The deiodination of thyroid hormone in rat liver

De dejodering van schildklierhormoon in de lever van de rat

PROEFSCHRIFT

Ter verkrijging van de graad
van doctor in de geneeskunde
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof. dr. M. W. van Hof
en volgens besluit van het college van dekanen.

De openbare verdediging zal plaatsvinden op
woensdag 12 juni 1985
te 15.45 uur

door

Jan Adrianus Mol
geboren te Dordrecht

BEGELEIDINGSCOMMISSIE

PROMOTOR   : PROF. DR.   G. HENNEMANN

OVERIGE LEDEN   : PROF. DR. W.C. HüLSMANN
                   PROF. DR. H.J. VAN DER MOLEN
                   PROF. DR. H.J. VAN EIJK

Kennis, zij zal afgedaan hebben....

Zo blijven dan: Geloof, hoop en liefde....

(I Korintiers 13)

aan mijn Ouders

aan Ellen,
Gerben en Jurjan

C O N T E N T S

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
$BrAcT_3$	N-bromoacetyl-3,3',5-triiodothyronine
BSA	bovine serum albumin
D-I	type I deiodinase
5'D-II	specific 5'-deiodinase type II
5D-III	specific 5 -deiodinase type III
DEP	diethylpyrocarbonate
DHL	dihydrolipoamide
DIT	diiodotyrosine
E-SH	enzyme with free sulfhydryl group
E-SI	enzyme-sulfenyl iodide
GSH	reduced glutathione
GSSG	oxidized glutathione
IAc	iodoacetate
RB	rose bengal
RIA	radioimmunoassay
Tetrac	3,3',5,5'-tetraiodothyroacetic acid
T_4	thyroxine; 3,3',5,5'-tetraiodothyronine
T_3	3,3',5-triiodothyronine
rT_3	reverse T_3; 3,3',5'-triiodothyronine
$3,3'-T_2$	3,3'-diiodothyronine
Triac	3,3',5-triiodothyroacetic acid
TBA	thyroxine-binding albumin
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin
TRH	TSH-releasing hormone
TSH	thyroid-stimulating hormone; thyrotropin

C H A P T E R I

GENERAL INTRODUCTION

I.A Thyroid hormone synthesis

The thyroid is the production site of 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) which is synthetized by iodination of tyrosine residues in thyroglobulin and subsequent coupling of two diiodotyrosine residues within the thyroglobulin molecule. Thyroxine is liberated by proteolytic degradation of the thyroglobulin in the follicular cells of the thyroid (DeGroot and Niepomniszcze 1977). Coupling of a monoiodotyrosine residue with a diiodotyrosine residue yields the biologically active thyroid hormone 3,3',5-triiodothyronine (T_3). The secretion of T_3 by the thyroid gland is, however, a minor source of circulating T_3 (Chopra et al 1978[a]). The production and release of iodothyronines by the thyroid are under control of thyroid stimulating hormone (TSH) of the pituitary gland (Fig. 1). The rate of TSH secretion is subject to neuroendocrine regulation by hypothalamic peptides. The tripeptide TRH (TSH-releasing hormone) has a stimulatory effect on TSH secretion whereas somatostatin has an inhibitory effect (Reichlin 1978). In addition, thyroid hormone exerts a negative feedback on TSH secretion (Larsen 1982, Larsen et al 1981). The ultimate inhibition of TSH release by T_3 binding to the nuclear receptor of the pituitary depends largely on intracellular conversion of T_4 to T_3 (see chapter I.B). Thus although a linear correlation exist between T_3 binding to the nucleus and the degree of TSH suppression (Silva and Larsen 1978) the actual serum TSH concentration correlates better with the serum T_4 concentration (Larsen 1982). Little is known about the potential role of changes in TRH release in the physiological regulation of TSH scretion and thyroid function. Long term glucocorticoid excess as seen in Cushing's disease may reduce the pituitary sensitivity for TRH (Sowers et al 1977, Visser and · Lamberts 1981) or inhibit the secretion of TRH by the hypothalamus (Singer et al 1978) while estrogen may potentiate the

TRH-induced TSH release (Reichlin 1978). Finally, dopamine administration results in a suppression of serum TSH probably by a direct effect on the pituitary (Kaptein et al 1980). Also dopamine receptor agonists suppress serum TSH whereas dopamine receptor antagonists stimulate TSH secretion (Foord et al 1983).
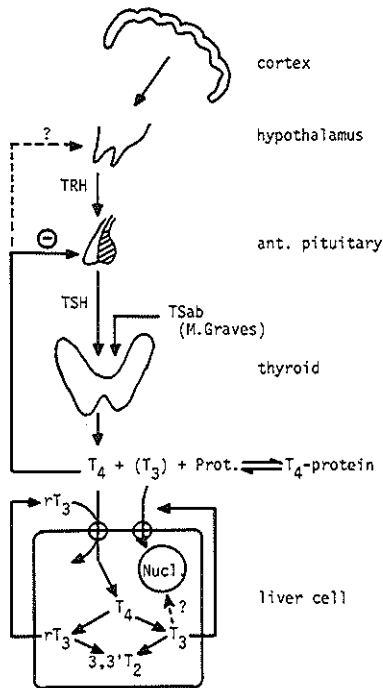
Fig.1. Schematic outline of the hypothalamus-pituitary- thyroid axis together with the liver cell as a potential production site for T_3.

I.B Thyroid hormone metabolism.

After secretion of T_4 into the circulation it becomes largely bound to specific serum proteins. In humans T_4 is mainly bound to T_4-binding globulin (TBG) and to lesser extents to T_4-binding prealbumin (TBPA) and albumin, resulting in a free concentration of approximately 0.03%

(Hoffenberg and Ramsden 1983). Recently, the presence of a specific T_4-binding albumin (TBA) has been reported as a normal constituent of human serum although in limited quantities (Docter et al 1984). Inherited elevation of TBA results just like in inherited TBG excess in an increase of serum total T_4 levels with normal free concentrations (Docter et al 1981, Barlow et al 1982). The free iodothyronine concentration is suggested to regulate the rate of undirectional transfer of hormone from plasma to tissue (Oppenheimer et al 1967). This transport phenomenon has been thought to be a passive diffusion-controlled process (Hillier 1970). Experiments with cultured rat hepatocytes have however shown the presence of an active transport system for iodothyronines (Krenning et al 1983).

A variety of metabolic pathways for thyroid hormone has been published. Figure 2 summarizes these reactions. The deiodination of T_4 is the most

1. Conjugation → Sulfate or glucuronide
2. Outer ring deiodination → T_3 as initial product
3. Etherbond cleavage → Diiodotyrosine, monoiodotyrosine
4. Inner ring deiodination → Reverse T_3 as initial product
5. Deamination → Tetrac, triac, diac
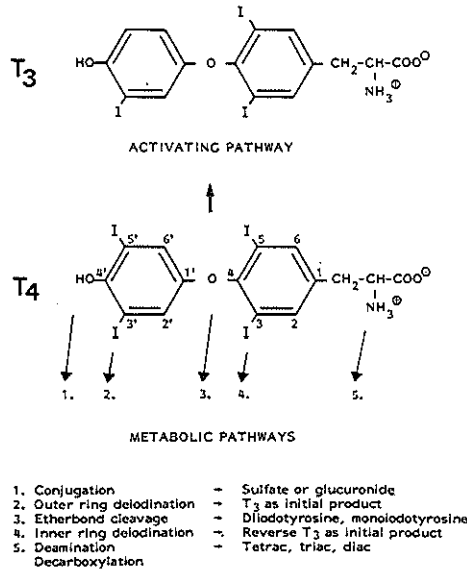   Decarboxylation

Fig.2. Pathways of T_4 metabolism.

important pathway and it may be calculated that in euthyroid humans approximately 80% of the total T_4 produced daily is metabolized by monodeiodination (Engler and Burger 1984). Outer ring or 5'-deiodination of T_4 gives rise to the production of T_3. Because the intrinsic thyromimetic activity of T_4, if anything, is low in comparison with that of T_3 it is nowadays generally accepted that T_3 is the biologically active thyroid hormone and T_4 serves merely as a prohormone (Chopra et al 1978[a], Visser 1978, Larsen et al 1981). About 80% of the serum T_3 concentration is derived from the 5'-deiodination of T_4 in peripheral tissues (Larsen et al 1981, Engler and Burger 1984). In this way about 30% of produced T_4 is converted to T_3. The remaining 20% is secreted by the thyroid. This secreted T_3 may in part be derived from intrathyroidal enzymatic deiodination of T_4 by an enzyme with similar characteristics as the 5'-deiodinase found in liver and kidneys (Erickson et al 1981, Ishii et al 1982, Wu 1983, Sugawara et al 1984).

A roughly equal proportion of T_4 is converted by inner ·ring or 5-deiodination to the biologically inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3). Thyroidal secretion of rT_3 is negligible so that peripheral deiodination accounts for 97.5% of the daily rT_3 production (Chopra 1976). Both triiodothyronines are further metabolized by stepwise deiodination to a series of diiodothyronines (T_2), monoiodothyronines (T_1) and finally thyronine (T_0). A central metabolite in this cascade is 3,3'-T_2, which is generated by both 5-deiodination of T_3 and 5'-deiodination of rT_3 (Gavin et al 1978, Chopra et al 1978[b]). In several clinical situations of non-thyroidal illness typical changes in the metabolism of T_4 are observed. These changes are characterized by a reduction of serum T_3 together with an increase of serum rT_3 concentration while T_4 levels often remain constant. Analysis of serum kinetic parameters of the triiodothyronines have demonstrated that the production of T_3 is decreased but its clearance is unaffected. Conversely, the production of rT_3 is apparently not changed but its metabolic clearance rate is decreased (Engler and Burger 1984, Hesch 1981). This situation is commonly refered to as the "low T_3 syndrome" and may be induced by caloric deprivation, systemic illness, surgical stress and the administration of certain drugs, i.e. propylthiouracil (PTU), propranolol, dexamethasone and

12

X-ray contrast agents. The teleological significance of the "low T_3 syndrome" may be that of an energy-sparing defense mechanism in situations of stress (Chopra et al 1978). The decrease of serum T_3 production with an unaltered metabolic clearance and the opposite effects on the kinetics of rT_3 has led to the hypothesis that the monodeiodination of T_4 is catalyzed by two different enzymes, i.e. a 5'- and a 5-deiodinase, which may be regulated independently. Recent findings have shown that this is very unlikely for the liver (Fekkes et al 1982[b], Chopra and Chua Teco 1982), which possesess a single enzyme capable of catalyzing both 5'- and 5-deiodinations. Furthermore, it has become clear that the mechanism and regulation of enzymatic deiodination differs among tissues (see chapter II.B).

Besides monodeiodinations some 20% of T_4 is metabolized by other pathways. These include conjugation of the phenolic hydroxyl group with glucuronic acid or sulfuric acid, ether link cleavage and oxidative deamination of the alanine side chain. Of these processes the iodothyronine conjugation is probably the major pathway. The conjugates are formed enzymatically by sulfotransferases in the cytoplasm or UDP-glucuronyl transferases in the endoplasmic reticulum of mainly liver and kidney. Both conjugating activities represent a panel of isoenzymes (Sekura et al 1981, Chowdurry et al 1983) with overlapping substrate specificities. Reviews on thyroid hormone conjugation have appeared recently (Engler and Burger 1984, Otten 1984[b]). Renewed interest in especially sulfation arises from recent findings that iodothyronine sulfates are much better substrates for the liver deiodinase in comparison with the native compounds (see chapter II).

Iodothyronine metabolism in the phagocytosing human leucocyte follows mainly the pathway of ether link cleavage, resulting in the formation of mainly diiodotyrosine and iodide. Ether link cleavage is associated with the "respiratory burst" of phagocytes, involving the generation of superoxide anion radicals and subsequently H_2O_2 which cause the oxidative breakdown of iodothyronines (Engler and Burger 1984, Burger et al 1983). Although ether link cleavage is normally a minor pathway it may be increased during infectious diseases and thereby accelerate T_4 turnover (Woeber 1971). Ether link cleavage requires enzymatic production of

radicals and H_2O_2 but is in nature a non-enzymatic process (Burger et al 1983). The ability of iodothyronines to react with free radicals make them excellent antioxidants (Tseng and Latham 1984).

Finally, the alanine side chain of iodothyronines may be converted by oxidative deamination or decarboxylation. Very little is known about the occurrence and effects of decarboxylated iodothyronines. They may have a β-adrenergic effect as revealed by 3,3',5-triiodothyronamine in an assay of cAMP production in turkey erythrocytes (Meyer and Rokos 1983). Also T_3 may have a neurotransmitter effect as is suggested by its synaptosomal accumulation (Dratman and Crutchfield 1978). More is known about the oxidative deamination resulting in the formation of acetic acid derivatives of iodothyronines. Experiments with dogs have shown that the acetic acid analogue of T_4 (tetrac) may be deiodinated in vivo (Flock et al 1962). These experiments have also shown that the acetic acid analogues may be glucuronidated and sulfated as determined by their appearance in the bile. The occurrence of reverse triac in canine serum after administration of radiolabeled tetrac in contrast with undetectable concentrations reverse triac in human serum (Engler and Burger 1984) may be caused by a remarkable difference between rT_3 deiodination as measured in vitro by dog liver microsomes (Laurberg and Boye 1982) or human and rat liver microsomes (Visser et al 1979[a], 1983[a]). Reverse T_3 is rapidly deiodinated by human and rat microsomes whereas rT_3 is more or less stable in incubations with dog liver microsomes. From the daily production rate of tetrac in humans it has been calculated that oxidative deamination is a very minor pathway and accounts for only 1 to 2% of the daily T_4 degradation (Engler and Burger 1984). Studies of Gavin et al (1980) have shown that up to 14% of the total T_3 amount produced daily may be converted to triac.

I.C **Scope of the present study**

As mentioned in the preceding paragraphs, enzymatic deiodination of T_4 is the most important route for the production of the biologically active thyroid hormone, T_3. The liver is regarded as the principal site for the peripheral production of T_3. Besides deiodination the liver is also most

active in the conjugation of the different iodothyronines with either glucuronic acid or sulfuric acid. Chapter II deals with a review of recent literature concerning cellular uptake and enzymatic deiodination of iodothyronines from experiments with cultured rat hepatocytes or subcellular fractions. The regulation and nature of the enzymatic deiodination in the liver will be compared with deiodinases found in other tissues. In order to gain more insight in the mechanism of enzymatic deiodination, attempts have been made to purify the deiodinating enzyme of rat liver and kidneys. Our efforts towards deiodinase purification will be discussed in conjunction with data available from the literature. Finally, some remarks will be made concerning the importance of the liver in thyroid hormone metabolism.

The following chapters describe investigations related to the mechanism of rat liver iodothyronine deiodinase. Chapters III and IV concern the development of convenient methods for the chemical synthesis of iodothyronine sulfate esters and sulfamates, and the subsequent study of the deiodination of these compounds, especially T_4 sulfate ester. The chemical modification of the iodothyronine deiodinase by diethylpyrocarbonate or photo-oxidation with Rose Bengal is reported in chapter V. These results strongly suggest the presence of an active site histidine residue. Chapter VI describes the use of N-bromoacetyl-T_3 as an affinity label for the deiodinase, while chapters VII and VIII deal with the partial purification of the enzyme from rat liver microsomal fraction. Basic knowledge of the deiodinating enzymes will help us to understand the alterations of serum iodothyronine concentrations in health and disease.

C H A P T E R   II

THE LIVER, A CENTRAL ORGAN FOR IODOTHYRONINE METABOLISM?

II.A Iodothyronine uptake in cultured rat hepatoytes.

