactivity was accounted for by the high affinity system, 25% by the low affinity system, and only a small fraction by diffusion.

Iodoacetic acid inhibited uptake of T_3, but not that of T_4, which could indicate that different transport systems for these hormones are present in the plasma membrane, as we also found in rat hepatocytes (16). In the experiments where inhibition of uptake was studied, inhibition was expressed as percent change in total cellular uptake. T_3 and T_4 uptakes were strongly dependent on temperature; they were much lower at 22 C. It is known that diffusion is dependent on temperature, but because uptake by diffusion is small compared to total uptake at 37 C, any diminution of diffusion at 22 C cannot explain the much lower T_3 uptake at 22 C. This dependence of uptake on temperature was also found in rat hepatocytes (17).

From the data shown in Table 2 it cannot be concluded which uptake system, *e.g.* the low K_m or the high K_m system, was affected, but several findings point to the involvement of the low K_m system. Firstly, the data in Table 1 show that T_4 inhibited the low K_m system of T_3 uptake. Secondly, after inhibition of the high affinity, low K_m system for T_3 uptake with high doses of T_4, no further inhibition occurred after the addition of any of the compounds listed in Table 2 to the incubation medium, indicating that the low K_m system was indeed affected (data not shown).

We previously reported that T_4 and T_3 uptakes into rat hepatocytes by the high affinity system were dependent on the cellular ATP concentration (16, 21), and compounds that lowered cellular ATP inhibited uptake of thyroid hormones. Preincubation of hepatocytes with *d*-propranolol or KCN or in the absence of glucose lowered the ATP concentration and subsequently inhibited hormone uptake by the cells (21). Table 2 shows that the conditions that lower ATP and thyroid hormone uptake in rat hepatocytes inhibited thyroid hormone uptake in human fibroblasts, suggesting that a similar energy-dependent mechanism is operative.

A similar resemblance between fibroblasts and hepatocytes was found with ouabain. In both hepatocytes (16, 21) and fibroblasts thyroid hormone uptake was inhibited by this compound. As ouabain inhibits the activity of Na^+,K^+-ATPase, the importance of a sodium gradient over the plasma membrane in the transport process is apparent, analogous to transport of other amino acids (24).

Thus, there appears to be a striking similarity between the mechanism of uptake of thyroid hormone by human fibroblasts and that by rat hepatocytes. We recently found that in rat hepatocytes the transport process was rate limiting in total cellular uptake and metabolism (25). This finding underlines the potential importance of the transport system in the regulation of thyroid hormone metabolism and bioavailability.

We have found that transport of thyroid hormone into tissues in humans may be modulated by dietary manipulation (26). Specifically, T_4 transport into cells was diminished during caloric deprivation, leading to a lower intracellular T_4 pool and, consequently, lower extrathyroidal T_3 production due to diminished substrate availability for 5′-deiodinase. One explanation for this decreased tissue accumulation of T_4 could be depressed activity of membrane transport during caloric deprivation (26). The study reported here and that of others (13) indeed suggest that in human cells thyroid hormone uptake is an active process and, therefore, subject to regulatory factors.

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Chapter VIII

Inhibition of uptake of thyroid hormone into rat hepatocytes by preincubation with N-bromoacetyl-3,3',5-triiodothyronine

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Inhibition of Uptake of Thyroid Hormone into Rat Hepatocytes by Preincubation with *N*-Bromoacetyl-3,3′,5-Triiodothyronine

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ABSTRACT. To investigate whether affinity coupling of *N*-bromoacetyl-T_3 ($BrAcT_3$) to the T_3 membrane carrier results in an inhibition of transport of T_3 into the cell, rat hepatocytes in monolayer were incubated for 2 h at 21 C with 1.3 μmol/liter $BrAcT_3$ in medium without protein. After extensive washing, cells were incubated during 20 h at 37 C with [^{125}I]T_3 in medium with 0.5% BSA, and products in supernatants were analyzed by LH-20 column chromatography. In addition the apparent affinity constant (K_m) and maximal uptake velocity (V_{max}) of the high affinity uptake process were estimated using 1 min incubations of hepatocytes with various concentrations of T_3.

In control experiments (*i.e.* without $BrAcT_3$ affinity coupling) about 57% of the added T_3 was cleared from the medium and further metabolized, 86% of the cleared T_3 reappeared in the medium as I^-, 15% as conjugates. Addition of propylthiouracil during the 20 h incubation with T_3 strongly inhibited deiodination, without a change in T_3 clearance. Because T_3 is sulfated before deiodination, a concomitant rise in conjugates was observed. Addition of ouabain to control cells during the 20 h incubation with T_3 strongly inhibited uptake, with a parallel decrease in I^- and conjugate formation.

After affinity coupling of $BrAcT_3$, T_3 clearance was inhibited (by 30% $P < 0.001$). Since I^- production was more depressed (by 73%) than T_3 clearance, with some rise in conjugate formation ($P < 0.001$), inhibition of deiodinase by $BrAcT_3$ also took place. The effects of $BrAcT_3$ and ouabain on uptake of T_3 appeared to be additive as were the effects of propylthiouracil and $BrAcT_3$ on deiodination.

After affinity coupling of $BrAcT_3$, the K_m of T_3 uptake did not change significantly; however V_{max} was 54% lower ($P < 0.025$) indicating a noncompetitive inhibition of the transport system. Preincubation of the cells with *N*-acetyl-T_3 does not alter the characteristics of uptake of T_3 by rat hepatocytes as compared to controls, indicating that no binding of this compound occurs.

It is concluded that preincubation of hepatocytes with $BrAcT_3$ diminished I^- formation from T_3; 50% of this inhibition is due to decreased membrane transport and 50% by reduction of deiodination. Inhibition of membrane transport by $BrAcT_3$ is substantiated by a 54% lower V_{max} without a significant change in K_m as compared to control. The effect of transport of thyroid hormone on metabolism stresses the importance of the membrane carrier in the translocation process. (*Endocrinology* **123**: 1520–1525, 1988)

BINDING-site directed reagents such as *N*-bromoacetyl-T_3 ($BrAcT_3$) or *N*-bromoacetyl-T_4 ($BrAcT_4$) have provided information about the mechanism of binding of thyroid hormones to T_4-binding prealbumin (1) and T_4-binding globulin (2). Covalent binding of $BrAcT_4$ to a lysine residue in T_4-binding prealbumin (1), or to a methionine residue in the case of T_4-binding globulin (2), in or near the binding site occurs slowly (a few percent binding after 2 h of incubation) and can be diminished by coincubation with T_4. Reaction of $BrAcT_3$ with the T_3 binding site of the nuclear receptor is also slow (3). On the other hand, reaction of $BrAcT_3$ with the type I deiodinase of rat liver is extremely fast, being almost complete within 10 min (4). Binding to the enzyme is accompanied by a complete loss of activity, indicating that the active site of the enzyme is involved in the binding process.

Before thyroid hormones reach the nuclear receptor (5) and the deiodinating enzymes, which are located in the endoplasmic reticulum (6), they have to be transported from the extracellular compartment through the plasma membrane. In recent years increasing evidence has been accumulated (7, 8) that transport of thyroid hormone into rat hepatocytes is an energy-dependent and saturable process, which is inhibited by a monoclonal antibody directed against a plasma membrane protein (9). This inhibition leads to a greatly diminished clearance of thyroid hormone from the incubation medium by

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cultured hepatocytes, and a concomitant decrease in metabolism as estimated from production of I^- (10).

Recently, $BrAcT_3$ has been used as an affinity label of the T_3 plasma membrane binding protein of GH_3 cells (11) and of human placenta cells (12). The affinity labeling was found to be specific for a membrane protein of 55,000 daltons in GH_3 cells, and of 65,000 daltons in human placenta membranes. However, no direct correlation was shown between the affinity labeling of these membrane proteins and inhibition of uptake of T_3, although such an effect was hypothesized (12). Here we show that preincubation of cultured rat hepatocytes with $BrAcT_3$ results in inhibition of transport of T_3 into the cell, and, in addition, inhibition of deiodination.

Materials and Methods

Materials

$BrAcT_3$ and *N*-acetyl-T_3 (AcT_3) were synthesized and purified as previously described (4). The radioactive labeled compounds were made by substituting [3′-^{125}I]T_3 (specific activity > 1200 μCi/μg; the Radiochemical Centre, Amersham, U.K.) for unlabeled T_3. BSA, piperazine-*N*,*N*′-bis[2-ethane sulfonic acid], HEPES, *N*,*N*-bis[2-hydroxyethyl]-2-amino ethane sulfonic acid, and 2-bromoacetic acid (BrAc) were purchased from Sigma Chemical Co. (St. Louis MO). Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden). The sources of all other materials not mentioned here were described previously (13, 14).

Methods

Cell culture. Parenchymal cells were isolated from livers of male Wistar rats as previously described (14). Hepatocytes (2×10^6 cells) were incubated in culture medium (Ham's F-10, 10.6 mmol/liter piperazine-*N*,*N*′-bis[2-ethane sulfonic acid], 11.2 mmol/liter *N*,*N*-bis[2-hydroxyethyl]-2-amino ethane sulfonic acid, 8.9 mmol/liter HEPES, 12 mU/ml insulin, 2 mmol/liter $CaCl_2$, 10 U/ml penicillin/streptomycin, pH 7.4) with 10% fetal calf serum at 37 C during 4 h. The cells were then washed with rinsing medium (136.9 mmol/liter NaCl, 2.7 mmol/liter KCl, 8.1 mmol/liter Na_2HPO_4, 1.5 mmol/liter KH_2PO_4, 0.9 mmol/liter $CaCl_2$, 0.5 mmol/liter $MgCl_2$, 6.7 mmol/liter glucose, pH 7.4) and used for the experiments.