The research into the enzymatic deiodination of thyroid hormone by rat liver has predominantly been carried out with tissue homogenates or the microsomal fraction. Some investigators have made use of whole-cell preparations, i.e. primary cultured hepatocytes, hepatoma cell lines or liver perfusions. For a long time little attention has been paid to the uptake process of iodothyronines into the liver cell as it was thought to be only a passive diffusion-controlled process without a regulatory function (Hillier 1970). However, increasing evidence has been obtained that the uptake of thyroid hormone into isolated rat liver cells is at least in part carrier-mediated (Krenning et al 1983). This specific uptake depends on the cellular ATP concentration and may be associated with the sodium gradient over the plasma membrane. As T_3 production is predominantly dependent on intracellular T_4 deiodination, it follows that any process that lowers the intracellular concentration of T_4 will reduce the production of biologically active thyroid hormone. Such a process that regulates the uptake of T_4 by tissue cells may also influence the metabolic clearance of T_3. Recently, tracer kinetic studies in obese subjects showed that the transport of T_4 and T_3 into tissues is diminished during caloric deprivation. This phenomenon was much more pronounced for T_4 than for T_3 (Van der Heyden et al 1985).

The similarity between cellular uptake of T_4 and T_3 is still controversial. Studies using cultured hepatocytes suggest that rat liver plasma membranes contain two different sites for transport of iodothyronines, i.e. a common T_4 and rT_3 site and a different T_3 site (Krenning et al 1981). Other studies with freshly isolated hepatocytes suggest that T_4 enters the cell by diffusion and that only T_3 transport is carrier-mediated (Rao and Rao 1983). Recent studies with a monoclonal antibody against rat liver cells show an equal inhibitory effect on T_4 and T_3 uptake by cultured hepatocytes without affecting the sodium gradient

over the plasma membrane, suggesting that T_4 and T_3 share a common carrier in the plasma membrane. By electrophoretic analysis of immunoprecipitated proteins it has been shown that the uptake is mediated by a 52,000 molecular weight protein of the plasma membrane (Mol et al 1984[c]). The concept of a regulatory role for the cell membrane in cellular T_4 metabolism merits full exploration.

II.B Enzymatic deiodination

Deiodination of iodothyronines in vitro was initially investigated by using tissue homogenates of both liver and kidney (Hesch et al 1975, Visser et al 1975, Hüfner et al 1977, Chiraseveenuprapund et al 1978). Several studies have shown that deiodinase activity is most abundant in liver and kidney (Chopra 1977, Kaplan et al 1979). Subcellular fractionation of these homogenates has shown that the enzymatic deiodination requires the presence of a microsomal enzyme and a cytoplasmic cofactor (Visser et al 1976, Leonard and Rosenberg 1978[a]). Until now no ultimate purification of the iodothyronine deiodinating enzyme nor the cytosolic cofactor has been reported. Our present knowledge of enzymatic deiodination has gained widely from the observation that enzyme activity is greatly enhanced by addition of sulfhydryl group-containing compounds such as dithiothreitol (DTT) and 2-mercaptoethanol to the microsomal fraction (Visser et al 1976, Leonard and Rosenberg 1978[a], Chopra 1978) (see also the section on cofactor requirements). The exact localization of the deiodinating enzyme is somewhat controversial. Most studies report on an endoplasmic reticulum localization (Auf dem Brinke et al 1979, 1980, Fekkes et al 1979, Saito et al 1980), while another report suggested an association with the plasma membranes although substantial copurification with marker enzymes of the endoplasmic reticulum was observed (Maciel et al 1979). The kidney deiodinating enzyme seems to be associated with the plasma membrane fraction (Leonard and Rosenberg 1978[a]).

Using a crude microsomal fraction in the presence of DTT it has been shown that at physiological pH T_4 is converted in roughly equal proportions by 5'-deiodination to T_3 or by 5-deiodination to rT_3 (Visser et al 1979[a]). Among the native iodothyronines rT_3 is by far the prefered substrate for

the deiodinase. It undergoes rapid 5'-deiodination to 3,3'-T_2, which is also produced slowly by 5-deiodination of T_3 (Visser et al 1979[a], Leonard and Rosenberg 1980[b], Fig. 3). Studies with cultured rat hepatocytes have shown that sulfation and deiodination of T_3 and 3,3'-T_2 are strongly related processes (Otten et al 1983, 1984, Visser et al 1983[b]). Inhibition of the sulfotransferase activity of these cells results in a diminution of the metabolism of T_3 and 3,3'-T_2 as measured by their disappearance from the culture medium and $^{125}I^-$ formation from radiolabeled substrates.
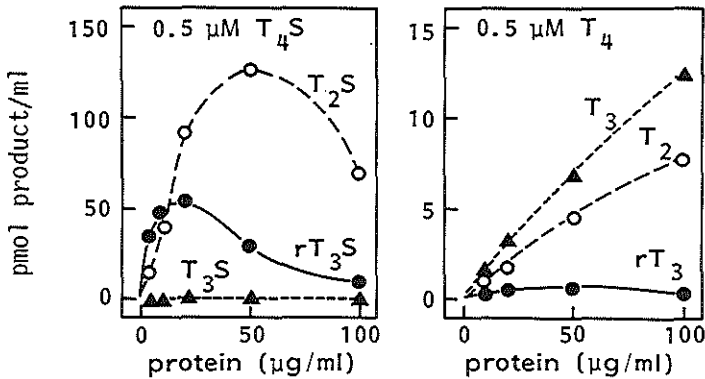

Fig.3. *Comparison of the deiodination of T_4 or T_4 sulfate in rat liver as a function of the enzyme concentration (See chapter IV).*

Inhibition of deiodinase activity with propylthiouracil (PTU) results in an increase of sulfated T_3 or 3,3'-T_2 in the culture medium. The effect of sulfation was further investigated using synthetic iodothyronine sulfate esters which were prepared either by reaction with concentrated H_2SO_4 or chlorosulfonic acid in dimethylformamide as is reported in chapter III. Sulfation of T_3 results in an increase of the V_{max} of approximately 30 times (Visser 1983[b]). The effect of sulfation on subsequent deiodination of T_4 and rT_3 is described in chapter IV. Although T_4 is normally converted to both T_3 and rT_3 (at pH 7.2), sulfation seems to inhibit the 5'-deiodinative pathway to T_3 sulfate whereas 5-deiodination to rT_3 sulfate is greatly enhanced (Fig. 4). The efficiency of the 5-deiodination of T_4

is stimulated 200-fold by sulfation due to an increase of V_{max} with a concomitant decrease of the K_m. Table I summarizes the measured kinetic parameters of the deiodination of both native and sulfated iodothyronines by rat liver deiodinase (see also the section on enzyme mechanism).

Nowadays consensus is achieved in that rat liver contains a single enzyme which is involved in 5-deiodination and 5'-deiodination of iodothyronines, called type I deiodinase. The hypothesis of a single enzyme was originally based on the equal competitive inhibition of the 5'-deiodination of $3',5'-T_2$ and the 5-deiodination of T_3 by a series of iodothyronines and radiographic contrast agents (Fekkes et al 1982[b]). Similar results were obtained by Chopra and Chua Teco (1982). Further arguments for one deiodinating enzyme are the co-purification of both 5- and 5'-deiodinating activities in a series of chromatographic steps after solubilization as described in the chapters VII and VIII, and an equal reaction mechanism (vide infra). Comparison of the 5-deiodination of T_4 sulfate and 5'-deiodination of rT_3 also strongly suggests that sulfated iodothyronines are converted by the same enzyme as the native iodothyronines (chapter IV).

Finally, a variety of competitive inhibitors of iodothyronine deiodinating activity have been reported, mainly compounds structurally related to the substrates. These include iodinated radiographic contrast agents (Bürgi et al 1976, Chopra et al 1978[a], Fekkes et al 1982[a]), and halogenated derivatives of the dyes phenolphthalein (Fekkes et al 1982[c]) and fluorescein (Ruiz and Ingbar 1982, Mol et al 1984[b]). The latter may also give rise to formation of oxygen radicals which lead to oxidative destruction of iodothyronine substrates or to oxidation of essential amino acid residues of the deiodinase. Furthermore, deiodinase inhibition has also been reported with phenolic compounds such as salicylic acid (Chopra et al 1980), diiodosalicylic acid (Fekkes et al 1982[a]), iodine-free polyphenolic plant extracts (Auf'mkolk et al 1984) and the 4-hydroxy metabolite of propranolol (Jørgensen et al 1984). Inhibition has also been reported with dicoumarol (Goswami et al 1982) and bilirubin (Fekkes et al 1982[c]). A review of the effects of drugs on iodothyronine metabolism in vivo has been published (Cavalieri and Pitt-Rivers 1981).

Effect of sulfation on the kinetic parameters of iodothyronine
deiodination by rat liver microsomes.

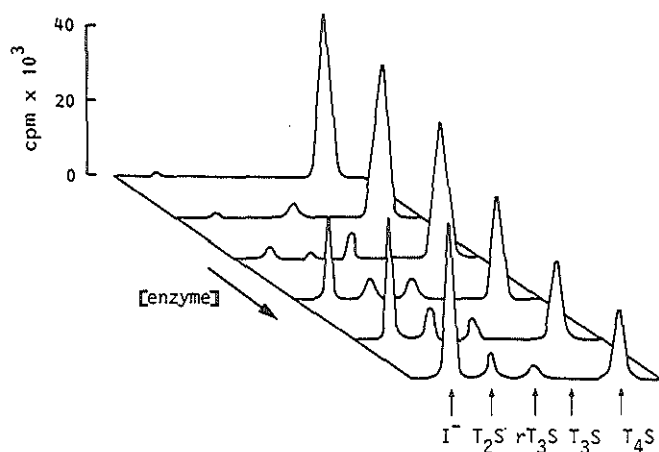| Substrate	Reaction	K_m [a]	V_{max} [b]	V_{max}/K_m
T_4	IRD	1.9	18	9
T_4S	IRD	0.3	530	1820
rT_3	ORD	0.06	560	8730
rT_3S	ORD	0.06	516	8600
T_3	IRD	6.2	36	6
T_3S	IRD	4.6	1050	230

[a] μM; [b] pmol/min/mg protein.

Fig.4. Analysis of the sequential deiodination of T_4 sulfate in rat liver by high performance liquid chromatography of the products formed as a function of the enzyme concentration (See chapter IV).

Enzyme mechanism

The present knowledge about the mechanism of type I iodothyronine deiodination is obtained from experiments with the crude microsomal fraction since purified enzyme preparations are still not available.
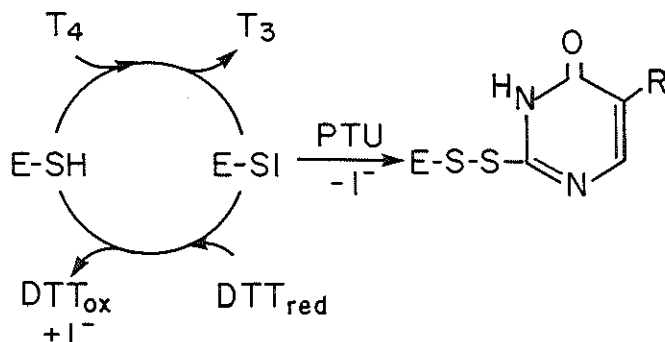

Fig.5. Mechanism of the enzymatic deiodination of iodothyronines in rat liver, and inhibition by PTU. The 5'-deiodination of T_4 is shown as an example.

Analysis of the stimulation of enzymatic deiodination with DTT as the cofactor showed parallel lines in the Lineweaver Burk plot of $3,3'-T_2$ formation from rT_3 at various fixed thiol concentrations. This suggested a ping-pong reaction mechanism with rT_3 acting as the first substrate that is converted to $3,3'-T_2$ with a concomitant generation of a modified enzyme form. Reaction of the second substrate, reduced DTT, with the intermediate enzyme form results in the regeneration of the native enzyme and the second product, oxidized DTT and I^-. This is also shown for the 5'-deiodination of T_4 to T_3 as depicted in Fig. 5 (Leonard and Rosenberg 1978[a], 1980[a], Visser 1979). Support for this proposed mechanism was obtained by studies with propylthiouracil (PTU), a competitive inhibitor of deiodinase activity with respect to DTT and an uncompetitive inhibitor with respect to iodothyronines (Leonard and Rosenberg 1978[b], Visser 1979). It has been shown that thiouracil reacts selectively with sulfenyl iodides (SI) forming mixed disulfides (Cunningham 1964). The reactivity of PTU towards the modified enzyme suggests that the latter contains a SI group. This is in agreement with the mechanism of inhibition by PTU and that of stimulation

by DTT and it is supported by the observation that covalent incorporation of labeled PTU into microsomes is stimulated in the presence of substrate (Visser and Van Overmeeren 1979, Leonard and Rosenberg 1980[a]). Studies with selective reagents for sulfhydryl (SH) groups, especially iodoacetate or N-ethylmaleimide, have indicated the presence of an active site SH group (Leonard and Rosenberg 1980[a], Leonard and Visser 1984). It was, therefore, concluded that the essential SH group probably acts as an acceptor of the leaving iodonium ion. In the absence of PTU, the resultant SI group is reduced to the SH form by DTT (Fig. 5). Other indications for the catalytic mechanism of iodothyronine deiodination by rat liver type I deiodinase stem from either modification reactions with the enzyme or from studies of the structure-activity relations using substrate analogues and cofactor analogues. The cofactor requirements will be described in the following section.

Reaction of microsomes with diethylpyrocarbonate (DEP) results in a rapid loss of deiodinase activity (chapter V). Inactivation with DEP follows pseudo first order reaction kinetics. The inactivation reaction is inhibited by substrate and modified enzyme is reactivated by treatment with hydroxylamine. The effect of pH suggests the modification of an amino acid residue with a pK value of 7.3. Together with the observation of a rose bengal-sensitized photo-oxidative inhibition of enzyme activity the location of an essential histidine residue at or near the active site is suggested (Mol et al 1984[b]). It is speculated that this histidine residue forms a hydrogen bond with the catalytically important SH group. This would explain the high reactivity of the latter.

Modification of the alanine side chain of iodothyronines shows that the deiodination of T_4 is inhibited with increasing potency when the side chain is modified from alanine, to N-acetyl-alanine or to acetic acid (Köhrle 1983). A special case is the T_3 analogue N-bromoacetyl-3,3',5-tri-iodothyronine (BrAcT$_3$) as reported in chapter VI. Incubation of rat liver microsomes with extremely low concentrations (<1 nM) of BrAcT$_3$ results in the irreversible loss of deiodinase activity due to covalent binding to the active site of the enzyme. Apparently, the reactive bromoacetyl group is in a very close proximity of a nucleophilic amino acid residue in the enzyme. The reaction of BrAcT$_3$ with the deiodinase is optimal at pH 7.25

although it is difficult to interpret the pH profile in terms of the dissociation of a single amino acid residue.

The effect of pH on the enzymatic deiodination of iodothyronines has been reported. The 5'-deiodination has its optimum around pH 6.5 while 5-deiodination is favoured by a slightly alkaline pH of around pH 8 (Köhrle 1983). The different optimal pH values for the various deiodinations have been used as arguments to speculate on the existence of two different deiodinating enzymes, a 5'-deiodinase and a 5-deiodinase, respectively. The latter possibility rejected, it has been suggested that the direction of the deiodination by a single liver enzyme is determined by a pH-induced alteration of the binding site for iodothyronines on the deiodinase rather than by the difference in the pK_a value of the phenolic OH-group of the substrates (Köhrle 1983). It has been speculated that the intracellular pH determines whether T_3 or rT_3 is formed from the prohormone T_4 (Köhrle 1983). The esterification of the phenolic OH-group with sulfuric acid shows that in general the negative charge of the sulfate does not influence explicitly the direction of the deiodination. The 3'-deiodination of $3,3'-T_2$ is stimulated 50 times mainly due to a decrease in K_m, whereas the 5-deiodination of T_3 is accelerated 40 times mainly as the result of an increase in V_{max} (Otten et al 1983, Visser et al 1983[b]). The 5'-deiodination of rT_3 is hardly changed after sulfation. Sulfation of T_4 results in a 200 fold acceleration of the 5-deiodination, chiefly as the result of an increase in V_{max}, whereas the 5'-deiodination of T_4 sulfate is not detectable (chapter IV). An answer to the question as to how the 5-deiodination and 5'-deiodination are regulated has to await the availibility of pure enzyme and cofactor preparations.

Finally the inhibition of deiodinase activity with thiouracil has been further investigated using structurally related compounds (Visser et al 1979[b], Chopra et al 1982, Harbottle and Richardson 1984). It was generally found that methylation of the nitrogen atom at position 1 adjacent to the essential 2-mercapto group prevents the inhibitory activity. Introduction of a hydrophobic propyl group at C_5 or C_6 stimulates inhibitory activity approximately 2 times whereas 5-iodo-2-thiouracil is 13 times as potent as thiouracil (Chopra et al 1982). The goitrogenic drug

24

1-methyl-2-mercaptoimidazole (methimazole) does not inhibit deiodinase activity due to the introduction of a methyl group on N_1. Non-methylated analogues of methimazole are inhibitory, greatest effects being observed with 2-mercaptobenzimidazole (Visser et al 1979[b]). In general, modification of inhibiting thiols by the introduction of hydrophilic substituents reduces the inhibitory potency (Harbottle and Richardson 1984), suggesting that the catalytic center of the deiodinase forms a hydrophobic pocket which one would also expect from the hydrophobic nature of iodothyronines.

Cofactor(s)

Until now the nature of the cellular cofactor(s) of rat liver deiodinase is unknown. The addition of well-known cofactors to the microsomal fraction like reduced or oxidized nicotinamide adenine dinucleotides and flavins is without effect (Visser et al 1975, 1976, Hüfner et al 1977, Chiraseveenuprapund et al 1978). Based on the findings of an essential SH group in the active site of liver deiodinase and the requirement of thiol reductants for enzyme activity, it was postulated that the most abundant cellular thiol, reduced glutathione (GSH), would act as the physiological cofactor. However, incubation of iodothyronines with rat liver microsomes in the presence of GSH shows no stimulation of deiodination (Goswami and Rosenberg 1983[a]) not even if a GSH regenerating system is included, consisting of glutathione reductase and NADPH (Mol, unpublished results). Addition of GSH or NADPH to liver homogenates of fasted rats, however, stimulates the deiodination of T_4 (Balsam et al 1979[a], Sato et al 1982). Addition of diamide to cultured rat hepatocytes causes a 83% decrease of the cellular GSH content by oxidation to GSSG with a concomitant inhibition of the deiodinating activity (Sato et al 1983[b]). When hepatocytes are cultured with a cystine and methionine deficient medium, which results in a 90% reduction of both GSH and GSSG content, no inhibition of deiodinating activity is found (Sato and Robbins 1981[b]). When tested with the isolated microsomal fraction, GSSG inhibits T_4 deiodination, probably by formation of an enzyme-GSH mixed disulfide. These results, therefore, suggest a regulatory role of the GSH-GSSG ratio

(Sato et al 1983[b]).

The naturally occurring thiol, dihydrolipoamide (DHL), has been found to stimulate deiodinase activity approximately 6 times as effectively as DTT in rat kidney microsomes (Goswami and Rosenberg 1983[a]). Addition of oxidized DHL in the presence of NADPH to rat kidney homogenates also enhances deiodinase activity. However, it is likely that DHL occurs mainly in a protein-bound form.

As NADPH stimulates deiodinase activity in rat liver homogenates, it is interesting to consider how electrons from NADPH are transferred to the sulfhydryl group(s) at the catalytic site of iodothyronine deiodinase. Recently it has been shown that a cytosolic protein may mediate the GSH-dependent reduction of rat kidney deiodinase (Goswami and Rosenberg 1983[b]). The existence of a number of thiol: disulfide-interchanging enzymes with broad specificities have been reported (Battelli and Lorenzoni 1982, Eklund et al 1984). One such electron carrier is thioredoxin, a 12,000 dalton protein with two cysteine residues in close proximity which form a disulfide in the oxidized state (Holmgren 1981). Preliminary experiments using thioredoxin from E. Coli in the presence of thioredoxin reductase and NADPH showed no stimulation of microsomal deiodinase activity (Mol, unpublished results). Another interesting electron carrier is glutaredoxin which may transfer reductive equivalents of GSH to other enzymes (Holmgren, 1976). In the presence of GSSG-reductase and NADPH, this system clearly stimulates deiodinating activity although to a limited extent (Mol, unpublished results).

The concept that the reduction of the deiodinase is mediated by glutaredoxin or a related protein needs to be fully explored.

Purification of rat liver iodothyronine deiodinase.

In rat liver the deiodinating enzyme is a constituent of the endoplasmic reticulum (Fig. 6). In order to purify the deiodinase it has to be solubilized from the membranes of the endoplasmic reticulum using non-denaturating detergents. It has been shown possible to solubilize deiodinase activity with the ionic detergents deoxycholate as used for the kidney enzyme, and cholate as used for the liver enzyme (Leonard and

Rosenberg 1981, Fekkes et al 1980). The apparent molecular weights of these preparations amounted to 50,000 and 65,000 dalton, respectively, as determined by both gel filtration and sucrose gradient centrifugation. However, these preparations are unstable and enzyme activity rapidly deteriorates probably due to denaturation and aggregation. Leonard and Rosenberg (1981) have demonstrated that this is partially overcome by addition of soybean lipids to the dispersed kidney enzyme. Some progress has been made by Fekkes et al (1983) using the non-ionic detergent W-1 ether. Isoelectric focusing of a W-1 ether extract of microsomes revealed an isoelectric point near pH 6.4. However, after removal of phospholipids the W-1 ether-dispersed enzyme concentrated at pH 9.3, showing that the rat liver deiodinase is a basic protein. Further purification proved to be difficult.
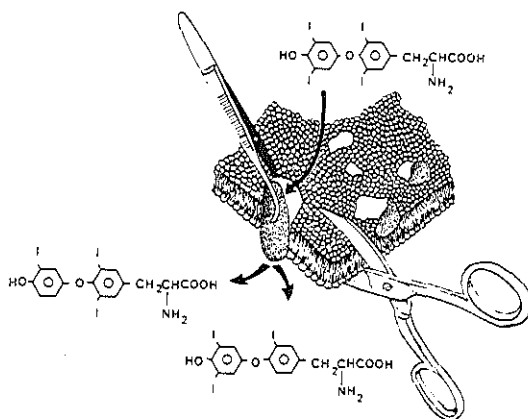

Fig.6. Purification of rat liver iodothyronine deiodinase.

Renewed investigations into which detergents are useful for subsequent purification are presented in chapter VII. In order to obtain a stable enzyme preparation high detergent concentrations are necessary. It appears that the non-ionic detergent Emulgen-911 is most suitable for column chromatography when used in the presence of 20% glycerol and a minimal NaCl concentration of 15 mM. Using rat liver microsomes from hyperthyroid rats

Table II Characteristics of the pathways of iodothyronine deiodination in the rat

	Type I	Type II	Type III
Deiodination site	phenolic and tyrosyl rings	phenolic ring only	tyrosyl ring only
Substrate preference	$rT_3 \gg T_4 \gg T_3$	$T_4 \geq rT_3$	$T_3 > T_4$
Kinetic pattern	ping-pong	sequential	sequential
K_m for T_4 in microsomal preparations	$\sim 1\,\mu M$ (at 1–5 mM DTT)	~ 1 nM (at 20 mM DTT)	~ 40 nM (at 50 mM DTT)
Tissue localization	highest in liver, kidney and thyroid, present in many other organs	pituitary, central nervous system, and brown adipose tissue	central nervous system, eye, and placenta
Thiol reductants	stimulatory	stimulatory	stimulatory
Propylthiouracil	inhibitory	no effect	no effect
Iopanoic acid	inhibitory	inhibitory	inhibitory
Hypothyroidism	reduced activity in liver and kidney, increased in thyroid	increased activity	decreased activity, in brain
Fetal/neonatal life	decreased in liver	increased in pituitary, complex in brain	increased in brain and eye

The identities of the pathways in the thyroid gland, eye and placenta are probable, but not definite. DTT = Dithiothreitol.

Michael M. Kaplan   (1984)

an ultimate purification of about 2400 times is achieved by means of a variety of chromatographic steps, details of which are presented in chapter VII and VIII. The thus obtained enzyme preparation is approximately 50% pure but shows to be labile. This may be due to dissociation of subunits since affinity-labeling experiments with $BrAcT_3$ (chapter VI) suggest that the deiodinase is composed of subunits.

Iodothyronine deiodination in other tissues.

The characteristics of T_4 and rT_3 deiodination in rat kidney are very similar to those of the rat liver enzyme (Kaplan et al 1979). However, clearly different deiodinase activities are found in other tissues. Until now three different iodothyronine deiodinases have been described (Table II). The enzyme predominantly found in liver, kidneys and also thyroid gland is called type I deiodinase (D-I). It is characterized by its susceptibility to inhibition by PTU and iodoacetate, by the postulated ping-pong reaction mechanism and by the preference for rT_3 as the substrate. Type II 5'-deiodinase (5'D-II) is mainly found in the central nervous system, pituitary gland, brown adipose tissue and placenta (Kaplan 1980, 1984, Melmed et al 1981, Visser et al 1981, 1982, 1983[c], Leonard et al 1983). Type II deiodinase is a specific 5'-deiodinase which requires high concentrations of DTT in comparison with type I, is not inhibited by PTU and less sensitive to inhibition with iodoacetate (Visser et al 1982, 1983[d]). Under the conditions used, type II deiodinase is also characterized by low K_m and V_{max} values. Analysis of the enzyme kinetics suggests a "sequential" reaction mechanism (Visser et al 1982). Furthermore, T_4 is the prefered substrate for the type II enzyme.

The third iodothyronine deiodinase is a true 5-deiodinase which only catalyzes the deiodination of the tyrosyl ring. Type III 5-deiodinase (5D-III) is mainly found in human and rat placenta (Roti et al 1981, 1982, Fay et al 1984) and in the central nervous system of the rat (Kaplan and Yaskoski 1980). It is characterized by intermediate K_m values, and it is not or only little affected by PTU. As proposed for 5'D-II this specific 5-deiodinase also shows "sequential" reaction kinetics with T_3 as substrate and DTT as cofactor (Kaplan et al 1983).

II.C Regulation of deiodinase activity in vivo.

Age related changes in enzyme content

The production of the biologically active thyroid hormone T_3 from T_4 by type I deiodinase (D-I) as found in liver, kidneys and thyroid, and by type II deiodinase (5'D-II) as found in the central nervous system, pituitary and brown adipose tissue depends largely on age, thyroid status, diet and exposure to a variety of drugs. In this section some remarks will be made concerning the regulation of the deiodinase activity in a variety of tissues with special emphasis on the contribution by the liver to plasma T_3. The regulation at the level of the deiodinase activities will be discussed here, although thyroid hormone metabolism may also be regulated by changes in the availibility of intracellular hormone through alterations in plasma binding or in cellular uptake (Hoffenberg and Ramsden 1983, Van der Heyden et al 1985). The content of D-I appears to follow a general maturational pattern for all tissues studied so far. Neonatal rat livers contain low 5'-DI activities which increase with age until an adult level is attained at 20-30 days of age (Köhrle 1983, Sato et al 1983[a]). No differences in the development of rat liver 5D-I and 5'D-I activities are detected (Köhrle 1983) which is additional evidence that both activities are intrinsic to a single enzyme (Fekkes et al 1982[b]). It has been suggested that the low D-I activity in the fetal and neonatal period is not only a function of D-I content but also caused by a reduced capacity of NADPH to support deiodination in cell homogenates (Sato et al 1983). In the female rat a significant decrease in hepatic T_3 generation is observed at the age of 30 days which remains constant thereafter at approximately 50% of the value in age-matched male rats. This sex-related difference is attributed to a decreased enzyme content rather than differences in cofactor concentrations (Harris et al 1979). 5'D-II activity in the hypothalamus increases also with age in contrast with 5D-III as measured with T_3 as the substrate which has the highest activity in fetal rats and shows a rapid decline during the first weeks of life (Kaplan 1984, McCann et al 1984). Similar observations have been made in rat retina during postnatal development (Ientile et al 1984).

Thyroid status

It has been shown that the content of deiodinase in the various tissues depends largely on the thyroid status. In general, D-I activity in especially liver is reduced by thyroidectomy to approximately 30% of the values found in the euthyroid rat whereas a 2-3 fold increase is observed in rats injected with T_4 to render them thyrotoxic (Kaplan 1979[b], Balsam et al 1979[b]). Results obtained with the microsomal fraction in the presence of DTT strongly suggest effects on enzyme content (Kaplan 1979[b]) rather than changes in cofactor concentrations as proposed by others (Balsam et al 1979[b]). The effect of hypothyroidism on D-I requires at least a few days before a decrease will be observed. Similar changes in enzymatic activity in altered thyroid states have been reported for the kidneys (Kaplan et al 1979, Larsen et al 1981). Interestingly, the D-I of rat thyroid depends on circulating TSH concentrations (Erickson et al 1982, Ishii et al 1983, Wu 1983). Ten days after hypophysectomy, when T_4 levels are maintained at control levels by T_4 replacement, no detectable D-I is present in the thyroid while the liver content remains normal (Erickson et al 1982). Inhibition of T_4 production by chronic ingestion of methimazole leads to a concomitant increase in serum TSH and D-I content of the thyroid. In this case rat liver D-I levels decrease due to the low plasma T_4 concentration.

In contrast with the slow adaption of the liver enzyme to an altered thyroid status, 5'D-II of the cerebral cortex responds rapidly to changes in thyroid hormone concentration (Leonard et al 1981). Within 24 hours after thyroidectomy a 2-4 fold increase in 5'D-II is observed. The increase in chronically hypothyroid animals is normalized within 4 hours after injection of T_3. It has been shown that in short-term hypothyroidism (12 days) the augmentation in 5'-deiodination is the result of an increase in the specific 5'-DII enzyme which is insensitive to inhibition by PTU (Visser et al 1981, 1982). The 5'D-II appears also to be present in secretory cells of the pituitary with highest activities in isolated lactotrophs and somatotrophs and a remarkably lower enzyme level in thyrotrophs (Koenig et al 1984). The increase in 5'D-II content of the cerebral cortex and pituitary in hypothyroid rats is achieved by a decrease in the degradation/inactivation rate rather than by an increase in the

31

synthesis of the enzyme (Leonard et al 1984). Conversely, administration of T_3 induces an increase in 5'D-II turnover, by an extra-nuclear action which requires no protein synthesis. This is consistent with reports on a rapid decrease of 5'D-II levels after administration of T_4 or rT_3 (Silva et al 1983, Kaiser et al 1984). The 5'D-II of brown adipose tissue increases also in hypothyroidism (Leonard et al 1983).

Finally, the specific 5D-III content of the cerebral cortex increases in hyperthyroid rats and decreases or remains constant in hypothyroid neonatal rats (Kaplan et al 1983).

Hormonal regulation of T_4 monodeiodination.