Preincubations. Cells were incubated for 2 h at 21 C with 1.3 μmol/liter $BrAcT_3$, AcT_3, or BrAc in culture medium without protein, and subsequently cells were washed extensively (six times 5 min) with culture medium with 0.5% BSA to remove any unreacted $BrAcT_3$, AcT_3, or BrAc.

Metabolic studies. Monolayers were preincubated as described and subsequently incubated during 20 h at 37 C with [^{125}I]T_3 (free T_3 concentration 2.1 pM; total concentration 100 pmol/liter) in culture medium with 0.5% BSA. Thereafter the supernatant was analyzed by LH-20 column chromatography. Because the medium contains 0.5% albumin, only a small amount (2–3%) of total added radioactivity is associated with the cells after incubation, and this amount has been neglected in the calculations.

Uptake studies. High affinity uptake of T_3 was measured as previously described (15). Monolayers of preincubated hepatocytes were incubated for 1 min at 37 C with rinsing medium with 0.5% BSA containing [^{125}I]T_3 and various concentrations of unlabeled T_3. Parallel incubations with the same T_3 concentrations in the presence of 100 μmol/liter T_4 were also performed. Calculation of transport parameters was based on the finding (13) that this high T_4 concentration completely inhibits high affinity T_3 uptake without inhibition of low affinity binding and diffusion. Therefore the difference in total T_3 uptake and uptake in the parallel incubations yields a measure of the active transport. This difference was plotted in a double reciprocal plot against the free T_3 concentration used, to obtain K_m and V_{max} values of the high affinity uptake process.

Efflux procedure. Efflux of T_3 from hepatocytes was measured as previously described (16), with modifications. Cells were preincubated during 2 h with $BrAc[^{125}I]T_3$, $Ac[^{125}I]T_3$, or [^{125}I]T_3 (±100,000 cpm/ml, equivalent to ~50 pmol/liter) at 7 C or 37 C. Subsequently the monolayers were washed briefly with rinsing medium, and incubated with culture medium with 0.5% BSA during 32 min. To avoid reuptake of labeled hormone once released in the medium, the culture medium was replaced by fresh medium every 4 min. Finally cell-associated radioactivity was determined. Efflux curves were constructed by plotting the logarithm of the cell-associated radioactivity (calculated from the cumulative radioactivity in the medium and the final cell-associated radioactivity and expressed as percent of the added tracer), against time. Best fit of the data points was obtained by the sum of two exponentials, *i.e.* as the sum of two first order components.

LH-20 chromatography. Columns 0.5 × 2 cm were prepared by pouring 2 ml of a 1:3 (wt/vol) slurry of Sephadex LH-20 in water in pasteur pipettes, plugged with some cotton wool, and equilibrated with 0.1 mol/liter HCl. One milliliter of incubation medium was acidified to pH 1.0 with HCl and applied to the column. $^{125}I^-$ was eluted from the column with 3 × 1 ml 0.1 mol/liter HCl. Subsequently the T_3 conjugates were eluted with 7 × 1 ml H_2O, and finally the iodothyronines were removed from the column with 3 × 1 ml 50% ethanol in 0.1 mol/liter NaOH. Radioactivity of the different fractions was expressed as percent of total radioactivity applied to the column.

Cell-associated radioactivity was estimated after lysis of the cells with 0.1 mol/liter NaOH. Free hormone concentrations of the media containing BSA were estimated by equilibrium dialysis (17). Statistical analysis of the data was performed with a one way classification, first performing an analysis of variance, followed by comparisons among class means (18), or with Student's *t* test (Table 1).

Results

Typical efflux curves obtained with cells preloaded with [^{125}I]T_3, $BrAc[^{125}I]T_3$, or $Ac[^{125}I]T_3$ are shown in Fig. 1. As can be seen the final efflux curve of T_3 can be described as the sum of two first order curves with a $t_{1/2}$

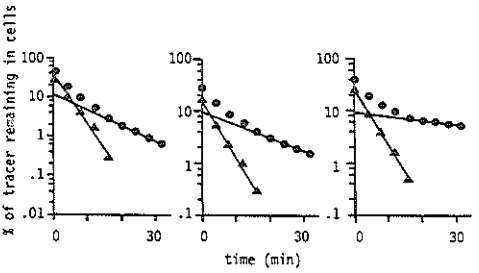

FIG. 1. Tracer efflux from cultured rat hepatocytes at 37 C, after the cells were preloaded with tracer during 120 min at 7 C. Percentage of added tracer remaining in the cells as a function of time. ●, Activity present in the cells at each time point, with the regression line on the final straight part of the curve. ▲, Activity in the cells minus the contribution of the slow phase with the regression line on the curve. *Left panel*, Efflux of [^{125}I]T_3, $t_{½}$ rapid phase 2.4 min, $t_{½}$ slow phase 7.8 min. *Middle panel*, Efflux of Ac[^{125}I]T_3, $t_{½}$ rapid phase 2.8 min, $t_{½}$ slow phase 11.4 min. *Right panel*, Efflux of BrAc[^{125}I]T_3, $t_{½}$ rapid phase 2.9 min, $t_{½}$ slow phase 31.4 min.

TABLE 1. Half-times of the rapid and slow phases of the efflux of $BrAcT_3$, AcT_3, and T_3 from rat hepatocytes, and the percent of tracer remaining in the cells after 32 min of efflux

	$BrAcT_3$ 37 C (n = 3)	$BrAcT_3$ 7 C (n = 5)	AcT_3 7 C (n = 5)	T_3 7 C (n = 5)
$t_{½}$ rapid phase (min)	4.8 ± 0.2[a]	3.0 ± 0.4	2.5 ± 0.3	2.8 ± 0.4
$t_{½}$ slow phase (min)	59.8 ± 4.1[b]	29.0 ± 6.3[b]	13.3 ± 2.0	12.2 ± 4.6
% in cells (32 min)	32.2 ± 2.5[b]	12.6 ± 2.9[b]	4.3 ± 1.0	3.3 ± 1.5

Cells were loaded during 2 h with tracer $BrAcT_3$, AcT_3, or T_3 at the indicated temperature. Thereafter efflux studies were performed at 37 C. Values are mean ± SD of the indicated number of experiments. Data were compared using Student's *t* test for unpaired groups (18).

[a] $P < 0.005$ *vs.* T_3.

[b] $P < 0.001$ *vs.* T_3.

of 7.8 and 2.4 min, respectively. The curves obtained with AcT_3 are similar to those with T_3. Incorporation of a bromine atom in AcT_3 does not affect the $t_{½}$ of the rapid phase, but markedly affects the $t_{½}$ of the slow efflux phase. Table 1 summarizes the results obtained with the efflux experiments with tracer $BrAcT_3$, AcT_3, and T_3.

Incubation of rat hepatocytes with tracer T_3 during 20 h at 37 C results in a 57% metabolism of the tracer, 85% of which reappears as iodide and 15% as conjugates in the medium (Table 2). Incubation of the cells with tracer T_3 and propylthiouracil (PTU) (100 μmol/liter) does not change the disappearance of T_3 significantly, but now only 20% of the metabolized tracer reappears in the medium as iodide and 79% as conjugates. Incubation of the cells with tracer T_3 and ouabain (1 mmol/liter) resulted in a 36% lower disappearance of T_3, without a relative change in the products formed. Preincubation of hepatocytes with $BrAcT_3$ resulted in a 30% lower disappearance of T_3; 66% of tracer T_3 cleared from the medium reappeared as conjugates and 33% as iodide (Table 2). PTU, if present during the 20 h incubation with tracer T_3 after preincubation with $BrAcT_3$, further stimulates the accumulation of conjugates, while ouabain only inhibits the disappearance of the tracer.

It appears that the effect of $BrAcT_3$ is concentration dependent (Table 2). The lower concentration of $BrAcT_3$ (*i.e.* 0.13 μmol/liter) had no effect on T_3 disappearance. Nevertheless, the production of iodide was reduced and conjugates increased. The results obtained with AcT_3 and BrAc (Table 2) show that both compounds do not have a persistent effect on T_3 metabolism in the concentrations used in the preincubation. It seems, therefore, that 1) the inclusion of a Br atom into AcT_3 is necessary to exhibit any effect, and 2) that the T_3 residue in $BrAcT_3$ is necessary to direct the reactive Br atom to its reaction site.

Figure 2 and Table 3 show the results of the measurement of the high affinity uptake of T_3 by hepatocytes preincubated with $BrAcT_3$ or AcT_3. Preincubation of the cells with AcT_3 does not significantly affect the uptake of T_3, in contrast to $BrAcT_3$, which compound significantly inhibits V_{max} without an influence on the K_m.

Discussion

We have previously argued that the component of the efflux curves with a $t_{½}$ of about 2.5 min (rapid phase) represents hormone bound to the outer cell surface, while the component with the longest half-time (slow phase) represents hormone efflux from the intracellular compartment (16). Because $t_{½}$ of the rapid phase is not significantly different between the cells loaded with $BrAcT_3$, AcT_3, or T_3 at 7 C, it can be concluded that $BrAcT_3$ bound to the outside of the cell is not covalently linked, and as easily removed as T_3 or AcT_3, which are not capable of covalent coupling to proteins under the circumstances used here. However, $t_{½}$ of the rapid phase is significantly longer in cells loaded with $BrAcT_3$ at 37 C. This is in contrast to the $t_{½}$ values after the cells are loaded with T_3 at 37 C [$t_{½}$ rapid phase 1.3 ± 0.17 min, $t_{½}$ slow phase 7.6 ± 0.41 min (16)], which are similar or even shorter as compared to loading at 7 C. The extension of the $t_{½}$ of both the rapid and the slow efflux phase, found after loading the cells with $BrAcT_3$ at 37 C as compared to 7 C, is therefore not due to the change in temperature *per se*, but indicates that under these circumstances covalent linking of $BrAcT_3$ to membrane protein has occurred.