States of hypoinsulinaemia, i.e. fasting or diabetes mellitus, are associated with decreased serum T_3 concentrations and elevated rT_3 concentration in man, the so-called "low T_3 syndrome" (Saunders et al 1978). Turnover studies have shown that during fasting serum T_3 is decreased due to lowered production rates, whereas plasma rT_3 increases rather as a result of decreased elimination rates (Suda et al 1978). Various hypotheses have been put forward to explain the altered production of T_3, namely a decrease in deiodinase content (Balsam et al 1981, Gavin et al 1981), a decrease in cytosolic cofactor(s) (Balsam et al 1979) or a reduced uptake of T_4 by the liver (Jennings et al 1979, Van der Heyden et al 1985). Fasting-induced changes of T_3 and rT_3 plasma levels are restored predominantly by refeeding with a carbohydrate-rich diet in the rat (Gavin et al 1981), indicating a glucose-mediated effect. Studies with rat hepatocytes in primary culture show that 5'-deiodinase activity is reduced if the GSSG/GSH ratio is increased by diamide. This effect is not seen in the presence of glucose in the medium (Sato et al 1983[b]). It has been suggested that glucose maintains enzyme activity by keeping glutathione in the reduced form, probably through the supply of NADPH as cofactor for GSSG-reductase. Furthermore, both in vitro (cultured hepatocytes) and in vivo administration of insulin enhances the deiodinase activity of the liver (Sato and Robbins 1981[a], Loos et al 1984). The findings reported by Loos et al (1984) strongly suggest that insulin acts directly on de novo synthesis of the deiodinase. The alteration in T_3 production consequent on

dietary restriction or diabetes are not mediated by high glucagon levels (Senga et al 1982, Gavin and Moeller 1983[a]) although glucagon may inhibit the insulin-mediated increase of deiodinase in cultured hepatocytes (Sato and Robbins 1981[a]). Finally, fasting and diabetes are associated with an increased pancreatic content of somatostatin. A continuous somatostatin infusion inhibits hepatic 5'D-I in a carbohydrate-fed rat and prevents the normalization of enzyme activity by carbohydrate-refeeding of fasted rats (Gavin and Moeller 1983[b]).

Fasting of adult rats results in decreased serum T_4 and TSH concentrations. Significantly higher 5'-deiodination rates in hypothalamic homogenates are observed in fasted rats (Kaplan and Yaskoski 1982). No effect was seen either on che 5-deiodinase activity in cerebral cortex and hypothalamus or on the 5'-deiodinase activity of cerebrocortical homogenates (Kaplan and Yaskoski 1982). Also the inability of somatostatin to inhibit pituitary 5'-deiodinase indicates that the 5'D-II deiodinase is regulated quite differently (Gavin and Moeller 1983[b]).

Obviously, the low T_3 syndrome has a complex etiology and may be caused by tissue specific effects on deiodinase content, cofactor or substrate availability. Also species differences may exist. When rats are fasted serum concentrations of TSH, T_4 and T_3 decrease whereas fasting in humans is accompanied only by a decrease in serum T_3 with a reciprocal rise in rT_3 concentrations. The rise in serum concentrations of rT_3, however, is not observed in fasting dogs (De Bruijne et al 1981, Laurberg and Boye 1984).

Contribution of rat liver deiodinase to plasma iodothyronine concentrations.

In euthyroid rats, PTU reduces the extrathyroidal conversion of T_4 to T_3 by almost 70%, stressing the importance of the type I deiodinase of liver and kidney for maintaining plasma T_3 concentrations (Silva et al 1984). In the hyperthyroid rat the contributions of the PTU-sensitive type I deiodinase may even be greater. The high specific activity of rat liver deiodinase together with the fact that the liver contains a major proportion of the total intracellular T_4 pool (Hennemann 1981) makes it reasonable that the liver is an important contributor to the circulating

T_3. Circumstantial evidence is further obtained from the observation of low T_3 concentrations during liver cirrhosis (Chopra 1981). Also in chronic renal failure reduced T_3 concentrations are observed (Kaptein et al 1983). However, during hypothyroidism the contribution of rat liver to serum T_3 concentrations diminishes and almost all circulating T_3 in thyroidectomized rats may be produced via a PTU-insensitive pathway (Silva et al 1984). The contribution of the PTU-sensitive deiodinase of the thyroid gland to plasma T_3 concentration during hypothyroidism remains, however, to be established.

The liver contributes not only to the production of T_3, but also regulates the elimination of iodothyronines from the plasma pool. From studies using cultured rat hepatocytes it has been shown that elimination of T_3 largely depends on sulfation prior to deiodination. It is, therefore, suggested that the rate of T_3 disposal may depend predominantly on enzymatic sulfation. It is speculated that the unaltered metabolic clearance of T_3 in cases of the low T_3 syndrome may reflect an intact sulfation and glucuronidation system. The metabolic clearance of rT_3 depends probably solely on deiodination and is consequently diminished if liver deiodinase activity is impaired. The fact that the production of plasma rT_3 is unaltered after administration of PTU makes it likely that rT_3 is mainly derived from 5D-III activities. From the tissue specific deiodinase activities, it is suggested that the low T_3 syndrome may especially affect tissues that depend on the plasma T_3 concentration, whereas the tissues that depend on local conversion of T_4 to T_3 are less affected.

REFERENCES

Auf dem Brinke, D., Hesch, R.D. and Köhrle, J. (1979) Reexamination of the subcellular localization of thyroxine 5'-deiodination in rat liver. Biochem. J. 180: 273–279.

Auf dem Brinke, D., Köhrle, J., Ködding, R. and Hesch, R.D. (1980) Subcellular localization of thyroxine 5-deiodinase in rat liver. J. Endocrinol. Invest. 3: 73–76.

Auf'mkolk, M., Köhrle, J., Gumbinger, H., Winterhoff, H. and Hesch, R.D. (1984) Antihormonal effects of plant extracts: iodothyronine deiodinase of rat liver is inhibited by extracts and secondary metabolites of plants. Horm. Metab. Res. 16: 188–192.

Balsam, A., Ingbar, S.H., and Sexton, F. (1979a) Observations on the factors that control the generation of triiodothyronine from thyroxine in rat liver and the nature of the effect induced by fasting. J. Clin. Invest. 63: 1145–1156.

Balsam, A., Sexton, F. and Ingbar, S.H. (1979b) The mechanism of impaired in vitro generation of 3,5,3'-triiodothyronine from thyroxine in livers of hypothyroid rats. Endocrinology 105: 1115–1121.

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

Barlow, J.W., Csicsmann, J.M., White, E.L., Funder, J.W. and Stockigt, J.R. (1982) Familial euthyroid thyroxine excess: characterization of abnormal intermediate affinity thyroxine binding to albumin. J. Clin. Endocrinol. Metab. 55: 244–250.

Battelli, M. and Lorenzoni, E. (1982) Purification and properties of a new glutathione-dependent thiol: disulphide oxidoreductase from rat liver. Biochem. J. 207: 133–138.

Burger, A.G., Engler, D., Buergi, U., Weissel, M., Ingbar, S.H., Rosin, R.E. and Babior, B.M. (1983) Ether link cleavage is the major pathway of iodothyronine metabolism in the phagocytosing human leukocyte and also occurs in vivo in the rat. J. Clin. Invest. 71: 935–949.

Bürgi, H., Wimpfheimer, C., Burger, A., Zaubauer, W., Rösler, H. and Lemarchand-Beraud, T. (1976) Changes of circulating thyroxine, triiodothyronine and reverse triiodothyronine after ·radiographic contrast agents. J. Clin. Endocrinol. Metab. 43: 1203–1210.

Cavalieri, R.R. and Pitt-Rivers, R. (1981) The effects of drugs on the distribution and metabolism of thyroid hormones. Pharmacol. Rev. 33: 55–80.

Chiraseveenuprapund, P., Bürgi, U., Goswami, A. and Rosenberg, I.N. (1978) Conversion of L-thyroxine to triiodothyronine in rat kidney homogenate. Endocrinology 102: 612–622.

Chopra, I.J. (1976) An assesment of daily turnover and significance of thyroidal secretion of reverse T3. J. Clin. Invest. 58: 32–40.

Chopra, I.J. (1977) A study of extrathyroidal conversion of thyroxine (T4) to 3,3',5-triiodothyronine (T3) in vitro. Endocrinology 101: 453–463.

Chopra, I.J. (1978) Sulfhydryl groups and the monodeiodination of thyroxine to triiodothyronine. Science 199: 904–905.

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

Chopra, I.J., Wu, S.Y., Nakamura, Y. and Solomon, D.H. (1978b) Monodeiodination of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine to 3,3'-diiodothyronine in vitro. Endocrinology 102: 1099–1106.

Chopra, I.J., Solomon, D.H., Chua Teco, G.N. and Nguyen, A.H. (1980) Inhibition of hepatic outer ring monodeiodination of thyroxine and 3,3',5'-triiodothyronine by sodium salicylate. Endocrinology 106: 1728–1734.

Chopra, I.J. (1981) Triodothyronines in health and disease. In: Monographs on Endocrinology, Springer-Verlag, New York.

Chopra, I.J. and Chua Teco, G.N. (1982) Characteristics of inner ring (3 or 5) monodeiodination of 3,5-diiodothyronine in rat liver: evidence suggesting marked similarities of inner and outer ring deiodinases for iodothyronines. Endocrinology 110: 89–97.

Chopra, I.J., Chua Teco, G.N., Eisenberg, J.B., Wiersinga, W.M. and Solomon, D.H. (1982) Structure-activity relationship of inhibition of hepatic monodeiodination of thyroxine to 3,5,3'-triiodothyronine by thiouracil and related compounds. Endocrinology 110: 163–168.

Chowdurry, J.R., Chowdurry, N.R., Moscioni, A.D., Tukey, R., Tephly, T. and Arias, I.M. (1983) Differential regulation by triiodothyronine of substrate specific uridine diphosphoglucuronate glucuronosyl transferases in rat liver. Biochim. Biophys. Acta 761: 58–65.

Cunningham, L.W. (1964) The reaction of -lactoglobulin sulfenyl iodide with several antithyroid agents. Biochemistry 3: 1629–1634.

DeBruijne, J.J., Altszuler, N., Hampshire, J., Visser, T.J. and Hackeng, W.H.L. (1981) Fat mobilization and plasma hormone levels in fasted dogs. Metabolism 30: 190–194.

DeGroot, L.J. and Niepomniszcze, R. (1977) Biosynthesis of thyroid hormone: basic and clinical aspects. Metabolism 26: 665–718.

Docter, R., Bos, G., Krenning, E.P., Fekkes, D., Visser, T.J. and Hennemann, G. (1981) Inherited thyroxine excess: a serum abnormality due to an increased affinity for modified albumin. Clin. Endocrinol. 14: 363–371.

Docter, R., Bos, G., Krenning, E.P. and Hennemann, G. (1984) Specific thyroxine binding albumin is a constituent of normal serum. The Lancet I : 50.

Dratman, M.B. and Crutchfield, F.L. (1978) Synaptosomal [125-I]triiodothyronine after intravenous [125-I]thyroxine. Am. J. Physiol. 235: E638–E647.

Eklund, H., Cambillau, C., Sjöberg, B.M., Holmgren, A., Jörnvall, H., Höög, J.O. and Bränden, C.I. (1984) Conformational and functional similarities between glutaredoxin and thioredoxins. EMBO Journal 3: 1443–1449.

Engler, D. and Burger, A.G. (1984) The deiodination of the iodothyronines and of their derivatives in man. Endocr. Rev. 5: 151–184.

Erickson, V.J., Cavalieri, R.R. and Rosenberg, L.L. (1981) Phenolic and nonphenolic ring iodothyronine deiodinases from rat thyroid gland. Endocrinology 108: 1257–1264.

Erickson, V.J., Cavalieri, R.R. and Rosenberg, L.L. (1982) Thyroxine 5'-deiodinase of rat thyroid, but not that of liver, is dependent on thyrotropin. Endocrinology 111: 434–440.

Fay, M., Roti, E., Fang, S.L., Wright, G., Braverman, L.E. and Emerson,

C.H. (1984) The effects of propylthiouracil, iodothyronines, and other agents on thyroid hormone metabolism in human placenta. J. Clin. Endocrinol. Metab. 58: 280-285.

Fekkes, D., Van Overmeeren-Kaptein, E., Docter, R., Hennemann, G. and Visser, T.J. (1979) Location of rat liver iodothyronine deiodinating enzymes in the endoplasmic reticulum. Biochim. Biophys. Acta 587: 12-19.

Fekkes, D., Van Overmeeren, E., Hennemann, G. and Visser, T.J. (1980) Solubilization and partial characterization of rat liver iodothyronine deiodinases. Biochim. Biophys. Acta 613: 41-51.

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

Fekkes, D., Hennemann, G. and Visser, T.J. (1982b) Evidence for a single enzyme in rat liver catalyzing the deiodination of the tyrosyl and the phenolic ring of iodothyronines. Biochem. J. 201: 673-676.

Fekkes, D., Hennemann, G. and Visser, T.J. (1982c) Inhibition of iodothyronine deiodinase by phenolphthalein dyes. FEBS Lett. 137: 40-44.

Fekkes, D., Hennemann, G. and Visser, T.J. (1983) Properties of detergent-dispersed iodothyronine 5- and 5'-deiodinase activities from rat liver. Evidence for a common enzyme. Biochim. Biophys. Acta 742: 324-333.

Flock, E.V., Bollman, J.L. and Stobie, G.H.C. (1962) Metabolic pathways of tetraiodothyroacetic acid, triiodothyroacetic acid, tetraiodothyropropionic acid and triiodothyropropionic acid. Biochem. Pharmacol. 11: 627-632.

Foord, S.M., Peters, J.R., Dieguez, C., Scanlon, M.F. and Hall, R. (1983) Dopamine receptors on intact anterior pituitary cells in culture: function association with the inhibition of prolactin and thyrotropin. Endocrinology 112: 1567-1576.

Gavin, L.A., Hammond, M.E., Castle, J.N. and Cavalieri, R.R. (1978) 3,3'-Diiodothyronine, a major pathway of peripheral iodothyronine metabolism in man. J. Clin. Invest. 61: 1276-1285.

Gavin, L.A., Livermore, B.M., Cavalieri, R.R., Hammond, M.E. and Castle, J.N. (1980) Serum concentration, metabolic clearance, and production rates of 3,5,3'-triiodothyroacetic acid in normal and athyreotic man. J. Clin. Endocrinol. Metab. 51: 529-535.

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

Gavin, L.A. and Moeller, M. (1983a) Glucagon does not modulate the alterations in T3 metabolism consequent to dietary manipulation and diabetes. Diabetes 32: 798-803.

Gavin, L.A. and Moeller, M. (1983b) Somatostatin inhibits rat hepatic T4 5'-deiodinase. The effect is independent on the associated hypoinsulinemia. J. Clin. Invest. 72: 2020-2030.

Goswami, A., Leonard, J.L. and Rosenberg, I.N. (1982) Inhibition by coumadin anticoagulants of enzymatic outer ring monodeiodination of iodothyronines. Biochem. Biophys. Res. Commun. 104: 1231-1238.

Goswami, A. and Rosenberg, I.N. (1983a) Stimulation of iodothyronine outer ring monodeiodinase by dihydrolipoamide. Endocrinology 112: 1180-1187.

Goswami, A. and Rosenberg, I.N. (1983b) A cytosolic protein mediates

GSH-responsiveness of a low K_m rat kidney microsomal iodothyronine 5'-deiodinase (5'D). 65th Annual Meeting of the Endocrine Society, San Antonio, Texas, p. 266.

Harbottle, R. and Richardson, S.J. (1984) Structural requirements of thiol compounds in the inhibition of human liver iodothyronine 5'-deiodinase. Biochem. J. 217: 485-491.

Harris, A.R.C., Vagenakis, A.G. and Braverman, L.E. (1979) Sex related differences in outer ring deiodination of thyroxine and 3,3',5'-triiodothyronine by rat liver homogenates. Endocrinology 104: 645-652.

Hennemann, G. (1981) Transport and distribution of thyroid hormones. In: The low T3 syndrome, Hesch, R.D. (Ed) Academic Press, New York, vol. 40: 41-47.

Hesch, R.D., Brunner, G. and Söling, H.D. (1975) Conversion of thyroxine (T4) and triiodothyronine (T3) and the subcellular localisation of the converting enzyme. Clin. Chim. Acta 59: 209-213.

Hesch, R.D. (Ed) (1981) The Low T3 Syndrome. Proceedings of the Serono Symposia, Vol. 40, Academic Press, New York.

Hillier, A.P. (1970) The binding of thyroid hormones to phospholipid membranes. J. Physiol. (London) 211: 585-597.

Hoffenberg, R. and Ramsden, D.B. (1983) The transport of thyroid hormones. Clin. Sci. 65: 337-342.

Holmgren, A. (1976) Hydrogen donor system for E. Coli ribonucleoside-diphosphate reductase dependent upon glutathione. Proc. Natl. Acad. Sci. USA 73: 2275-2279.

Holmgren, A. (1981) Thioredoxin: structure and functions. Trends Biochem. Sci. 6: 26-29.

Hufner, M., Grussendorf, M. and Ntokalou, M. (1977) Properties of the thyroxine monodeiodinating system in rat liver homogenates. Clin. Chim. Acta 78: 251-259.

Ientile, R., Macaione, S., Russo, P., Pugliese, G. and DiGiorgio, R.M. (1984) Phenolic and tyrosyl ring deiodination in thyroxine from rat retina during postnatal development. Eur. J. Biochem. 142: 15-19.

Ishii, H., Inada, M., Tanaka, K., Mashio, Y., Naito, K, Nishikawa, M., Matsuzuka, F., Kuma, K. and Imura, H. (1982) Sequential deiodination of thyroxine in human thyroid gland. J. Clin. Endocrinol. Metab. 55: 890-896.

Ishii, H., Inada, M., Tanaka, K., Mashio, Y., Naito, K., Nishikawa, M., Matsuzuka, F., Kuma, K. and Imura, H. (1983) Induction of outer ring and inner ring monodeiodinases in human thyroid gland by thyrotropin. J. Clin. Endocrinol. Metab. 57: 500-505.

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

Jørgensen, P.H.. Lumholtz, I.B., Faber, J., Kirkegaard, C., Siersbaek-Nielsen, K. and Friis, T. (1984) The effect of D,L-4-hydroxypropranolol on the thyroxine to 3,3',5-triiodothyronine conversion in rat renal and liver microsomes. Acta Endocrinol. 105: 205-210.

Kaiser, C.A., Goumaz, M.O. and Burger, A.G. (1984) In vivo inhibition of brain cortex 5'-deiodinase by rT3. Ann. Endocrinol. 45: 65.

Kaplan, M.M., Tato, J.B., Breitbart, R. and Larsen, P.R. (1979) Comparison of thyroxine and 3,3',5'-triiodothyronine metabolism in rat kidney and liver homogenates. Metabolism 28: 1139-1146.

Kaplan, M.M. (1979a) Subcellular alterations causing reduced hepatic thyroxine 5'-monodeiodinase activity in fasted rats. Endocrinology 104: 58-64.

Kaplan, M.M. (1979b) Changes in the particulate subcellular component of hepatic thyroxine 5'-monodeiodinase in hyperthyroid rats. Endocrinology 105: 548-554.

Kaplan, M.M. (1980) Thyroxine 5'-monodeiodination in rat anterior pituitary homogenate. Endocrinology 106: 567-576.

Kaplan, M.M. and Yaskoski, K.A. (1980) Phenolic and tyrosyl ring deiodination of iodothyronines in rat brain homogenates. J. Clin. Invest. 66: 551-562.

Kaplan, M.M. and Yaskoski, K.A. (1982) Effects of congenital hypothyroidism and partial and complete food deprivation on phenolic and tyrosyl ring deiodination in rat brain. Endocrinology 110: 761-767.

Kaplan, M.M., Visser, T.J., Yaskoski, K.A. and Leonard, J.L. (1983) Characteristics of iodothyronine tyrosyl ring deiodination by rat cerebral cortical microsomes. Endocrinology 112: 35-42.

Kaplan, M.M. and Shaw, E.A. (1984) Type II iodothyronine 5'-deiodination by human and rat placenta in vitro. J. Clin. Endocrinol. Metab. 59: 253-257.

Kaplan, M.M. (1984) The role of thyroid hormone deiodination in the regulation of hypothalamo-pituitary function. Neuroendocrinology 38: 254-260.

Kaptein, E.M., Spencer, C.A., Kamiel, M.B. and Nicoloff, J.T. (1980) Prolonged dopamine administration and thyroid hormone economy in normal and critical ill subjects. J. Clin. Endocrinol. Metab. 51: 387-393.

Kaptein, E.M., Feinstein, E.I., Nicoloff, J.T. and Massry, S.G. (1983) Serum reverse triiodothyronine and thyroxine kinetics in patients with chronic renal failure. J. Clin. Endocrinol. Metab. 57: 181-189.

Koenig, R.J., Leonard, J.L., Senator, D., Rappaport, N., Watson, A.Y. and Larsen, P.R. (1984) Regulation of thyroxine 5'-deiodinase activity by T3 in cultured rat anterior pituitary cells. Endocrinology 115: 324-329.

Köhrle, J. (1983) Schilddrusenhormonstoffwechsel-1983. Untersuchungen zur Charakterisierung der enzymatischen Dejodierung von L-Thyroxin und anderen Jodthyroninen durch Lebergewebe der Ratte. Enke-Verlag, Stuttgart.

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

Krenning, E.P., Docter, R., Visser, T.J. and Hennemann, G. (1983) Plasma membrane transport of thyroid hormone: its possible pathophysiological significance. J. Endocrinol. Invest. 6: 59-66.

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

Larsen. P.R., Silva, J.E. and Kaplan, M.M. (1981) Relationship between

circulating and intracellular thyroid hormones: physiological and clinical implications. Endocr. Rev. 2: 87-102.

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

Laurberg, P. and Boye, N. (1982) Outer and inner ring monodeiodination of thyroxine by dog thyroid and liver: a comparative study using a particulate cell fraction. Endocrinology 110: 2124-2130.

Laurberg, P. and Boye, N. (1984) Propylthiouracil, ipodate, dexamethasone and periods of fasting induce different variations in serum rT3 in dogs. Metabolism 33: 323-325.

Leonard, J.L. and Rosenberg, I.N. (1978a) Subcellular distribution of thyroxine 5'-deiodinase in the rat kidney: a plasma membrane location. Endocrinology 103: 374-380.

Leonard, J.L. and Rosenberg, I.N. (1978b) Thyroxine 5'-deiodinase activity of rat kidney: observations on activation by thiols and inhibition by propylthiouracil. Endocrinology 103: 2137-2144.

Leonard, J.L. and Rosenberg, I.N. (1980a) Characterization of essential enzyme sulfhydryl groups of thyroxine 5'-deiodinase from rat kidney. Endocrinology 106: 444-451.

Leonard, J.L. and Rosenberg, I.N. (1980b) Thyroxine 5'-deiodinase from rat kidney: substrate specificity and the 5'-deiodination of reverse triiodothyronine. Endocrinology 107: 1376-1383.

Leonard, J.L., Kaplan, M.M., Visser, T.J., Silva, J.E. and Larsen. P.R. (1981) Cereral cortex responds rapidly to thyroid hormones. Science 214: 571-573.

Leonard, J.L. and Rosenberg, I.N. (1981) Solubilization of a phospholipid-requiring enzyme, iodothyronine 5'-deiodinase, from rat kidney membranes. Biochim. Biophys. Acta 659: 205-218.

Leonard, J.L., Mellen, S.M. and Larsen, P.R. (1983) Thyroxine 5'-deiodinase activity in brown adipose tissue. Endocrinology 112: 1153-1155.

Leonard, J.L., Silva, J.E., Kaplan, M.M., Mellen, S.A., Visser, T.J. and Larsen, P.R. (1984) Acute posttranscriptional regulation of cerebrocortical and pituitary iodothyronine 5'-deiodinases by thyroid hormone. Endocrinology 114: 998-1004.

Leonard, J.L. and Visser, T.J. (1984) Selective modification of the active center of renal iodothyronine 5'-deiodinase by iodoacetate. Biochim. Biophys. Acta 787: 122-130.

Loos, U., Grau, R., Keck, F.S. and Pfeiffer, E.F. (1984) Increased hepatic microsomal T3 production in vitro induced by insulin administration in rats in vivo. Clin. Physiol. Biochem. 2: 56-59.

Maciel, R.M.B., Ozawa, Y. and Chopra, I.J. (1979) Subcellular localization of thyroxine and reverse triiodothyronine outer ring monodeiodinating activities. Endocrinology 104: 365-371.

McCann, U.D., Shaw, E.A. and Kaplan, M.M. (1984) Iodothyronine deiodination reaction types in several rat tissue: effects of age, thyroid status, and glucocorticoid treatment. Endocrinology 114: 1513-1521.

Melmed, S., Nelson, M., Kaplowitz, N., Yamada, T. and Hershman, J. (1981) Glutathione-dependent thyroxine 5'-monodeiodination modulates growth hormone production by cultured nonthyrotropic rat pituitary cells. Endocrinology 108: 970-976.

Meyer, T. and Rokos, H. (1983) Beta-adrenergic effect of iodinated

tyramines and thyronamines on cAMP production in turkey erythrocytes. Ann. Endocr. 44: 77A.

Mol, J.A., Docter, R., Kaptein, E., Jansen, G., Hennemann, G. and Visser, T.J. (1984a) Inactivation and affinity-labeling of rat liver iodothyronine deiodinase with N-bromoacetyl-3,3',5-triiodothyronine. Biochem. Biophys. Res. Commun. 124: 475-483.

Mol, J.A., Docter, R., Hennemann, G. and Visser, T.J. (1984b) Modification of rat liver iodothyronine 5'-deiodinase activity with diethylpyrocarbonate and rose bengal; evidence for an active site histidine residue. Biophys. Res. Commun. 120: 28-36.

Mol, J.A., Krenning, E.P., Docter, R., Rozing, J., Bernard, H.F. and Hennemann, G. (1984c) Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. Program of the 60th Meeting of the American Thyroid Association, New York, T-17.

Oppenheimer, J.H., Bernstein, G. and Hasen, J. (1967) Estimation of rapidly exchangeable cellular thyroxine from the plasma disappearance curves of simultaneously administered thyroxine-131I and albumin-125I. J. Clin. Invest. 46: 762-777.

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

Otten, M.H., Hennemann, G., Docter, R. and Visser, T.J. (1984) Metabolism of 3,3'-diiodothyronine in rat hepatocytes: interaction of sulfation with deiodination. Endocrinology 115: 887-894.

Otten, M.H. (1984b) Aspects of peripheral thyroid hormone metabolism. Thesis, Erasmus University, Rotterdam.

Rao, G.S. and Rao, M.L. (1983) L-Thyroxine enters the rat liver cell by simple diffusion. J. Endocr. 97: 277-282.

Reichlin, S. (1978): Neuroendocrine control. In: Werner, S.C., Ingbar, S.H. (Eds) The Thyroid, Harper and Row, New York, pp. 151-173.

Roti, E., Fang, S.L., Green, K., Emerson, C.H. and Braverman, L.E. (1981) Human placenta is an active site of thyroxine and 3,3',5-triiodothyronine tyrosyl ring deiodination. J. Clin. Endocrinol. Metab. 53: 498-501.

Roti, E., Fang, S.L., Braverman, L.E. and Emerson, C.H. (1982) Rat placenta is an active site of inner ring deiodination of thyroxine and 3,3',5-triiodothyronine. Endocrinology 110: 34-37.

Ruiz, M. and Ingbar, S.H. (1982) Effects of erythrosine (2',4',5',7'-tetraiodofluorescein) on the metabolism of thyroxine in rat liver. Endocrinology 110: 1613-1617.

Saito, K., Yamamoto, K., Takai, T. and Yoshida, S. (1980) Thyroxine 5'-monodeiodinase activity in regenerating liver of triiodothyronine treated rats. J. Biochem. (Tokyo) 88: 1595-1600.

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

Sato, K. and Robbins, J. (1981b) Glutathione deficiency induced by cystine and/or methionine deprivation does not affect thyroid hormone deiodination in cultured rat hepatocytes and monkey hepatocarcinoma cells. Endocrinology 109: 844-852.

Sato, T., Maruyama, S., Saida, K. and Takata, I. (1982) Correlation of hepatic thyroxine 5'-monodeiodination with hexose monophosphate shunt

in young rats. Pediatr. Res. 16: 377–380.

Sato, T., Kato, T., Kajiwara, S., Miyamori, C. and Kita, K. (1983a) Maturational changes in the regulatory mechanisms of hepatic thyroxine 5'-deiodination in growing rats. Pediatr. Res. 17: 562–566.

Sato, K., Mimura, H., Wakai, K., Tomori, T., Tsushima, T. and Shizume, K. (1983b) Modulating effect of glutathione disulfide on thyroxine 5'-deiodination by rat hepatocytes in primary culture: effect of glucose. Endocrinology 113: 878–886.

Saunders, J., Hall, S.E.H. and Sönksen, P.H. Thyroid hormones in insulin requiring diabetes before and after treatment. Diabetalogica 15: 29–32.

Sekura, R.D., Sato, K., Cahnmann, H.J., Robbins, J. and Jakoby, W.B. (1981) Sulfate transfer to thyroid hormones and their analogues by hepatic aryl sulfotransferases. Endocrinology 108: 454–456.

Senga, S., Pittman, C.S., Lindsay, R.H., Chambers, J.B. and Hill, J.B. (1982) Comparison of peripheral thyroid hormone metabolism in normal rats and rats receiving prolonged glucagon infusion. Endocrinology 110: 2011–2017.

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

Silva, J.E., Leonard, J.L., Crantz, F.R. and Larsen, P.R. (1982) Evidence for two tissue specific pathways for in vivo thyroxine 5'-deiodination in the rat. J. Clin. Invest. 69: 1176–1184.

Silva, J.E., Leonard, J.L. and Larsen, P.R. (1983) Relative in vivo potency of iodothyronines to inhibit cerebral cortex (Cx) and pituitary (P) iodothyronine 5'-deiodinase activity in hypothyroid rats. Program of the 65th Annual Meeting of the Endocrine Society, San Antonio, p 202.

Silva, J.E., Gordon, M.B., Crantz, F.R., Leonard, J.L. and Larsen, P.R. (1984) Qualitative and quantitative differences in the pathways of extrathyroidal triiodothyronine generation between euthyroid and hypothyroid rats. J. Clin. Invest. 73: 898–907.

Singer, P.A., Nicoloff, J.T., Stein, R.B. and Jaramillo, J. (1978) Transient TRH deficiency after prolonged thyroid hormone therapy. J. Clin. Endocrinol. Metab. 47: 512–518.

Smallridge, R.C., Wartofsky, L. and Burman, K.D. (1982) The effect of experimental hyperthyroidism and hypothyroidism on 5'-deiodination of 3,3',5'-triiodothyronine and 3',5'-diiodothyronine by rat liver and kidney. Endocrinology 111: 2066–2069.