Loading the cells with $BrAcT_3$, but not with AcT_3 or T_3, prolonged the $t_{½}$ of the slow phase of efflux significantly, both at 7 C and at 37 C. Incorporation of a bromine atom in AcT_3 markedly affects the $t_{½}$ of the

TABLE 2. Formation of I^- and conjugates from T_3 and disappearance of T_3 as measured in the incubation medium after 20 h incubation of rat hepatocytes with tracer T_3 ([FT_3]= 2.1 pmol/liter) at 37 C

Preincubation	Incubation with T_3	Production of		Disappearance of T_3
		Iodide	Conjugates	
Control		48.2 ± 1.2	8.3 ± 0.3	56.9 ± 1.3
	+Ouabain	28.7 ± 1.1[a]	6.3 ± 0.3[a]	36.2 ± 1.1[a]
	+PTU	10.6 ± 0.5[a]	42.3 ± 0.9[a]	53.7 ± 0.8[b]
$BrAcT_3$ (1.3 μmol/liter)		13.0 ± 0.5[a]	26.3 ± 0.7[a]	39.6 ± 0.9[a]
	+Ouabain	6.2 ± 0.5[c]	19.6 ± 1.5[c]	25.9 ± 1.7[c]
	+PTU	3.8 ± 0.5[c]	30.2 ± 0.6[c]	34.5 ± 1.1[d]
$BrAcT_3$ (0.13 μmol/liter)		35.6 ± 0.7[a]	19.6 ± 0.5[a]	58.8 ± 0.5[c]
AcT_3 (1.3 μmol/liter)		48.0 ± 1.2[c]	7.2 ± 0.1[b]	54.8 ± 1.2[c]
	+Ouabain	34.2 ± 0.8[a]	10.0 ± 1.6[c]	43.0 ± 1.6[a]
	+PTU	8.0 ± 0.5[a]	43.4 ± 1.2[a]	55.8 ± 1.5[c]
BrAc (1.3 μmol/liter)		46.5 ± 0.9[c]	8.2 ± 0.4[c]	54.7 ± 1.2[c]
	+Ouabain	34.2 ± 0.8[a]	9.5 ± 0.5[c]	41.5 ± 1.5[a]
	+PTU	6.2 ± 0.8[a]	40.4 ± 1.9[a]	50.4 ± 2.5[b]

Cells were preincubated during 2 h at 21 C with culture medium without protein in the absence or presence of 1.3 μmol/liter or 0.13 μmol/liter $BrAcT_3$, 1.3 μmol/liter AcT_3, or 1.3 μmol/liter Br-acetate (BrAc). See also *Materials and Methods*. Values are mean ± SEM in percent of added [^{125}I]T_3 (9 exps in triplicate).

[a] $P < 0.001$ *vs.* control.

[b] NS *vs.* control; $0.05 < P < 0.1$.

[c] $P < 0.001$ *vs.* $BrAcT_3$.

[d] NS *vs.* $BrAcT_3$; $0.05 < P < 0.1$.

[e] NS *vs.* control; $P > 0.1$.

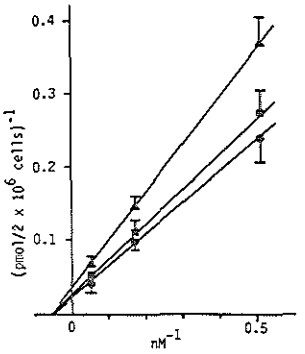

FIG. 2. Double reciprocal plot of the T_3 uptake (ordinate) against the free T_3 concentration in the medium (abcissa) by rat hepatocytes, corrected for diffusion and the low affinity uptake system. Data are the mean (±SEM) of six experiments performed in triplicate. ●, Cells preincubated with medium alone; ■, cells preincubated with AcT_3; and ▲, cells preincubated with $BrAcT_3$. See also Table 3.

TABLE 3. Characteristics of the uptake of T_3 by rat hepatocytes

	Preincubation with	
	K_m (nmol/liter)	V_{max} (pmol/2 × 10^6 cells)
Control	29 ± 9	83 ± 20
AcT_3	22 ± 4[a]	60 ± 14[a]
$BrAcT_3$	22 ± 5[a]	38 ± 12[b]

The effect of preincubation of the cells during 2 h at 21 C with culture medium without protein in the absence or presence of 1.3 μmol/liter $BrAcT_3$, or 1.3 μmol/liter AcT_3. See also *Materials and Methods*. Values are means ± SEM (6 exps).

[a] NS different from control; $P > 0.2$.

[b] $P < 0.025$ *vs.* control.

slow efflux phase. This finding could be an indication for coupling of $BrAcT_3$ to intracellular proteins as was recently shown to occur in GH_3 cells (11). Binding of $BrAcT_3$ to the nuclear envelope and endoplasmic reticulum of human carcinoma cells (19) and to rat liver microsomes, leading to the inactivation of 5′-deiodinase (4), has also been reported.

From the results in Table 2 it can be concluded that ouabain, a known inhibitor of uptake of T_3 (13), inhibits the disappearance of T_3 from the medium with a concomitant diminution of the production of I^- and conjugates. This indicates that intracellular metabolism is altered, due to only inhibited transport of T_3 over the plasma membrane. If PTU, a known inhibitor of liver 5′-deiodinase both in homogenates (20) and in cultured hepatocytes (21), is included in the incubation medium, no significant change in the disappearance of T_3 is observed, but now mainly conjugates are formed, and I^- production

is suppressed. This is in accordance with the recent finding that sulfation is the first step in T_3 degradation (21, 22). It appears, therefore, that production of iodide from T_3 can be inhibited by diminution of uptake of T_3 by hepatocytes, for instance by ouabain, or by inhibition of 5′-deiodinase using PTU, and that both processes can be influenced independently.

Treatment of the cells with $BrAcT_3$ during 2 h at 21 C results in a diminished disappearance of T_3 during a 20 h incubation period, indicating that $BrAcT_3$ is able to modify the uptake system of hepatocytes. However, the 5′-deiodinase is inhibited also. Iodide formation from T_3 is decreased by 73%: half of this diminution can be explained by a lower membrane transport; the remaining half can be explained by a inhibition of 5′-deiodination (4), resulting in a rise in conjugates in the medium. It appears that the enzyme is much more sensitive to $BrAcT_3$ than the uptake mechanism, because lowering the concentration of $BrAcT_3$ during the preincubation from 1.3 μmol/liter to 0.13 μmol/liter resulted in a loss of inhibitory activity on the uptake system, while the effect on the deiodinase is still present. The inhibitory effects of PTU and $BrAcT_3$ on 5′-deiodinase seem to be additive, as are the effects of ouabain and $BrAcT_3$ on the uptake system.

Preincubating the cells with 1.3 μmol/liter AcT_3 or BrAc and subsequent extensive washing have no effect on the uptake of T_3, or on intracellular metabolism of T_3, in contrast to the same concentration of $BrAcT_3$, which has a substantial effect. From this difference between AcT_3 and $BrAcT_3$ it can be concluded that the presence of a Br atom is essential for the inhibition of the T_3 uptake system and of the 5′-deiodinase in the cell. This strongly suggests that covalent coupling of $BrAcT_3$ is necessary before inhibition takes place. From the lack of effect of BrAc on metabolism of T_3 it can be concluded that the inhibition by $BrAcT_3$ is not due to a nonspecific reaction with SH groups, but that the T_3 residue specifically directs the reagent to the T_3 binding sites of the cell.

The K_m of the high affinity uptake of T_3 by hepatocytes is similar to the value reported previously for rat hepatocytes (14), and equal to the value found in human cultured fibroblasts (23). One minute uptake of T_3 was not changed after preincubation of the cells with AcT_3 and subsequent extensive washing, which indicates an adequate removal of AcT_3 from the cells, and the absence of a covalent reaction with the membrane carrier. On the other hand, $BrAcT_3$ significantly inhibits V_{max} without influence on the K_m. This noncompetitive type of inhibition is compatible with a diminution of transport units, caused by covalent coupling with $BrAcT_3$. Preincubation of the cells with $BrAcT_3$ diminished the V_{max} with 54% of control, which figure is higher than the inhibition of uptake (30%) in the metabolism studies. However initial uptake is measured during only 1 min after the 2 h preincubation step, while metabolism is measured during a further 20-h incubation with tracer T_3. This probably gives the cell an opportunity to restore the inactivated carrier protein, although it seems that this restoration is only partial.

The suppressive effect of $BrAcT_3$ on the translocation of T_3 over the plasma membrane and on 5′-deiodinase underline the importance of both these processes on metabolism of thyroid hormones.

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Chapter IX

Development and use of a mathematical two-pool model of distribution and metabolism of 3,3',5-triiodothyronine in a recirculating rat liver perfusion system: Albumin does not play a role in cellular transport

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Development and Use of a Mathematical Two-Pool Model of Distribution and Metabolism of 3,3′,5-Triiodothyronine in a Recirculating Rat Liver Perfusion System: Albumin Does not Play a Role in Cellular Transport*

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ABSTRACT. To describe the T_3 kinetics in a recirculating rat liver perfusion system, we have developed a mathematical two-pool model consisting of medium and liver. It appeared that all parameters of the model could be fully resolved by using the time-dependent disappearance of radioactive T_3 (2 nM) from the medium only. The model calculates the T_3 medium pool, the T_3 liver pool, and the amount of hormone metabolized at different times after the start of the perfusion. To check the validity of the model, metabolism was also estimated from the appearance of labeled metabolites (glucuronides, sulfates, and I^-) in the medium and the cumulative excretion of T_3 and metabolites into the bile. The medium pool was also estimated by the product of medium volume and remaining T_3 concentration, and the liver pool as the amount of T_3 at time zero minus medium pool minus T_3 metabolized). These results were in excellent agreement with the predicted values from the model.