Sowers, J.R., Carlson, H.E., Brautbar, N. and Hershman, J.M. (1977) Effect of dexamethasone on prolactin and TSH responses to TRH and metoclopramide in man. J. Clin. Endocrinol. Metab. 44: 237–241.

Suda, A.K., Pittman, C.S., Shimizu, T. and Chambers, J.B. (1978) The production and metabolism of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine in normal and fasting subjects. J. Clin. Endocrinol. Metab. 47: 1311–1319.

Sugawara, M., Lau, R., Wasser, H.L., Nelson, A.M., Kuma, K. and Hershman, J.M. (1984) Thyroid T4 5'-deiodinase activity in normal and abnormal human thyroid glands. Metabolism 33: 332–336.

Tanaka, K., Inada, M., Ishii, H., Naito, K., Nishigawa, M., Mashio, Y. and
    Imura, H. (1981) Inner ring monodeiodination of thyroxine and
    3,5,3'-L-triiodothyronine in rat brain. Endocrinology 109: 1619–1624.
Tseng, Y.L. and Latham, K.R. (1984) Iodothyronines: oxidative
    deiodination by hemoglobin and inhibition of lipid peroxidation.
    Lipids 19: 96–102.

Van der Heyden, J.T.M., Van Toor, H., Krenning, E.P., Wilson, J.H.P.,
    Hennemann, G. and Docter, R. (1985) Effects of caloric deprivation on
    transport of thyroid hormones into tissue cells and the generation of
    the low T3 syndrome. Submitted for publication.
Visser, T.J., Van der Does-Tobe, I., Docter, R. and Hennemann, G. (1975)
    Conversion of thyroxine into triiodothyronine by rat liver homogenate.
    Biochem. J. 150: 489–493.
Visser, T.J., Van der Does-Tobe, I., Docter, R. and Hennemann, G. (1976)
    Subcellular localization in rat liver of an enzyme converting thyroxine
    into triiodothyronine and possible involvement of essential thiol
    groups. Biochem. J. 157: 479–482.
Visser, T.J. (1978) A tentative review of recent in vitro observations of
    the enzymatic deiodination of iodothyronines and its possible
    physiological implications. Mol. Cell. Endocrinol. 10: 241–247.
Visser, T.J. (1979) Mechanism of action of iodothyronine 5'-deiodinase.
    Biochim. Biophys. Acta 569: 302–308.
Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1979a) Kinetics
    of enzymic reductive deiodination of iodothyronines; effect of pH.
    Biochem. J. 179: 489–495.
Visser, T.J., Van Overmeeren, E., Fekkes, D., Docter, R. and Hennemann, G.
    (1979b). Inhibition of iodothyronine 5'-deiodinase by thioureylenes;
    structure–activity relationship. FEBS Lett. 103: 314–318.
Visser, T.J. and Van Overmeeren, E. (1979) Binding of radioiodinated
    propylthiouracil to rat liver microsomal fractions; stimulation by
    substrates for iodothyronine 5'-deiodinase. Biochem. J. 183:
    167–169.
Visser, T.J. and Lamberts, S.W.J. (1981) Regulation of TSH secretion and
    thyroid function in Cushing's disease. Acta Endocr. 96: 480–483.
Visser, T.J., Leonard, J.L., Kaplan. M.M. and Larsen, P.R. (1981)
    Different pathways of iodothyronine 5'-deiodination in rat cerebral
    cortex. Biochem. Biophys. Res. Comm. 101: 1297–1304.
Visser, T.J. and Van Overmeeren-Kaptein, E. (1981) Substrate requirement
    for inactivation of iodothyronine 5'-deiodinase by thiouracil.
    Biochim. Biophys. Acta 658: 202–208.
Visser, T.J., Leonard, J.L., Kaplan, M.M. and Larsen, P.R. (1982) Kinetic
    evidence suggesting two mechanisms for iodothyronine 5'-deiodination in
    rat cerebral cortex. Proc. Nat. Acad. Sci. (USA) 79: 5080–5084.
Visser, T.J., Kaptein, E. and Krenning, E.P. (1983a) Deiodination of
    iodothyronines in human liver. Program of the 59th Meeting of the
    American Thyroid Association, New Orleans, 1983, T-16.
Visser, T.J., Mol, J.A. and Otten, M.H. (1983b) Rapid deiodination of
    triiodothyronine sulfate by rat liver microsomal fraction.
    Endocrinology 112: 1547–1549.
Visser, T.J., Kaplan, M.M., Leonard, J.L. and Larsen, P.R. (1983c)
    Evidence for two pathways of iodothyronine 5'-deiodination in rat
    pituitary that differ in kinetics, propylthiouracil sensitivity, and
    response to hypothyroidism. J. Clin. Invest. 71: 992–1002.
Visser, T.J., Frank, S. and Leonard, J.L. (1983d) Differential

sensitivity of brain iodothyronine 5'-deiodinases to sulfhydryl-blocking reagents. Mol. Cell. Endocrinol. 33: 321-327.

Weeke, J., Hansen, A.P., Jensen, S.E. and Lundbaek, K. (1976): Inhibition by somatostatin of TSH response to TRH and of basal TSH levels in man. In: Robbins, J., Braverman, L.E. (Eds) Thyroid Research: 15-17, American Elsevier, New York.

Woeber, K.A. (1971) Alterations in thyroid hormone economy during acute infection with Diplococcus pneumoniae in the resus monkey. J. Clin. Invest. 50: 378-384.

Wu, S.Y. (1983) Thyrotropin-mediated induction of the thyroidal iodothyronine monodeiodinases in the dog. Endocrinology 112: 417-424.

SYNTHESIS AND SOME PROPERTIES OF SULFATE ESTERS AND SULFAMATES OF IODOTHYRONINES.

Jan A. Mol and Theo J. Visser.

Department of Internal Medicine III and Clinical Endocrinology
Erasmus University Medical School, Rotterdam, The Netherlands.

ABSTRACT

In the present study convenient methods have been developed for the synthesis of sulfate derivatives of iodothyronines. Reaction with chlorosulfonic acid in dimethylformamide gave rise to the formation of the sulfate ester with the phenolic hydroxyl group. Reaction with the sulfurtrioxide-trimethylamine complex in alkaline medium afforded the sulfamate with the α-amino group of the alanine side chain. The sulfated products were isolated by adsorption onto Sephadex LH-20 in acidic medium followed by desorption with water. Iodide was not retarded on these columns while elution of native iodothyronines required alkaline ethanol mixtures. The yield of both reactions varied between 70% and 90%. The sulfates and sulfamates of thyroxine, 3,3',5-triiodothyronine, 3,3',5'-triiodothyronine and 3,3'-diiodothyronine could be separated by reversed phase, high-performance liquid chromatography. The sulfamates exhibited high cross-reactivities with antibodies against free iodothyronines in contrast to the low activities of the sulfates.

Products were further characterized by proton nuclear magnetic resonance, thin layer chromatography and hydrolysis by acid or sulfatase activity. The availability of large quantities of pure iodothyronine sulfates and sulfamates should facilitate the study of the importance of sulfate conjugation in the metabolism of thyroid hormone.

The main secretory product of the thyroid gland is the prohormone thyroxine (T_4) (1-3). In peripheral tissues T_4 is metabolized by outer ring deiodination to the biologically active 3,3',5-triiodothyronine (T_3), and by inner ring deiodination to the inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3). Further stepwise deiodination of T_3 and rT_3 leads to, among other things, 3,3'-diiodothyronine (3,3'-T_2) (4-7). Besides deiodination, iodothyronines are also subject to conjugation with either glucuronic acid or sulfate (8-11). The liver and kidneys are major sites for these metabolic reactions. Recent work in our laboratory has shown a close interaction between the sulfation and deiodination of iodothyronines in the liver. Sulfation by cytoplasmic phenol sulfotransferases greatly enhances the subsequent deiodination of T_3 and 3,3'-T_2 by the deiodinase located in the endoplasmic reticulum (12,13). This prompted us to search for a convenient method for the synthesis of the sulfate conjugates of the various iodothyronines. Here we describe the preparations of the sulfate ester as well as the sulfamate of these compounds.

MATERIALS AND METHODS

Reagents.

All iodothyronines were obtained from Henning, Berlin, FRG; [3',5'-^{125}I]T_4, [3'-^{125}I]T_3 and carrier-free Na^{125}I were from Amersham, UK. [3',5'-^{125}I]rT_3 and [3'-^{125}I]3,3'-T_2 were prepared by radio-iodination of 3,3'-T_2 and 3-iodothyronine, respectively, with the chloramine-T method and they were purified on Sephadex LH-20 (14). N-acetyl-iodothyronines were prepared as described by method B of Cheng et al (15). Chlorosulfonic acid (ClSO$_3$H), N,N-dimethylformamide (DMF), acetylchloride, and SO$_3$-pyridine complex were obtained from Merck; SO$_3$-trimethylamine complex (SO$_3$-TMA) was from Aldrich. Sulfatase (from Abalone Entrails type VIII) was obtained from Sigma. All other chemicals were of reagent grade.

Reaction of iodothyronines with different sulfurylating agents.

a. Reaction of iodothyronines with concentrated H_2SO_4 was performed as described previously (12).

b. Reactions involving chlorosulfonic acid were started by the slow addition of 200 μl $ClSO_3H$ (15 M) to 800 μl DMF or dioxane at $0^{\circ}C$. In another tube a solution of usually 100 pmol. unlabeled plus ^{125}I-labeled iodothyronine in ammoniacal ethanol was evaporated under a stream of nitrogen. To the residue of the latter tube 200 μl of the $ClSO_3H$ solution in DMF or dioxane was added at $0^{\circ}C$. Subsequently, the mixtures were allowed to attain room temperature, and, in general, reactions were continued overnight. After dilution with 800 μl icecold water, reaction products were analyzed by Sephadex LH-20 chromatography.

c. In the procedure involving SO_3 complexes, generally 100 μl of a solution of 2 mg SO_3-pyridine or SO_3-TMA in DMF were added to 100 pmol unlabeled plus ^{125}I-labeled iodothyronine in 100 μl 0.1 N NaOH. The reaction was allowed to proceed overnight at room temperature or for 1 h at $37^{\circ}C$. After addition of 800 μl 0.1 N HCl, reaction products were also analyzed by Sephadex LH-20 chromatography.

Analytical methods.

Because of their increased hydrophilicity the sulfated iodothyronines were expected to bind less tightly to Sephadex matrices than the native compounds. Reaction mixtures were, therefore, analyzed by hydrophobic interaction chromatography on Sephadex LH-20. The reaction products I^-, sulfate ester or sulfamate, and native iodothyronine were separated by successive elution with 0.1 N HCl, water, and 1 N ammonia in ethanol.

Reversed-phase, high performance liquid chromatography (HPLC) was performed on a radial-PAK μBondapak C_{18} column using a Model 6000A solvent delivery system and monitoring absorbance at 254 nm with a Model 440 fixed wavelength detector (Waters, Milford, MA, USA).

Thin-layer chromatography (TLC) was performed on silicagel 60 F 254 plates (Merck), using as solvent ethylacetate/acetic acid (9/1, v/v). After the plates were developed they were sprayed with either ninhydrin to

show a free amino group, or according to Pauly (16) to show a free phenolic hydroxyl group.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were acquired on samples dissolved in $NaOD/D_2O$ (pD 10). Spectra were recorded at 200 MHz on a Nicole NT 200 NMR-spectrometer.

Cross-reactivities of sulfated iodothyronines in the radioimmunoassays for the native iodothyronines were assessed using antisera to T_4, T_3 (Henning, Berlin), rT_3 (14) and $3,3'-T_2$ (17). Serial dilutions of the sulfated or non-sulfated iodothyronines were incubated overnight at room temperature with 20.000 cpm of the corresponding ^{125}I-outer ring labeled iodothyronine and an appropriate dilution of antiserum in 1 ml 0.06 M barbital/0.15 M NaCl/0.1% bovine serum albumin, pH 8.6. Antibody-bound radioactivity was precipitated with second antibody and polyethyleneglycol.

Acid hydrolysis of sulfated iodothyronines was performed by reaction of ^{125}I-labeled sulfate derivatives for varying time periods at 37°C, 50°C or 80°C in 0.1 or 1 N HCl. Reactions were stopped by placing the mixtures on ice, and hydrolysis was quantitated by Sephadex LH-20 chromatography as described above. Enzymatic hydrolysis was measured by incubation of ^{125}I-labeled sulfate derivative at 37°C with 125 ug sulfatase type VIII in 0.5 ml 50 mM sodium acetate (pH 5.0). After various times the reaction was terminated by addition of 0.5 ml icecold 1 N HCl, and products were again analyzed by LH-20 chromatography.

RESULTS

The potential reactions of sulfurtrioxide (SO_3) or related compounds with iodothyronines are depicted in Fig.1 (18,19). Since sulfonation of halogenated benzenes or phenolic compounds requires rigorous conditions, i.e. high temperatures and SO_3 as the sulfurylating agent, it seems unlikely to occur under the mild conditions used here. The reactivity of SO_3 is moderated by complex formation with a base. With increasing strength of the base, the stability of the complex increases and its reactivity decreases (18). Table I shows the results of the reaction of various sulfurylating agents with native or N-acylated iodothyronines. The

48

REACTION OF SO$_3$ WITH THYROXINE
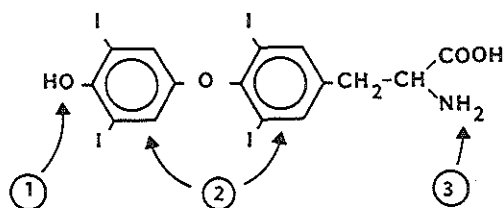
1. Sulfation   ⟶ ROSO$_3$H

2. Sulfonation ⟶ RSO$_3$H

3. Sulfamation ⟶ RNHSO$_3$H

Fig.1. Theoretical reactions of sulfurtrioxide or its organic complexes with thyroxine. The reactions include formation of 1) the sulfate ester, 2) sulfonic acid derivatives, or 3) the sulfamate (ref.18).

SO$_3$-TMA complex reacts in aqueous alkali with the native iodothyronines but not with N-acetyl iodothyronines indicating that only the amino group of the alanine side chain is modified by sulfamate formation. Because of the high stability of the SO$_3$-TMA complex it is usable at the high pH which is necessary for reaction with a dissociated amino group. At neutral or acidic pH, or in pyridine/DMF no product formation could be observed at the use of the SO$_3$-TMA complex. Reaction with the less stable SO$_3$-pyridine complex under the various conditions yielded also no sulfated products.

Chlorosulfonic acid dissolved in dioxane or, especially, in DMF reacted with both native and N-acylated iodothyronines indicating at least the formation of the sulfate ester with the phenolic hydroxyl group. The SO$_3$-DMF complex which is probably formed as an intermediate is one of the most reactive SO$_3$ complexes known so far (18). The advantage of the SO$_3$-DMF complex is that little or no ^{125}I$^-$ is formed due to oxidative breakdown of the iodothyronines in contrast to the methods using concentrated H$_2$SO$_4$ or ClSO$_3$H. The latter reactions are difficult to control, and although some T$_3$ sulfate ester could be synthesized using H$_2$SO$_4$ (12), reaction of T$_4$ with H$_2$SO$_4$ yielded merely iodide.

TABLE I

Reactivity of several sulfurylating agents with native or
N-acetylated iodothyronines.

		% product formation with:[a]		
Reagent	Solvent	T_3	T_4	$N-Ac-T_3$
SO_3-TMA	DMF/0.1 N NaOH	90	90	0
SO_3-TMA	DMF	7	8	0
SO_3-TMA	DMF/H_2O	1	0	N.D.
SO_3-TMA	DMF/0.1 N HOAc	0	0	N.D.
SO_3-Pyridine	DMF/0.1 N NaOH	3	5	0
$ClSO_3H$	---	iodide[b]	iodide	iodide
$ClSO_3H$	DMF	90	65	87
$ClSO_3H$	Dioxane	14	10	15
H_2SO_4	---	40	iodide	N.D.

[a] Reactions are positive if labeled product can be eluted with
water from the Sephadex LH-20 column. Results are the means of
two closely agreeing experiments each performed in duplicate.

[b] Iodide indicates the appearance of large quantities of $^{125}I^-$
in the HCl fraction of the LH-20 column.

  N.D. = not determined.

Fig.2 depicts a typical purification on Sephadex LH-20 of the reaction products of T_3 with the SO_3-TMA or the SO_3-DMF complex. With water as the second solvent, the sulfamates are usually eluted as a broad peak. In Fig.2 the elution of the sulfamate is improved by the use of 25% ethanol without effecting the elution of the native iodothyronine. Both products and native T_3 were further analyzed by TLC. In the system used T_3 had an Rf value of 0.08 and was coloured with both ninhydrin and Pauly reagent. The product of the reaction of T_3 with SO_3-TMA had an Rf value of 0.12. It was coloured by Pauly reagent but not with ninhydrin, indicating a blocked amino group. The reaction product of T_3 with SO_3-DMF (Rf 0.01) was ninhydrin-positive but Pauly-negative, showing that only the phenolic hydroxyl group was blocked. By reaction with the SO_3-TMA complex the sulfamates of the various iodothyronines were prepared in a yield of 90%. The sulfate esters were prepared with the SO_3-DMF complex in the following yields (mean of two closely agreeing experiments): 3,3'-T_2 (85%), T_3 (90%), rT_3 (70%) and T_4 (65%).
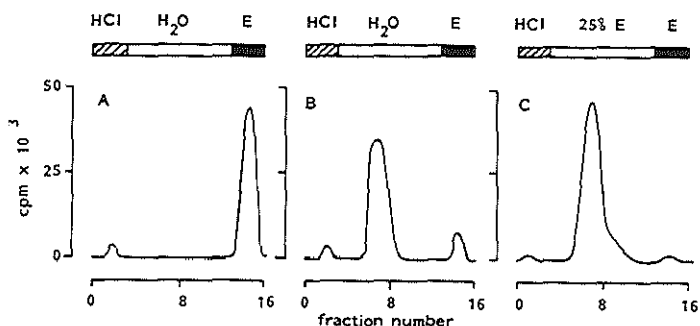

Fig.2. Chromatography of ^{125}I outer ring labeled T_3 before (A) and after the reaction with the SO_3-DMF complex (B) or the SO_3-TMA complex (C). Reaction mixture B was diluted with 800 μl B_2O and reaction mixture C was acidified by addition of 800 μl 0.1 N BCL (see Materials and Methods). Products were applied to a small 1 ml Sephadex LH-20 column. Serial elution was performed with 1 ml fractions of 0.1 N BCl, water or 25% ethanol in water and 1 N ammonia in ethanol.
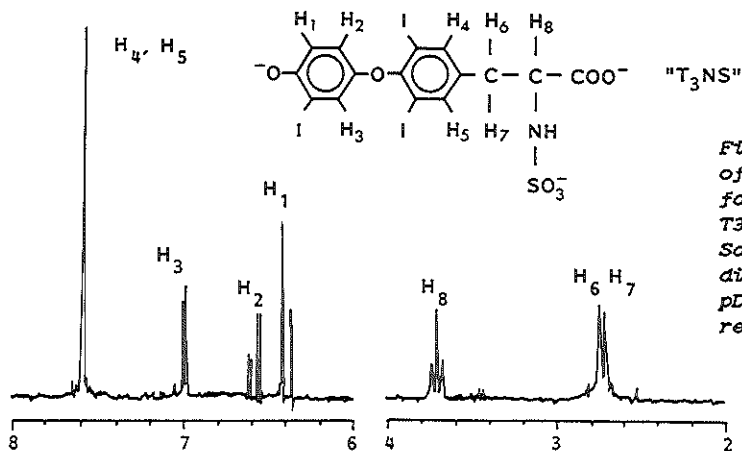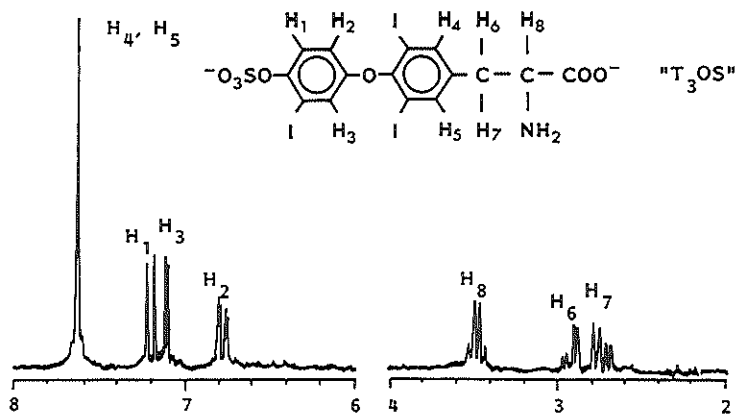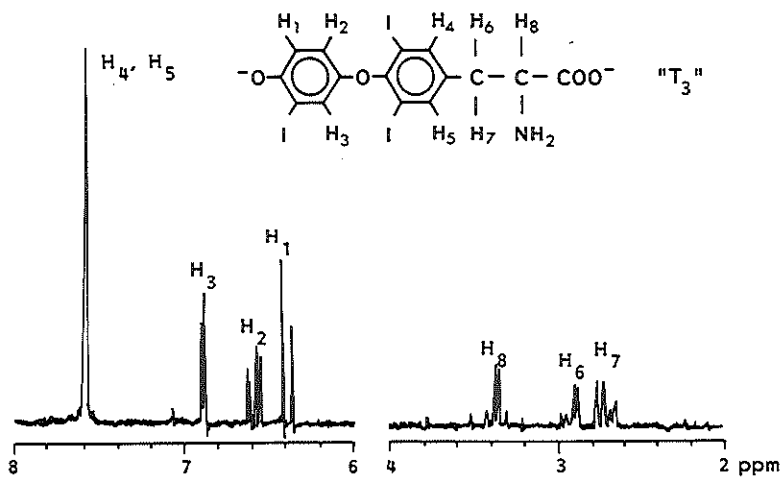
Fig.3. ¹H-NMR spectra of native T3, T3 sulfate ester (T3OS) and T3 sulfamate (T3NS). Samples of 4 mg were dissolved in D₂O/NaOD, pD=10 and spectra were recorded at 200 MHz.

The identity of the sulfated products was further substantiated by proton-NMR of the T_3 sulfate ester (T_3S) and of the T_3 sulfamate (T_3NS) (Fig.3). It should be noted that the numbering of the protons in Fig.3 is different from the conventional numbering of the carbons in the iodothyronine structure. Formation of the sulfate ester with the phenolic hydroxyl group (T_3S) leads to a downfield shift from 6.40 to 7.22 ppm in the signal of the proton H-1 in the ortho position with minor downfield shifts of the meta protons H-2 (6.55 to 6.78 ppm) and H-3 (6.93 to 7.12 ppm). The chemical shift of especially H-1 is explained by the conversion of the electron-donating hydroxyl group into the electron-withdrawing sulfate group. In contrast to the sulfate ester, the sulfamate of T_3 (T_3NS) shows no chemical shifts of the various ring protons. However, a downfield shift is observed from, 3.28 to 3.71 ppm of proton H-8 on the α-carbon atom, adjacent to the amino group. Furthermore, the signal of the protons H-6 and H-7 on the -carbon atom is now split into a doublet in contrast to the two quartets as seen in the case of native T_3. The NMR data, therefore, confirm the identity of the sulfated T_3 derivatives, and show that no sulfonation did occur by substitution of a ring proton by a sulfate group.

Fig.4. Reversed-phase HPLC of a mixture of the sulfamates of 3,3'-T2, T3, rT3 and T4, 1 nmol each. Products were eluted isocratically with a mixture of acetonitril and 20 mM ammonium acetate pH 4 (30/70, v/v) at a solvent flow of 2 ml/min. Absorbance was recorded at 254 nm. The peak A_{245} of 3,3'-T2 sulfamate amounted to 0.06 absorbance units.
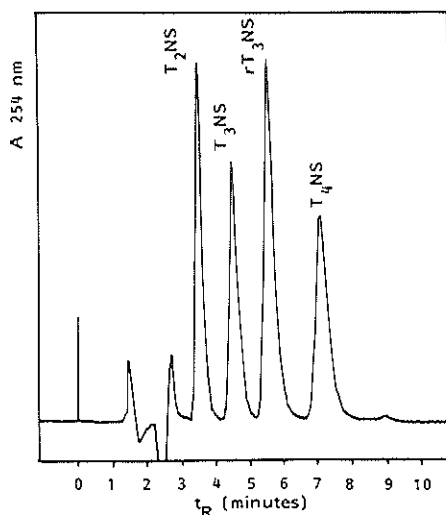
The sulfate esters and the sulfamates of the various iodothyronines were further analyzed by HPLC. Fig.4 depicts the isocratic elution of the sulfamates with a mixture of acetonitril and 20 mM ammonium acetate, pH 4 (30/70, v/v). The sequence of elution of the various sulfamates was identical to that of the native iodothyronines although elution of the latter required a higher acetonitril concentration. In contrast,
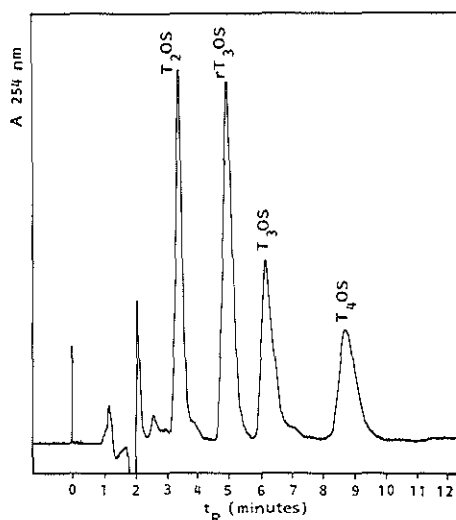

Fig.5. Reversed-phase HPLC of a mixture of the sulfates of 3,3'-T2, T3, rT3 and T4, 1 nmol each. Products were eluted isocratically with a mixture of acetonitril and ammonium acetate, pH 4 (22/78, v/v), at a solvent flow of 2 ml/min. Absorbance was recorded at 254 nm. The peak A_{254} of 3,3'-T2 sulfate ester amounted to 0.07 absorbance units

for separation of the various sulfate esters at pH 4 (fig. 5), solvent with less acetonitril was needed, indicating that the sulfate esters are less hydrophobic in comparison with the sulfamates. Notable is also that in the case of the sulfate esters rT_3 elutes earlier than T_3, in contrast to the sulfamates and the native compounds. This may be due to the fact that in rT_3 sulfate the most hydrophobic part of the rT_3 molecule, i.e. the outer ring, is made more hydrophilic. With the sulfamate the alteration is confined to the alanine side chain where the normally occurring zwitter-ion is replaced by a double negative charge at the pH used. The retention time of the sulfate ester of T_3 prepared with the SO_3-DMF method or with concentrated H_2SO_4 was identical.

We investigated the possibility to regenerate the original iodothyronines by enzymatic or acid-catalyzed hydrolysis. The sulfate esters and the sulfamates of the various iodothyronines were almost

completely (>95%) hydrolyzed by treatment for 1 h at 80°C with 1N HCl. The intact structure of the iodothyronine liberated was demonstrated by HPLC. The susceptibility to acid hydrolysis again shows that no sulfonation has occurred. Radioiodide production was less than 1%, indicating that oxidative breakdown was negligible under the circumstances used. The rate of hydrolysis by 0.1 or 1N HCl at different temperatures or by sulfatase digestion at 37°C was analyzed by Sephadex LH-20 chromatography. The results are summarized in Fig.6. The rate of acid hydrolysis of the sulfamates was independent of the structure of the iodothyronine. It was slower than the hydrolysis of any of the sulfate esters, e.g. half the rate observed with T_3 and $3,3'-T_2$ sulfate. Acid hydrolysis of the sulfate esters of T_4 and rT_3 was considerably faster than hydrolysis of T_3 and $3,3'-T_2$ sulfate. Only the sulfate esters of $3,3'-T_2$ and T_3 were hydrolyzed by treatment with sulfatase activity from Abalone Entrails.
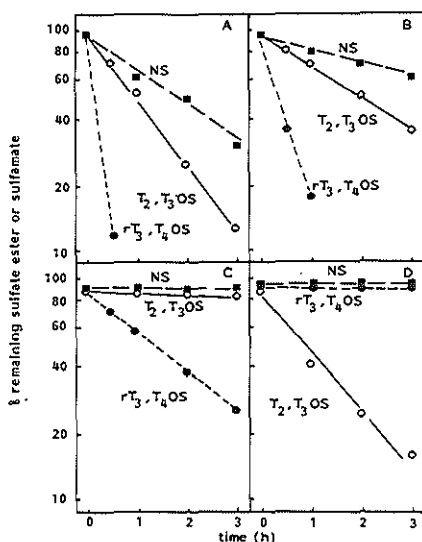

Fig.6. Hydrolysis of iodothyronine sulfates and sulfamates. A tracer quantity of the various compounds was incubated for the indicated time periods with: A, 0.1 N HCl at 80 °C; B, 1 N HCl at 50 °C; C, 1 N HCl at 37 °C; and D, 250 µg/ml sulfatase in 50 mM sodium acetate, pH 5. The results are derived from 2 to 3 experiments each performed in duplicate. Depicted are the means of $3,3'-T_2$, T_3, rT_3 or T_4 sulfamate (■), $3,3'-T_2$ or T_3 sulfate (○) and rT_3 or T_4 sulfate (●). The variation within these groups was less than 10%.

Finally, the dose-response curves of the sulfated compounds in the radioimmunoassays for native iodothyronines are shown in Fig.7. In general, the sulfamates exhibit 100% cross-reactivity except that T_4 sulfamate shows a somewhat reduced affinity for the T_4 antibody. Of the sulfate esters, only rT_3 sulfate exhibits a cross-reactivity of approximately 1% while that of the other sulfates is below 0.1%. It is not excluded that this low degree of cross-reactivity is due to contamination with small quantities of non-sulfated iodothyronines.
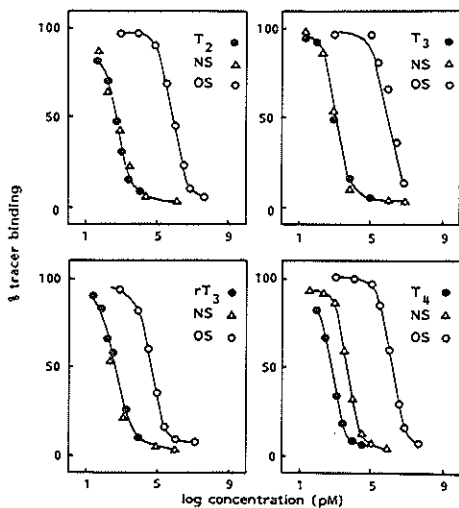
Fig.7. Dose-response curves of sulfated iodothyronines in the radioimmunoassay for the corresponding native iodothyronine. The displacement of a tracer quantity of tracer iodothyronine from the antibody was measured by incubation with serial dilutions of non-labeled native iodothyronine (●) or with the corresponding sulfamate (△) or sulfate ester (○). The results are the means of 2 experiments with two preparations of the various sulfated iodothyronines. Each experiment was performed in triplicate.