Taking the metabolites appearing in medium and bile together, about 38% of the total amount of T_3 metabolized during 60 min was converted into T_3 glucuronide, 12% into T_3 sulfate, and 48% into I^-, respectively, while about 3% was excreted in the bile unaltered. The results show that not all T_3 transported to the liver is being metabolized, but part is bound outside the cellular compartment. This latter pool of T_3 is dependent on the albumin concentration in the medium. The amount of T_3 metabolized is solely determined by the free T_3 concentration and is independent of total T_3 or albumin concentration in the medium (*Endocrinology* **126**: 451–459, 1990)

RECENTLY, we have used a three-compartmental model of distribution and metabolism for T_3 and T_4, consisting of a plasma compartment, a rapidly equilibrating pool (REP), and a slowly equilibrating pool (SEP), as proposed by DiStefano III *et al.* for the rat (1, 2). The model has been adapted for humans and describes the changes in thyroid hormone metabolism during caloric deprivation (3) and treatment with *d*-propranolol (4). Both conditions elicit a low T_3 syndrome in humans, but it appeared that the mechanism that is the cause of the low T_3 syndrome is different. During caloric restriction transport of T_4 and T_3 into tissues is diminished, and this phenomenon is much more pronounced for T_4 than for T_3. It was postulated that regardless of any possible change in 5′-deiodinase activity, inhibition of T_4 transport *per se* may contribute to low T_3 production and, therefore, low T_3 serum levels, due to less substrate (*i.e.* T_4) availability for T_3 production in tissues (3). On the other hand, no inhibition of T_4 or T_3 transport into the tissues could be found during *d*-propranolol treatment, although the production of T_3 from T_4 was substantially diminished. It was, therefore, concluded that the *d*-propranolol-induced changes in thyroid hormone metabolism, resulting in a low T_3 syndrome, are due to inhibition of thyroid hormone deiodination.

A drawback of the use of the three-compartmental model is the fact that an assumption has to be made about where the site of the disposal of the hormone under study is located before the parameters of the model can be calculated. Recently we could locate the site of disposal for rT_3 (5), which is situated completely or nearly completely in the liver, an organ that is part of the REP (1). No such data are at present available for T_4 or T_3.

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and therefore, we calculated all parameters of the T_4 and T_3 models, with the disposal arbitrarily divided equally between REP and SEP. It was shown that the conclusions drawn under these circumstances are not different from those obtained after recalculation of the model with the disposal located in either the SEP or REP (4). This finding indicates that the location of the site of the disposal is not a major determinant in the calculation of the transport parameters from the plasma to the REP and SEP for these two hormones, in contrast to rT_3 (4). So far, it has been impossible to validate the compartmental model directly by comparing the parameters calculated from the model with the actual parameters measured by direct analysis.

To validate the calculation technique, we have used a recirculating liver perfusion system, instead of an *in vivo* (rat) model, because the perfusion system consists of only two compartments. These are a plasma (medium) compartment and one tissue compartment, equivalent to the REP (1) of the three-pool model. Distefano and Mori (6) have shown that a unique solution for a two-compartmental model is possible if there is not disposal from the plasma pool, although no practical mathematical solution was given. Furthermore, it is easy to sample medium, liver tissue, and bile. Thus, after chromatographic analysis of the samples, it is possible to measure the amount of hormone metabolized and the fate of the products formed, and to compare the results with the values predicted by the model.

Materials and Methods

Materials

L-T_3 was obtained from Sigma Chemical Co. (St. Louis, MO) 3′-L-[^{125}I]T_3 (SA >1200 μCi/μg) was purchased from the Radiochemical Centre (Amersham, United Kingdom). Albumin (Boseral), used in the perfusion medium, was a product of Organon Teknika (Oss, The Netherlands). All other reagents were of the highest purity commercially available.

Methods

Livers of male Wistar rats, 200–250 g BW, were isolated and perfused in a recirculating system at 37 C as described by Meijer *et al.* (7), using 150 ml Krebs-Ringer buffer (118 mmol/liter NaCl, 5 mmol/liter KCl, 1.1 mmol/liter $MgSO_4$, 2.5 mmol/liter $CaCl_2$, 1.2 mmol/liter KH_2PO_4, and 25 mmol/liter $NaHCO_3$) supplemented with 10 mM glucose and 1% albumin. The pH of the medium was maintained at 7.43 by gassing with carbogen (95% O_2-5% CO_2; 400 ml/min). The function of the liver was monitored by its outer appearance, measurement of the hydrostatic pressure necessary to maintain a medium flow of 40 ml/min, bile flow, pH of the medium, and enzyme release (GOT) into the perfusion medium.

Livers were preperfused for 30 min with medium without T_3. Then, the experiment was started by the addition of 300 pmol T_3 together with 4 μCi [^{125}I]T_3 (7.6×10^6 cpm; ~5 pmol) to the medium, resulting in a final T_3 concentration of 2 nmol/liter. Subsequently, 0.5-ml samples of the medium were taken at 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, and 120 min. Bile was collected at 10-min intervals. The experiment was ended at 60 or 120 min, the liver was weighed and homogenized in 3 vol 0.1 mol/liter NaOH. Total radioactivity was estimated in all samples, and the distribution of the radioactivity over T_3 and the various metabolites (i.e. the amount present in the sample as I^-, T_3 glucuronide (T_3G), or T_3 sulfate (T_3S)) was measured by Sephadex LH-20 chromatography. Mixtures of 0.5 ml medium and 0.5 ml 1 M HCl were applied to small Sephadex LH-20 columns (bed volume, 1 ml) equilibrated with 3 ml 0.1 M HCl. I^- was eluted with 3 ml 0.1 M HCl, T_3G with 8 ml ammonium acetate (0.1 M; pH 4.0), T_3S with 6 ml H_2O, and T_3 with 3 ml NaOH (0.1M)-ethanol (50:50, vol/vol). Fractions of 1 ml were collected and counted for radioactivity. Bile was analyzed in a similar way after the addition of 950 μl 0.1 M HCl to a 50-μl aliquot.

Free hormone concentrations in the media containing albumin were estimated by equilibrium dialysis (8) during and at the end of the perfusion. Statistical analysis of the data was performed with a one-way analysis of variance, followed by comparisons among class means. (9).

Calculations

1) Directly from the measured data the following parameters can be calculated (see *Glossary* for an explanation of the symbols used):

$$V_m(t) = 150 - \sum_0^t V_{sample} \text{ (ml)}$$

$$Q_1(t) = V_m(t) * [T_3]_f \text{ (pmol)}$$

$$\sum_0^t D(t)dt = [0.01*V_m(t)*(100 - \%T_3(t)_m)*C_m(t) + \sum_0^t V_b(t)*C_b(t)]/SA_{T_3} \text{ (pmol)}$$

$$Q_2(t) = 300 - Q_1(t) - \sum_0^t D(t)dt \text{ (pmol)}$$

where $[T_3] = \%T_3(t)_m * C_m(t)/SA_{T_3}$ (pmol/ml), and SA_{T_3} = total added activity/total added cold T_3 (cpm/pmol).

2) By a two-compartmental model, the two-pool model structure in Fig. 1 was used to describe the T_3 kinetics in the liver perfusion system. The model consists of a medium pool and a tissue (liver) pool, in which the metabolism of the hormone takes place. The only assumption made, that metabolism in the liver is much slower than the exchange of hormone from the medium to the liver, *e.g.* $k_{02} \ll k_{12}$, is actually found (Table 1).

The medium radioactivity of T_3 is expressed as a percentage of the total added tracer T_3 per ml. The change of the activity as a percentage of the dose per ml as a function of time could then be described as the sum of two exponentials as:

$$y(t) = \sum_{i=1}^{2} A_i e^{\lambda_i t} \text{ (\% dose/ml)}$$

where t is time, A_i is the coefficient and λ_i is the constant of

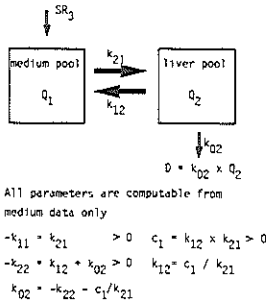

FIG. 1. Two-pool model of iodothyronine distribution and metabolism. k_{ij} values ($i \neq j$; i, j= 1,2; all $k_{ij} > 0$) are fractional transport rates from pool j to pool i. k_{ii} ($i = 1,2$) are fractional turnover rates of pool Q_i. D, Disposal in the tissue pool. All parameters listed *below the figure* can be uniquely calculated from medium data only.

TABLE 1. T_3 kinetics in perfused rat livers after 60 min of perfusion

	Calculated with model	Measured directly
Total disposal (pmol)	156 (17.9)	146 (19.7)
Disposal rate at 60 min (pmol/min)	1.6 (0.08)	
MCR (ml/min)	4.88 (0.94)	
Medium compartment		
Free hormone conc. (pmol/liter)		5.6 (1.0)
Total hormone conc. (nmol/liter)		0.34 (0.06)
Size (ml)	129 (10.2)	140
Pool (pmol)	44.4 (10.1)	49.4 (9.2)
Liver compartment		
Size (ml)	310 (65.2)	
Pool (pmol)	104 (15.4)	105 (17.3)
Mass transfer rate from medium (pmol/min)	15.6 (2.8)	
Fractional transfer rates		
k_{21} (min^{-1})	0.359 (0.067)	
k_{12} (min^{-1})	0.137 (0.031)	
k_{02} (min^{-1})	0.016 (0.003)	

Values are the mean ± SD (n = 12).

the exponent of the i-th exponential. A peel-off system of curve fitting was used, of which procedure a detailed outline can be found in Fig. 2. Together with the parameters estimated by linear regression on the straight parts of the curve (Fig. 2), the SDs of these parameters were also calculated. Finally, it was determined whether the estimated parameters were significantly different from zero. From the A_i and λ_i thus obtained, the parameters, depicted in Fig. 1, of a two-pool model of T_3 distribution and metabolism could be calculated, as outlined in the *Appendix*.

The mean medium T_3 during the 60-min perfusion period was calculated from the fitted two-exponential curve as follows:

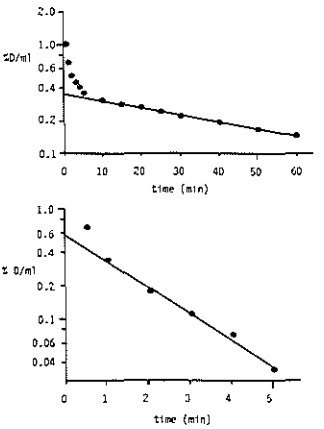

FIG. 2. Typical example of the curve fitting of a T_3 disappearance curve by a two-exponent model. *Upper panel*, Plot of log y_t (percent dose per ml) against time, with the least squares regression line on the final straight part of the curve, giving estimates of coefficient $A_2 = 0.35$ [±0.004 (±SD)] %D/ml and exponent $\lambda_2 = -0.015$ (±0.0003) min^{-1}. *Lower panel*, Plot of log $[y(t) - A_2e^{\lambda_2 t}]$ against time, with the least squares regression line on the straight curve, giving estimates of coefficient $A_1 = 0.57$ (±0.056) %D/ml and exponent $\lambda_1 = -0.55$ (±0.028) min^{-1}. The residual sum of squares is 0.0004; the total sum of squares is 1.661.

$$\text{mean dose/ml} = \left(\int_{t=0}^{\infty} y(t) \cdot t \, dt\right)/60 \text{ (\% dose/ml)}$$

mean $[T_3]$

= (mean dose/ml)*0.01*dose*1000/medium vol. (nmol/liter)

The mean free T_3 was calculated by multiplying the mean T_3 concentration with the dialyzable fraction.

The amount of hormone entering the cells was calculated as follows. During the perfusion with 1% albumin

$$T_{(1\% \text{ alb})} = X + Y$$

where X is the amount of T_3 entering the cells, Y is the amount of T_3 transported to the outside of the cell, and $T_{(1\% \text{ alb})}$ is the total amount of T_3 transported to the liver during the perfusion with 1% albumin. During perfusion with 4% albumin in the medium

$$T_{(4\% \text{ alb})} = (FT_{3(4\% \text{ alb})}/FT_{3(1\% \text{ alb})})X + 4(FT_{3(4\% \text{ alb})}/FT_{3(1\% \text{ alb})})Y$$

This equation holds when transport into the cells is linearly related to the free hormone concentration, and transport to the outside of the cell is linearly related to the albumin concentration and the free hormone concentration (for a justification of these assumptions see *Discussion*).

In this way two equations with two unknowns are obtained, of which both unknowns can be solved.

Results

In all experiments the disappearance of tracer T_3 from the medium could be described as the sum of two exponentials. A typical example is shown in Fig. 2. The quality of curve fitting was estimated by expressing the residual sum of squares as a percentage of the total sum of squares. This figure never exceeded 0.18% [0.09 ± 0.049 (mean ± SD)]. Furthermore, the fitted A_i and λ_i ($i = 1,2$) are highly significantly different from zero ($P < 0.001$ in all experiments; see also Fig. 2), which indicated that the use of a two-exponential disappearance curve is adequate to describe the changes in tracer T_3 in the medium.

With the parameters of this two-exponential disappearance curve the two-pool model of distribution and-metabolism was calculated for each experiment. For medium total and free T_3 concentrations the values at 60 min of perfusion were used, except for calculation of the total disposal during the perfusion, in which case the integral T_3 concentration from time zero to the end of the study was used. The mean results are depicted in Table 1, together with the mean values of the parameters that were calculated from direct measurements (*i.e.* of T_3 and T_3 metabolites in medium, bile, and liver) without use of the two-compartmental model. There was no significant difference between these two sets of data, indicating that the model adequately describes the fate of T_3 in this perfusion system after 60 min of perfusion, including fractional transfer rates and mass transport values, because these parameters are implicitly used to calculate the correlated model parameters.

Figures 3, 4 and 5 show the correlation among the medium pool (Fig. 3), the liver pool (Fig. 4), and disposal (Fig. 5) at the end of the perfusion, at 60 min, or at 120 min, respectively, as calculated from the two-compartmental model and as obtained from direct measurements without the use of the two-compartmental model. From the excellent correlations, and the slopes of unity, it can be concluded that the model adequately corrects for changes during the perfusion.

Normally the medium contains 1% BSA, which results in a dailyzable fraction of T_3 of 1.63 ± 0.16% (mean ± SD; n = 22). Raising the albumin concentration to 4% decreases the dialyzable fraction to 0.45 ± 0.08% (n = 4), and with the same total amount of hormone added, decreases the free hormone concentration accordingly. Dialyzable fractions were measured at the end of the perfusions. Despite these variations in the free hormone concentration in the medium, the model adequately calculates the medium pool, liver pool, and total disposal (Figs. 3, 4, and 5, respectively).

The production of metabolites from T_3 during a 60-min perfusion is summarized in Table 2 for livers perfused with 1% and 4% albumin in the medium. The amount of metabolites appearing in medium and bile are taken together, and the total amount is shown. Regardless of the amount of albumin or the total amount of T_3 in the medium, about 38% of the amount of T_3 metabolized is converted into T_3 glucuronide, 12% into T_3 sulfate, and 48% into I^-, respectively, while 3% of the T_3 metabolized is excreted in the bile without modification. The mean medium T_3, free T_3, and total amount of hormone metabolized during the perfusion are also summarized in Table 2 together with the total amount of T_3 transported to the liver (the last two parameters calculated from the model).

Discussion

The data in this paper show that a two-compartmental model adequately describes the kinetics of T_3 in a recir-

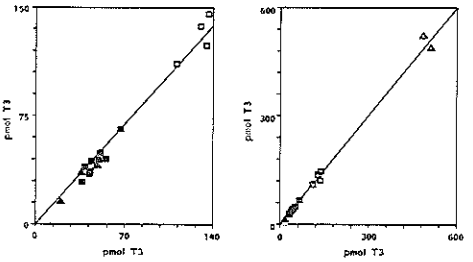

FIG. 3. *Right panel*, Correlation between the medium pool T_3 calculated by the model (*ordinate*) and the medium pool T_3 measured directly (*abscissa*) at the end of perfusion [n = 18; r = 0.997; slope = 1.01 ± 0.02 (mean ± SD)]. ■, After 60-min perfusion; ▲, after 120 min perfusion, with 1% albumin and 300 pmol T_3 in the medium; □, after 60-min perfusion with 4% albumin and 300 pmol T_3; △, after 60-min perfusion with 4% albumin and 1200 pmol T_3 added to the medium. *Left panel*, Same correlation calculated with omission of the two highest data points (n = 16; r = 0.993; slope = 0.98 ± 0.04).

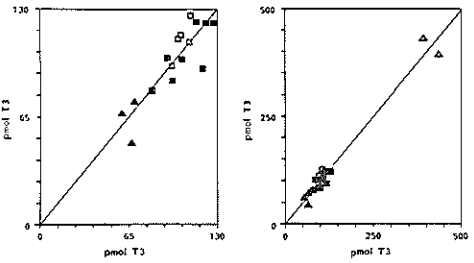

FIG. 4. *Right panel*, Correlation between the liver pool T_3 calculated by the model (*ordinate*) and the liver pool T_3 measured directly (*abscissa*) at the end of perfusion [n = 18; r = 0.987; slope = 0.99 ± 0.04 (mean ± SD)]. See Fig. 3 for explanation of the symbols. *Left panel*, Same correlation calculated with omission of the two highest data points (n = 16; r = 0.870; slope = 1.00 ± 0.14).

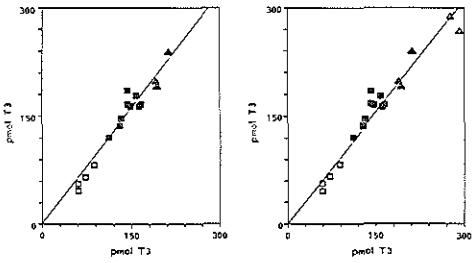

FIG. 5. *Right panel*, Correlation between the disposal of T_3 calculated by the model (*ordinate*) and the disposal of T_3 measured directly (*abscissa*) at the end of perfusion [n = 18; r = 0.972; slope = 1.03 ± 0.06 (mean ± SD)]. See Fig. 3 for explanation of the symbols. *Left panel*, Same correlation calculated with omission of the two highest data points (n = 16; r = 0.975; slope = 1.07 ± 0.07).

culating liver perfusion system. It is possible to obtain results from medium measurement of tracer and unlabeled hormone concentrations that cannot be otherwise calculated. In this way transport of hormone into and out of the liver can be calculated, besides metabolism of hormone in the liver, *i.e.* as the total amount of hormone irreversibly removed from the system. To break this total figure down, further chromatographic analysis of both medium and bile is necessary.

It is generally accepted that kinetic studies can be interpreted only under so-called equilibrium conditions, *i.e.* that there is no change in hormone concentration during the period of measurement. Here T_3 is added to the medium at the start of the experiment. Due to metabolism, the concentration of T_3 (both free and total) in the medium decreases gradually, without reaching a steady state in the sense of a constant hormone concentration during the metabolic study. Nevertheless, the good correlation between the parameters calculated by the model and those actually measured indicates that this constant hormone concentration is apparently not necessary to obtain correct results. This finding can be interpreted in two ways. It could indicate that indeed compartmental analysis is allowed even if there is not a steady state present in the system. This is in accordance with the findings of Meinhold *et al.* (10), who showed that turnover of diiodotyrosine measured by tracer kinetics or injection of a large dose of unlabeled diiodotyrosine and subsequent measurement of the disappearance of the material from the circulation by RIA gave identical results in both dogs and humans, and with the fact that already in 1956 Sterling and Chodos (11) showed that in humans an iv bolus injection of 2 mg T_4 (twice the total body pool) during measurement of T_4 tracer kinetics with a single compartmental analysis does not change the kinetic parameters.

The other interpretation might be that although during the perfusion studies the medium concentration of

TABLE 2. Production of metabolites from T_3 during 60 min by the perfused liver

	Livers perfused with medium containing		
	1% albumin, 300 pmol T_3	4% albumin, 300 pmol T_3	4% albumin, 1200 pmol T_3
No. of exp.	12	4	2
Mean medium T_3 (nmol/liter)	0.54 (0.07)	1.03 (0.10)	4.13 (0.24)
Mean medium FT_3 (pmol/liter)	8.9 (1.16)	4.7 (0.97)	20.6 (2.12)
T_3 metabolized[a] (pmol/60 min)	156 (17.9)	58.5 (20.8)	276 (15.2)
T_3 metabolized/pmol free T_3	17.6 (4.0)	12.4 (5.7)	13.4 (1.7)
Metabolites formed			
T_3G (pmol/60 min)	60.2 (13.5)	25.0 (10.9)	105.7 (0.4)
T_3S (pmol/60 min)	15.6 (2.8)	8.4 (0.7)	36.0 (5.4)
I^- (pmol/60 min)	66.4 (11.1)	36.8 (3.6)	132.1 (13.9)
T_3 (in bile) (pmol/60 min)	4.2 (2.5)	1.3 (0.4)	8.7 (3.7)
Total (pmol/60 min)	146.3 (19.7)	71.4 (10.3)	282.5 (12.0)
As % of the T_3 transported into the cells	20.8 (2.5)	19.3 (3.1)	19.5 (1.2)
T_3 transported			
Total to liver (pmol/60 min)	1488 (212)	2017 (200)	9467 (758)
Total to liver/pmol free T_3	167 (32)	429 (74)	460 (45)
Into cells[b] (pmol/60 min)	705	371	
To surface[b] (pmol/60 min)	783	1646	
Into cells[c] (pmol/60 min)	625		1449
To surface[c] (pmol/60 min)	864		8018

Values are the means, with SDs in parentheses.

[a] As calculated from the model.

[b] Calculated from the data of columns 1 and 2.

[c] Calculated from the data of columns 1 and 3.

hormone decreases gradually, at each time point during the experiment the liver is in equilibrium with the medium because transport of hormone over the membrane by influx and efflux is much greater than metabolism in the liver itself. Therefore, the liver reaches equilibrium with the medium within a period in which there is only a small change in T_3 concentration due to metabolism. Table 1 shows that this is actually the case; about 15% of the total liver pool is replaced by hormone from the medium per min, while only 1.5% of the total liver pool is metabolized per min.

During the perfusion a constant fraction of the metabolized T_3 is converted into T_3G, T_3S, and I^-, while a constant fraction is excreted in bile as T_3. The relative size of these fractions is not dependent on the amount of albumin or free T_3 present in the medium. This indicates that the amount of hormone metabolized by the cell is only dependent on the amount of hormone taken up by the cells. We have previously shown (12) that in rat hepatocytes in culture T_3 is also metabolized to I^- and conjugates. However, much less conjugates (9%) and much more I^- (85% of the metabolized amount of T_3) are formed than in the perfused liver, due to the fact that hepatocytes in culture excrete the conjugates into the medium. We have previously shown (13) that these conjugates are taken up by the cells anew and are further metabolized, in contrast to the liver perfusion, where a large part of the conjugates is eucreted to the bile, which is collected, removing these conjugates from the system. Our data correspond more closely with *in vivo* studies in rats (14). Twenty-four hours after injection of [^{131}I]T_3 in rats with biliary fistulas 37% of the dose was metabolized to I^- and 21% was excreted in the bile in the glucuronide region, 5% in the sulfate region, and 1.5% as unchanged T_3. Expressed as a percentage of the amount of T_3 metabolized, almost the same figures are obtained as in the liver perfusion studies reported here (Ref. 14 *vs.* this study: I^-, 57% *vs.* 48%; T_3S, 8% *vs.* 12% T_3G, 33% *vs.* 38%; T_3 in bile, 1.5% *vs.* 3%). The products in the sulfate and glucuronide region were not fully characterized. However, in a recent paper (15) it was shown that the radioactive products of injected T_3 appearing in the bile of control rats are almost exclusively composed of T_3G and T_3S, with only minor amounts of I^-, diiodothyronine, and T_3.

The amount of hormone entering the cells is only dependent on the free T_3, not the albumin, concentration, because there is no significant difference between the amount of T_3 metabolized divided by the medium free T_3 in the three groups. This suggests that in the perfused liver of normal rats the amount of hormone transported into the cell is only dependent on the free hormone concentration, in agreement with a recent report (16), and that metabolism is only dependent on the amount of hormone entering the cells.

Table 2 shows that there is no correlation between the total amount of T_3 transported to the liver and the amount of T_3 metabolized during 60 min. Neither is there a relation between the amount of T_3 transported to the liver and the medium free T_3, when the albumin concentrations in the medium are different. Thus, raising the albumin concentration in the medium from 1% to 4% lowers the mean free T_3 concentration (at the end of the perfusion) from 8.9 to 4.7 pmol/liter ($P < 0.001$), but increases transport to the liver from 1488 to 2017 pmol/60 min ($P < 0.001$). However, T_3 metabolism decreases from 156 to 58 pmol/60 min. If only the mean medium free T_3 concentration is changed (with constant albumin levels), total transport to the liver changes in parallel, leading to a constant total transport to the liver per pmol free T_3 (Table 2, *last two columns*; 429 *vs.* 460 pmol T_3/pmol free T_3; $P > 0.5$) and to a constant amount T_3 metabolized per pmol free T_3 (Table 2, *last two columns*; 12.4 *vs.* 13.4 pmol T_3/pmol free T_3; $P > 0.5$). Thus, lowering the free T_3 concentration, regardless of differences in albumin concentrations, leads to a decrease in T_3 metabolism. Furthermore, raising the albumin concentration increases total T_3 transport to the liver despite a decrease in free T_3. Thus, albumin *per se* has an influence on T_3 transport to the liver, independent of the free T_3 concentration. This transport does not lead to metabolism and, therefore, does not represent translocation to the intracellular compartment.

Two explanations for this albumin-dependent transport of T_3 to the liver are possible. Firstly, we have previously shown (17), that binding sites are present on the outside of cultured rat hepatocytes, and that transport of hormone to the pool composed by these sites can be stimulated by raising the albumin concentration in the medium. Secondly, there may be pools of albumin in the liver which bind hormone, but are slowly exchangable with the albumin in the medium, for instance albumin sequestered in the space of Disse (18) or loosely bound to the surface of the cells (19–21). Transport to and size of these pools will be linearly correlated with the albumin concentration in the medium. If we assume that binding and transport to these pools outside the liver cells are 4 times higher in livers perfused with 4% albumin than in livers perfused with medium containing 1% albumin, then it is possible to calculate the amount of hormone actually transported into the cells, as outlined in *Materials and Methods*. The results of these calculations are presented at the *bottom* of Table 2. About 45% of the hormone transported to the liver enters the cell when the livers are perfused with 1% albumin, in contrast to about 17% with a medium containing 4% albumin. Thus, 20% of the hormone actually transported into the cells is metabolized, regardless of the albumin or free hormone

concentrations in the medium. These results make clear that perhaps a three-pool model would give a better description of the system. The finding of a two-exponential curve to describe the tracer kinetics in the medium does not necessarily imply that there are only two pools in the system. It merely indicates that there are at least two pools present. In the case of a three-pool model, formula IV of the *Appendix*, describing the medium tracer disappearance, contains three exponential terms. However, the curve-fitting procedure used is not able to resolve all three terms if two of the λ values are about equal, in which case these two terms are lumped together, and only two exponentials are found, even in a three-pool system.

Due to the presence of these large extracellular hormone-binding pools, it is clear that it is not possible to measure hormone uptake into liver cells with the single pass injection technique used by Pardridge and Mietus (22, 23). In this technique tracer hormone is injected together with tritiated water, a freely diffusable substance, in various media into the portal vein. After one pass through the liver, cellular uptake of thyroid hormone is calculated from the difference between the amount of tritiated water and thyroid hormone retained by the liver. However, besides methodological problems, which were previously discussed (24, 25), injected hormone will for the greater part spread over these extracellular pools and not enter the cells. Therefore, uptake seems to follow diffusion kinetics in this system, because binding of hormone to these pools is linearly related to the free hormone concentration. The distribution of hormone over the injected plasma proteins and these extracellular pools will be governed by the relative affinities and number of binding sites in the injected fluid and the extracellular pools. We have shown that the binding sites on the outside of the liver cell have a K_d of 10^{-6} mol/liter for T_3 (26), the same order of magnitude or a little higher than that for albumin. Thus, hormone bound to albumin in the injected fluid will distribute over all binding sites in both the extracellular pools and the injected bolus, in contrast to hormone injected bound to, for instance, T_4-binding globulin, which has a much higher affinity for T_3. Therefore, it seems that albumin plays a role in the transport of thyroid hormone to the liver, but this is only in transport to the extracellular pools.

Because albumin plays a role in transport of thyroid hormone to extracellular, but not intracellular, T_4 pools and because it is not possible with the technique used by Pardridge and Mietus (22, 23) to distinguish between those two pools, it cannot be concluded from the studies of Pardridge *et al.* that albumin plays a role in translocation of thyroid hormone into the cell.

Appendix

The equations, describing the change of tracer masses [$q_i(t)$ representing the tracer concentration in pool i at time t_k] are:

$$\frac{dq_1(t)}{dt} = -(k_{21})q_1(t) + k_{12}q_2(t) \quad \text{(I)}$$

$$\frac{dq_2(t)}{dt} = k_{21}q_1(t) - (k_{12} + k_{02})q_2(t) \quad \text{(II)}$$

$$y(t_k) = \frac{q_1(t_k)}{V_{medium}}, \text{ with } 0 < t_k < T = t_N \; k = 1, \ldots N \quad \text{(III)}$$

All of the individual fractional transport or disposal rate parameters can be determined uniquely from this model and medium data only, as is outlined below. Medium tracer disappearance can be described as a two-exponential fitted function giving two coefficients A_i and two exponents λ_i ($i = 1,2$):

$$y(t) = \sum_{i=1}^{2} A_i e^{\lambda_i t} \quad \text{(IV)}$$

where $\lambda_1 < \lambda_2$. An analytical solution of the model output $y(t)$ in terms of the k_{ij} can be obtained by Laplace transformation of the model Eq I–III:

$$L[y(t)] = \left(\frac{u}{V_{medium}}\right)\left(\frac{s - k_{22}}{s^2 + a_2 s + a_1}\right) \quad \text{(V)}$$

with $a_1 = k_{11}k_{22} - k_{12}k_{21}$, and $a_2 = -k_{11} - k_{22}$. The Laplace transformation of Eq IV gives:

$$L[y(t)] = (A_1 + A_2)\left(\frac{s - (A_1\lambda_2 + A_2\lambda_1)/(A_1 + A_2)}{(s - \lambda_1)(s - \lambda_2)}\right) \quad \text{(VI)}$$

These two functions (V and VI) are identical if $A_1 + A_2 = u/V_m$, $s - k_{22} = s - (A_1\lambda_2 + A_2\lambda_1)/(A_1 + A_2)$, and $s^2 + a_2 s + a_1 = (s - \lambda_1)(s - \lambda_2)$.

These equations will result after algebraic manipulation of the data in expression of all k_{ij} in terms of A_i and λ_i:

$$k_{22} = (A_1\lambda_2 + A_2\lambda_1)/(A_1 + A_2)$$

$$a_1 = k_{11}k_{22} - k_{12}k_{21} = \lambda_1\lambda_2$$

$$a_2 = -(k_{11} + k_{22}) = -(\lambda_1 + \lambda_2)$$

$$k_{11} = -k_{21} = \lambda_1 + \lambda_2 - k_{22}$$

$$k_{12} = (k_{11}k_{22} - \lambda_1\lambda_2)/k_{21}$$

Further parameters of the model can be calculated as follows:

$$V_m = 100/(A_1 + A_2)$$

$$MCR = -100/\left(\sum_{i=1}^{2} A_i/\lambda_i\right)$$

The total concentration of T_3 diminishes during the perfusion study, but because $k_{02} \ll k_{12}$ there exists equi-

librium between liver and medium throughout the study, and the following parameters can be calculated also:

$$TR_{ml}(t) = V_m k_{21} [T_3]_t$$

$$MDR(t) = MCR[T_3]_t$$

$$Q_1(t) = V_m[T_3]_t$$

$$Q_2(t) = -TR_{ml}(t)/k_{22}$$

$$D_{liver}(t) = k_{02}Q_2(t) = V_m k_{21} k_{02}[T_3]_t/-k_{22}$$

and the total disposal during the experiment

$$D_{total} = (V_m k_{21} k_{12} k_{22}) \int_0^\infty [T_3]_t dt$$

Glossary

(i = 1,2; j = 1,2; i ≠ j)	
t	Time (min)
y(t)	Medium activity at time t (% dose/ml)
A_i	Coefficient of the i-th exponential component (% dose)
λ_i	Exponent of the i-th exponential component (min^{-1})
q_1	Size of pool 1 (medium pool; % dose)
q_2	Size of pool 2 (liver pool; % dose)
u	Dose of tracer introduced in medium (100%)
k_{ij}	Fractional transport rate from pool j to pool i (min^{-1})
k_{02}	Fractional disposal rate in pool 2 (min^{-1})
k_{ii}	Fractional turnover rate of pool i (min^{-1})
$V_m(t)$	Medium volume (ml) at time t
$V_b(t)$	Bile volume (ml) at time t
L[y(t)]	Laplace transform of y(t)
s	Laplace transform variable
$[T_3]_t$	Medium T_3 concentration (pmol/ml) at time t
FT_3	Free T_3 concentration
MCR	Medium clearance rate (ml/min)
$TR_{ml}(t)$	Medium to liver transport rate at time t (pmol/min)
MDR(t)	Medium disappearance rate at time t (pmol)
$Q_1(t)$	Size of pool 1 (medium pool) at time t (pmol)
$Q_2(t)$	Size of pool 2 (liver pool) at time t (pmol)
$D_{liver}(t)$	Disposal in the liver at time t (pmol/min)
$\int_0^t D(t)dt$	Total disposal during t min (pmol)
$\%\ T_3(t)_m$	% of total counts present in medium as T_3
$C_m(t)$	Counts/ml present in the medium at time t
$C_b(t)$	Counts/ml present in the bile at time t
SA_{T_3}	Specific activity of added T_3 (cpm/pmol)

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SUMMARY

Thyroid hormone plays an important role in the development and in the regulation of metabolic processes in vertebrates. The hormone is produced by the thyroid, mainly as the biological inactive product thyroxine (T_4), and secreted into the blood. Normally more than 99.97% of the total amount of T_4 is bound to three plasma proteins i.e. 75% to thyroxine binding globulin, 15% to thyroxine binding prealbumin and 10% to albumin. In chapter III it is shown that a strongly elevated binding of T_4 to albumin occurs in subjects with "familial disalbuminemic hyperthyroxinemia" (FDH). This biochemical syndrome, characterized by elevated total serum T_4 and normal FT_4 concentrations, and originally described by us, is caused by albumin with high affinity for T_4. This albumin is also present in low concentrations in normals. From our disposal studies in FDH subjects it can be concluded that this elevated serum binding does not influence cellular transport and metabolism of thyroid hormone.

Up till 1978 it has been assumed that thyroid hormone enters the cell by diffusion. However since that time a large body of evidence has been accumulated, indicating that transport of thyroid hormone into rat liver cells is an energy (ATP) dependent process, mediated by specific transport systems in the plasma membrane. In this way it could be shown that both T_3 and T_4 bind to two different sets of binding sites, of which the binding sites with the highest affinity specifically mediate the uptake of either T_4 or T_3. To investigate whether similar processes are present in human cells, membrane transport of thyroid hormone into human erythrocytes and fibroblasts was measured. Chapter IV shows that in erythrocytes a carrier mediated transport for T_3 is present, which is not energy (ATP) dependent. Chapter VII shows that human cultured fibroblasts possess energy dependent transport systems both for T_4 and T_3 which are comparable with those in rat hepatocytes.

As efflux of thyroid hormone out of the cell appears to be a passive mechanism (Chapter V), it is not likely, that a regulatory role with regard to the free hormone concentration is attributed to this process. However the uptake process is regulatory in this respect, because inhibition of uptake of thyroid hormone leads to a lower

occupancy of the T_3 receptors in the nucleus and a to a lower metabolism of hormone (Chapters V and VI), due to a lower substrate availability to the deiodinating and conjugating enzymes.

Chapter VIII shows that chemical modification of the membrane carrier protein with an affinity label leads to a diminished transport into the cell, and consequently to a diminished metabolism of hormone. However the affinity label used also inhibits intracellular deiodinase, which leads to an even more decreased metabolism. Both inhibitions appeared to be additive.

Finally we have shown with recirculating liver perfusions, that total thyroid hormone transport to the liver has to be devided in two components (Chapter IX). It could be shown that not all T_3 transported to the liver is being metabolized, but that part is bound outside the cellular compartment. This pool of T_3 is not only dependent on the free T_3 concentration, but also on the albumin concentration in the medium. The amount of T_3 metabolized is solely determined by the free medium T_3 concentration and is independent of total T_3 or albumin concentration in the medium. With 1% albumin in the medium, transport of T_3 into the cells is about 50% of the total transport to the liver, but with 4% albumin in the medium transport to the intracellular compartment is only 15% of the total transport to the liver. This implicates that at physiological albumin concentrations a much larger part of the hormone actually entering the cell is metabolized than was assumed up til now, and that even a large change in the transmembranal transport process will result in only a small change in total transport to the liver, the only parameter which can be measured by turnover studies in vivo.

SAMENVATTING

De schildklier maakt voornamelijk het prohormoon thyroxine (3,3',5,5'- tetrajodothyronine, T_4) en slechts een kleine hoeveelheid van het biologisch aktieve hormoon 3,3',5-trijodothyronine (T_3). Deze produktie van T_4 staat onder invloed van het TSH (thyroid stimulating hormone), een peptide hormoon, dat geproduceerd wordt door de thyrotrofe cellen in de hypofyse voorkwab. Deze cellen worden op hun beurt gestimuleerd tot de synthese en afgifte van TSH door TRH (TSH releasing hormone) uit de hypothalamus. Plasma T_4 daarentegen remt de afgifte van TSH door de hypofyse. Dit proces verloopt via binding van T_3, gevormd door een actieve lokale dejodering van T_4 in de hypofyse, aan de kernreceptoren voor T_3. Ook plasma T_3 kan door binding aan de kernreceptor in de thyrotrofe cel de afgifte van TSH remmen. Er is hier dus sprake van een regelkring die bij euthyreote personen de plasma T_4 concentratie constant houdt. Ongeveer 80% van het circulerende T_3 wordt gevormd door buitenring-dejodering van T_4 elders in het lichaam (fig. 1). De buitenring is de ring met de fenolische hydroxyl-groep, de binnenring die met de alanine zijketen. Beide ringen zijn door middel van een etherbrug met elkaar verbonden. Door perifere binnenring-dejodering van T_4 ontstaat de biologisch inaktieve metaboliet 3,3',5'-trijodothyronine (reverse T_3, rT_3). Dejodering is ook een belangrijke route voor het verdere metabolisme van T_3 en rT_3. Hierbij ontstaat vrijwel altijd het inaktieve 3,3'-dijodothyronine (3,3'-T_2), zowel door binnenring-dejodering van T_3 als door buitenring-dejodering van rT_3 (fig. 1). Dus, buitenring-dejodering is een aktiveringsreaktie (produktie aktief T_3) en binnenring-dejodering is een inaktiveringsreaktie (produktie inaktief rT_3 en afbraak T_3). Jodothyronines worden verder ook geconjugeerd met glucuronzuur (T_4, T_3) of sulfaat (T_3, 3,3'-T_2), waarna de glucuronides worden uitgescheiden in de gal, en de sulfaten een snelle verdere dejodering ondergaan, met name in de lever. Aangezien de hier geschetste processen intracellulair plaatsvinden, moet het hormoon dus via het plasma naar de cellen getransporteerd worden, en daarna via het plasmamembraan worden opgenomen.

Met het in dit proefschrift beschreven onderzoek is getracht inzicht te krijgen in de volgende problemen: Heeft de varierende serum binding van schildklier hormoon

invloed op het opname in de cel van het hormoon. Zijn de opnameprocessen zoals aangetoond in rattehepatocyten ook aanwezig in cellen van humane oorsprong. Wordt de efflux van hormoon uit de cel ook gereguleerd net zoals de opname. En heeft modulatie (remming) van het opnameproces invloed op het metabolisme, activatie zowel als afbraak, van schildklier hormoon.

Van de totale hoeveelheid T_4 in plasma is meer dan 99.97 % aan drie plasma eiwitten gebonden, n.l. 75 % aan TBG (thyroxine bindend globuline), 15 % aan TBPA (thyroxine bindend prealbumine) en 10 % aan albumine. In hoofdstuk III wordt aangetoond dat hiernaast bij sommige personen een autosomaal dominant overerfbare, sterk verhoogde binding aan albumine optreedt. Dit albumine met hoge affiniteit voor T_4 is in lage concentraties ook aanwezig in de normale populatie. In de latere literatuur is voor dit door ons voor het eerst beschreven biochemische syndroom de naam "familial disalbuminemic hyperthyroxinemia" (FDH) algemeen gebruikelijk geworden. Uit meting van het hormoon verbruik bij personen met FDH (hoofdstuk III) en uit literatuur gegevens kan worden geconcludeerd dat deze verhoogde serum binding geen invloed op het hormoon verbruik en de opname door de cel heeft.

Op grond van de hydrofobe eigenschappen van jodothyronines en het feit dat in vivo geen verzadiging van de opname kon worden gevonden, werd aangenomen dat schildklierhormoon via diffusie de cel binnen kwam. In de laatste jaren is echter ondubbelzinnig aangetoond dat transport van schildklierhormoon door het plasma membraan van levercellen van de rat een energie (ATP) afhankelijk proces is, dat gemedieerd wordt door specifieke transport systemen in het plasma membraan. Aldus is aangetoond dat zowel T_3 als T_4 reageren met twee sets bindingsplaatsen, waarvan die met de hoogste affiniteit hetzij T_4 hetzij T_3 specifiek de cel in transporteren. Om te onderzoeken of dezelfde processen ook aanwezig zijn in humane cellen, werden bij erythrocyten en fibroblasten de membraan transport processen gemeten. Hoofdstuk IV laat zien dat in humane erythrocyten een opname systeem aanwezig is, echter ook dat dit niet energie (ATP) afhankelijk is. Hoofdstuk VII laat echter zien dat menselijke fibroblasten wel een energie afhankelijk opname systeem voor

schildklier hormoon bezitten, dat vergelijkbaar is met dat van ratte hepatocyten.

Efflux van schildklier hormoon uit de cel blijkt een passief proces te zijn (hoofdstuk V) en kan dus geen rol spelen in de regulatie van de intracellulaire vrije hormoon concentratie. Het opname proces is wel regulerend op deze intracellulaire hormoon concentratie. Zo blijkt een remming van de opname van schildklierhormoon te leiden tot een vermindering van de bezetting van de T_3 receptoren in de kern en van het metabolisme van schildklierhormoon (hoofstukken V en VI).

Chemische modificatie van het membraan transport eiwit met een affiniteits label leidt ook tot een verminderd transport van hormoon de cel in, en daardoor tot een verminderd metabolisme van hormoon (hoofdstuk VIII). Echter het gebruikte affiniteits label remt ook het intracellulaire dejodase, waardoor het metabolisme nog verder wordt geremd. Deze beide remmende processen blijken additief te zijn.

Tenslotte hebben wij met behulp van recirculerende leverperfusies kunnen aantonen dat het schildklierhormoon transport naar de lever bestaat uit twee deeltransporten (hoofdstuk IX). Eén naar het intracellulaire metabool actieve compartiment, welk transport alleen afhankelijk is van de vrije hormoon concentratie, (uiteraard bij een onveranderde kwaliteit van het membraan transport mechanisme als zodanig), en een tweede deeltransport naar een metabool inactief compartiment. Dit laatste transport is afhankelijk van de vrije hormoon concentratie én de albumine concentratie in het medium. Bij 1 % albumine is dit deeltransport ongeveer de helft van het totale transport, maar bij 4 % albumine in het medium al 85 % van het totale transport naar de lever. De implicatie van deze bevinding is dat een veel groter deel van het hormoon dat de cel binnen komt wordt gemetaboliseerd dan tot nu toe werd aangenomen. Onze bevindingen betekenen ook dat in de fysiologische situatie, waarin de albumine concentratie 4 % bedraagt, een verandering van het membraan transport slechts een kleine verandering in het totaal transport veroorzaakt.

NAWOORD

Velen hebben direct of indirect meegewerkt aan de totstandkoming van dit proefschrift.

Voor een groot deel is het verschijnen ervan te danken aan de soms wat sarcastische vragen van de ene keer Eric en de andere keer Marianne hoe het nu met "het boekje" stond, waarbij op hun gezicht te lezen was dat ze er eigenlijk niet meer in geloofden.

De inhoud is ontstaan tijdens mijn jarenlange nauwe samenwerking met Jorg Hennemann, Theo Visser en Eric Krenning, later aangevuld met Marion de Jong. Hierbij is voortgebouwd op het fundament dat met Eric's proefschrift gelegd is, gekombineerd met de kennis van het metabolisme dat door Theo is uitgewerkt. Verder zijn de studies met modellen zoals vastgelegd in het proefschrift van Hans van der Heyden voor mij de vingeroefeningen geweest voor de ontwikkeling van het leverperfusie model zoals beschreven in hoofdstuk 9. De perfusietechnieken zijn daarbij op vaardige wijze uitgewerkt door Marion. Jorg, mijn promotor, ben ik erkentelijk voor zijn stimulerende discussies en de bijna onbeperkte bewegingsvrijheid, Theo, mijn kamergenoot, met zijn haast encyclopedische kennis van de literatuur, voor de opbouwende kritiek en zijn hulp bij het opzetten van de metabolisme studies, en tenslotte Eric voor de plezierige dinsdagmorgen discussies. Verder waardeer ik het zeer dat Eric en Theo bij mijn promotie als paranimfen willen optreden.

De experimenten zijn op het laboratorium door een groot aantal analisten uitgevoerd, waarbij Grietje Bos, Bert Bernard en Hans van der Hoek in het bijzonder genoemd moeten worden. Hans van Toor heeft het merendeel van de vrije hormoon bepalingen verricht die noodzakelijk waren voor de berekening en de interpretatie van de resultaten.

Wim Hülsmann ben ik erkentelijk voor de kritische opmerkingen, die hij als lid van de promotiecommissie bij het proefschrift heeft gemaakt.

Tenslotte realiseer ik mij ten zeerste dat Marianne, Marcel en Dennis er onder hebben moeten lijden dat ik vele avonden en weekeinden in mijn studeerkamer heb doorgebracht, om dit proefschrift en de andere artikelen te schrijven en om alle computerprogramma's te ontwikkelen die nu op het laboratorium gebruikt worden.

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augustus 1967 - juli 1974	Aanstelling als wetenschappelijk medewerker Afdeling Inwendige Geneeskunde III Medische Faculteit Rotterdam
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