DISCUSSION

We have developed convenient methods for the synthesis of iodothyronine sulfates and sulfamates. Both reactions are easy to perform although especially in the case of reaction with $ClSO_3H$ in DMF care must be taken to avoid high temperatures which may cause oxidative breakdown of the iodothyronines. The sulfated products are completely separated from native iodothyronines and iodide by Sephadex LH-20 chromatography. The selectivity of the reactions and the identity of the products are demonstrated by TLC, HPLC, and ^{1}H-NMR. Supporting evidence is provided by the different susceptibilities of the derivatives to enzymatic and acid-catalyzed hydrolysis as well as by their discriminating activities in the radioimmunoassays.

In general, the rate of acid hydrolysis of aryl sulfates is increased by the presence of electron-withdrawing substituents in the aromatic ring (19). This is also the case for the sulfate esters of T_4 and rT_3 with two iodine atoms in the outer ring. They are hydrolyzed about 5 times faster than the sulfate esters of $3,3'-T_2$ and T_3 having one iodine atom in the outer ring. Enzymatic hydrolysis with sulfatase activity is expected to be selective for sulfate esters. However, not only the sulfamates but also the sulfate esters of rT_3 and T_4 resist enzymatic hydrolysis, even at prolonged reaction times. Apparently, steric hindrance by two iodine atoms in the outer ring precludes the accessibility of the sulfate ester to the active centre of the sulfatase.

The high cross-reactivity of the sulfamates in the various radioimmunoassays is explained by the fact that the antibodies have been raised against iodothyronines coupled via the alanine side chain to a carrier protein. Such antibodies are not expected to recognize alterations in this side chain such as those introduced by sulfamation. Since the outer ring is part of the antigenic determinant, it is not surprising that the sulfate esters proved to be poor competitors in each of our radioimmunoassays. Since iodothyronine antisera are produced according to the same general principles, the low cross-reactivity of sulfate conjugates is probably a common property of iodothyronine radioimmunoassays.

The above findings merit further comments related to clinical

measurements of native and sulfated iodothyronines. Firstly, our results suggest that the possible occurence of sulfate conjugates in the plasma of patients will not interfere with the radioimmunoassay of non-conjugated iodothyronines. Secondly, we have shown that, although the sulfatase preparation from Abalone Entrails hydrolyzes the sulfate conjugates of T_3 and $3,3'-T_2$, it is ineffective with T_4 and rT_3 sulfate. Attempts to quantitate iodothyronine conjugates by radioimmunoassay following enzymatic hydrolysis should, therefore, be interpreted with caution. This is especially true for those studies where the necessary control experiments could not be performed because of the lack of standard preparations of the conjugates (20,21). With the present method large quantities of pure sulfate conjugates are conveniently obtained.

Previous studies have shown that deiodination of T_3 and $3,3'-T_2$ by rat hepatocytes requires the prior sulfation of these compounds. Biosynthetic $3,3'-T_2$ sulfate as well as the sulfate esters of $3,3'-T_2$ and T_3 prepared with concentrated H_2SO_4 proved to be much better substrates for the rat liver deiodinase than the native compounds (12,13). In preliminary experiments, we found that $3,3'-T_2$ and T_3 sulfate obtained by the present method using chlorosulfonic acid are equally effective deiodinase substrates. In addition, the preparation of the sulfate esters of all iodothyronines enabled us to study the deiodination of rT_3 and T_4 sulfate which cannot be synthesized with H_2SO_4 (22). Deiodination of the various iodothyronine sulfamates remains to be investigated.

REFERENCES

1. Chopra IJ, Solomon DH, Chopra U, Wu SY, Fisher DA, Nakamura Y 1978 Pathways of metabolism of thyroid hormones. Rec Prog Horm Res 34: 521
2. Larsen PR, Silva JE, Kaplan MM 1981 Relationship between circulating and intracellular thyroid hormones: physiological and clinical implications. Endocr Rev 2: 87
3. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5: 151
4. Hüfner M, Grussendorf M 1978 Investigations on the deiodination of thyroxine (T4) to 3,3'-diiodothyronine (3,3'-T2) in rat liver homogenate. Clin Chim Acta 85: 243
5. Chopra IJ, Wu SY, Nakamura Y, Solomon DH 1978 Monodeiodination of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine to 3,3'-diiodothyronine in vitro. Endocrinology 102: 1099
6. Visser TJ, Fekkes D, Docter R, Hennemann G 1979 Kinetics of enzymic reductive deiodination of iodothyronines. Biochem J 179: 489
7. Leonard JL, Rosenberg IN 1980 Iodothyronine 5'-deiodinase from rat kidney: substrate specificity and the 5'-deiodination of reverse triiodothyronine. Endocrinology 107: 1376
8. Flock EV, David D, Stobie GH, Owen CA 1963 3,3',5'-Triiodothyronine and 3,3'-diiodothyronine: partial deiodinated intermediates in the metabolism of the thyroid hormones. Endocrinology 73: 442
9. Van Middlesworth L 1974 Metabolism and excretion of thyroid hormones. In: Greer MA, Solomon DH (Eds) Handbook of. Physiology, section 7: Endocrinology, vol. III Thyroid. American Physiological Society, Washington, p 215
10. Takai NA, Rapoport B, Yamamoto M 1980 Biliary excretion of iodothyronines in rats as determined by high pressure liquid chromatography: effect of starvation. Endocrinology 107: 176
11. Sato K, Robbins J 1981 Thyroid hormone metabolism in primary cultured rat hepatocytes. J Clin Invest 68: 475
12. Visser TJ, Mol JA, Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112: 1547
13. Otten MH, Mol JA, Visser TJ 1983 Sulfation preceding deiodination of iodothyronines in rat hepatocytes. Science 221: 81
14. Visser TJ, Docter R, Hennemann G 1977 Radioimmunoassay of reverse triiodothyronine. J Endocrinol 73: 395
15. Cheng SY, Wilchek M, Cahnmann HJ, Robbins J 1977 Affinity labeling of human serum prealbumin with N-bromoacetyl-L-thyroxine. J Biol Chem 252: 6076
16. Krebs KG, Heuser D, Wimmer H 1967 In: Stahl E (Ed) Dünschicht Chromatographie, ein Laboratoriumhandbuch. Springer-Verlag, Berlin, p 851
17. Visser TJ, Krieger-Quist LM, Docter R, Hennemann G 1978 Radioimmunoassay of 3,3'-diiodothyronine in unextracted serum: the effect of endogenous triiodothyronine. J Endocrinol 79:357
18. Gilbert EE 1962 The reactions of sulfurtrioxide, and its adducts, with organic compounds. Chem Rev 62: 549

19. Roy AB 1981 The chemistry of sulfate esters and related compounds.
    In: Mulder GJ (Ed) Sulfation of Drugs and Related Compounds CRC
    Press, Boca Raton, Florida, p 5
20. Faber   J,   Busch-Sorensen   M,   Rogowski   P,   Kirkegaard   C,
    Siersbaek-Nielsen K, Friis T 1981 Urinary excretion of free and
    conjugated 3',5'-diiodothyronine and 3,3'-diiodothyronine. J Clin
    Endocrinol Metab 53:   587
21. Földes O, Langer P, Straussova K, Brozmanova H, Gschwendtova K
    1982 Direct quantitative estimation of several iodothyronines in
    rat bile by radioimmunoassay and basal data on their biliary
    excretion. Biochim Biophys Acta 716:   383
22. Mol JA, Visser TJ 1985 Rapid and selective deiodination of
    thyroxine   sulfate   by   rat   liver   deiodinase.   Endocrinology,
    accepted for publication

Endocrinology, accepted for publication.

CHAPTER IV

RAPID AND SELECTIVE INNER RING DEIODINATION OF THYROXINE SULFATE
BY RAT LIVER DEIODINASE.

Jan A. Mol and Theo J. Visser

Department of Internal Medicine III and Clinical Endocrinology,
Erasmus University Medical School, Rotterdam, The Netherlands.

ABSTRACT

Previous studies have shown that the inner ring deiodination (IRD) of T_3 and the outer ring deiodination (ORD) of 3,3'-diiodothyronine are greatly enhanced by sulfate conjugation. This study was undertaken to evaluate the effect of sulfation on T_4 and rT_3 deiodination. Iodothyronine sulfate conjugates were chemically synthetized. Deiodination was studied by reaction of rat liver microsomes with unlabeled or outer ring ^{125}I-labeled sulfate conjugate at $37^\circ C$ and pH 7.2 in the presence of 5 mM dithiothreitol. Products were analyzed by high performance liquid chromatography or after hydrolysis by specific radioimmunoassays. T_4 sulfate (T_4S) was rapidly degraded by IRD to rT_3 sulfate with an apparent K_m of 0.3 µM and a V_{max} of 530 pmol/min/mg protein. The V_{max}/K_m ratio of T_4S IRD is increased 200-fold compared with that of T_4 IRD. However, formation of T_3 sulfate by ORD of T_4S could not be observed. The rT_3 sulfate formed is rapidly converted by ORD to 3,3'-T_2 sulfate with an apparent K_m of 0.06 µM and a V_{max} of 516 pmol/min/mg protein. The enzymic mechanism of the IRD of T_4S is the same as that of the deiodination of non-sulfated iodothyronines as shown by the kinetics of stimulation by dithiothreitol or inhibition by propylthiouracil. The IRD of T_4S and the ORD of rT_3 are equally affected by a number of competitive inhibitors suggesting a single enzyme for the deiodination of native and sulfated

iodothyronines. In conjunction with previous findings on the deiodination of T_3 sulfate, these results suggest that sulfation leads to a rapid and irreversible inactivation of thyroid hormone.

In healthy humans, about 80% of the active thyroid hormone T_3 is produced by phenolic ring or outer ring deiodination (ORD) of T_4 in peripheral tissues (1-3). A roughly equal proportion of T_4 is metabolized by tyrosyl ring or inner ring deiodination (IRD) to biologically inactive rT_3. A further metabolite in the stepwise deiodination of T_4 is 3,3'-diiodothyronine (3,3'-T_2) which is generated by IRD of T_3 as well as by ORD of rT_3. The liver is an important site for these processes, where the ORD and IRD of the various iodothyronines are catalyzed by a single enzyme located in the endoplasmic reticulum (4-6). This enzyme requires thiols for activity (7) and is most effective in the ORD of rT_3 (8). The liver also metabolizes iodothyronines by conjugation of the phenolic hydroxyl group with sulfate or glucuronic acid (9-11). We have recently shown that the IRD of T_3 and the ORD of 3,3'-T_2 in the liver are greatly facilitated by sulfate conjugation (12,13). A recently developed method for the chemical synthesis of pure sulfate esters of iodothyronines (14) enabled us to investigate the effects of sulfation on the deiodination of T_4 and rT_3. Here we present the results of this study.

MATERIALS AND METHODS

All iodothyronines were obtained from Henning Berlin GmbH (West Berlin, West Germany). $[3',5'-^{125}I]T_4$, $[3'-^{125}I]T_3$ and carrier-free $Na^{125}I$ were from Amersham International (Amersham, UK). $[3',5'-^{125}I]rT_3$ and $3,[3'-^{125}I]T_2$ were prepared by radio-iodination of 3,3'-T_2 and 3-iodothyronine, respectively (14). Sulfate esters were prepared by reaction of iodothyronines with chlorosulfonic acid in dimethylformamide, and they were purified by Sephadex LH-20 chromatography (14).

Rat liver microsomes were prepared as described previously (8).

Deiodination was studied by reaction of microsomes with unlabeled or outer ring ^{125}I-labeled T_4 sulfate (T_4S) at 37°C in 200 µl 0.1 M sodium phosphate (pH 7.2), 3 mM EDTA and 5 mM dithiothreitol (DTT). Products were analyzed in different ways. Firstly, reactions with labeled T_4S were stopped by addition of 30 µl 10 mM 6-n-propyl-2-thiouracil (PTU), and the unextracted radioactive products were separated by reversed-phase, high performance liquid chromatography (HPLC) on a Waters Radial-PAK C_{18} column. Isocratic elution was performed with a mixture of acetonitril and 0.02 M ammonium acetate, pH 4, (22/78, v/v) at a flow of 1.5 ml/min. Fractions of 0.5 min (750 µl) were collected and counted for radioactivity. Secondly, reactions with unlabeled T_4S were terminated by addition of 500 µl 1 N HCl, followed by treatment for 1 h at 80°C to hydrolyze the sulfated products. After addition of 550 µl 1 N NaOH, products were estimated in duplicate in 50 µl aliquots of the resulting mixtures by specific RIA's (8). Products were not measurable without hydrolysis, in keeping with the low cross-reactivities of the sulfate conjugates in the RIA's (14). Thirdly, in parallel incubations ^{125}I$^-$ production from $[3',5'-^{125}$I$]T_4$S was quantitated after addition of 1 ml 1 N HCl by ion-exchange chromatography on Dowex 50W-X2 columns (15). The ORD of unlabeled or outer ring ^{125}I-labeled rT_3 sulfate (rT_3S) was quantitated in the same way after hydrolysis with a $3,3'-T_2$ RIA or by measurement of the release of radioiodide by ion-exchange chromatography. The products of T_4 or rT_3 deiodination were also determined by specific RIA's (8). Protein determinations were done by the method of Bradford using bovine serum albumin as the standard (16).

RESULTS

Figure 1 shows the effects of incubation time and enzyme concentration on the formation of deiodination products from T_4 or T_4S as determined by RIA. As demonstrated previously (8), approximately equal amounts of T_4 are converted by ORD to T_3 and by IRD to rT_3. Little rT_3 accumulates, however, as it is rapidly metabolized further by ORD to $3,3'-T_2$, whereas T_3 is stable in such incubations (Fig. 1, ref. 8).

outer ring-labeled substrate) are produced in equal amounts. Eventually, also the levels of $3,3'-T_2S$ reach a plateau while those of $^{125}I^-$ continue to increase. Rapid ORD of $3,3'-T_2S$ has also been observed previously (12,13). Under all circumstances T_3 sulfate (T_3S) production was below the detection limit of the RIA representing less than 0.1% conversion of T_4S by ORD. As is depicted in Table 1, added rT_3S was found to undergo rapid ORD with an apparent K_m value of 0.06 ± 0.01 µM and a V_{max} of 516 ± 70 pmol T_2S formed/min/mg protein (mean $\pm$ S.E., n=6), which is similar to the deiodination parameters for non-conjugated rT_3 in the presence of 3 mM DTT. It is unlikely that the lack of T_3S accumulation in reaction mixtures with T_4S is due to rapid further deiodination of this product. The V_{max}/K_m ratio of T_3S deiodination is 37-fold lower than that of rT_3S deiodination (Table 1). Nevertheless, rT_3S is readily observed as an intermediate in the deiodination of T_4S whereas formation of T_3S is not detectable.

TABLE I

Kinetic parameters of the deiodination of native and sulfated iodo-
thyronines by rat liver microsomes and 3-5 mM DTT at pH 7.2 and 37°C.

Substrate	Reaction	K_m[a]	V_{max}[b]	V_{max}/K_m	Ref.
T_4	ORD	2.3	30	13	8
T_4	IRD	1.9	18	9	8
T_4S	ORD	N.D.[c]			this paper
T_4S	IRD	0.29	527	1820	this paper
rT_3	ORD	0.06	560	8730	8
rT_3S	ORD	0.06	516	8600	this paper
T_3	IRD	6.2	36	6	8
T_3S	IRD	4.6	1050	230	10

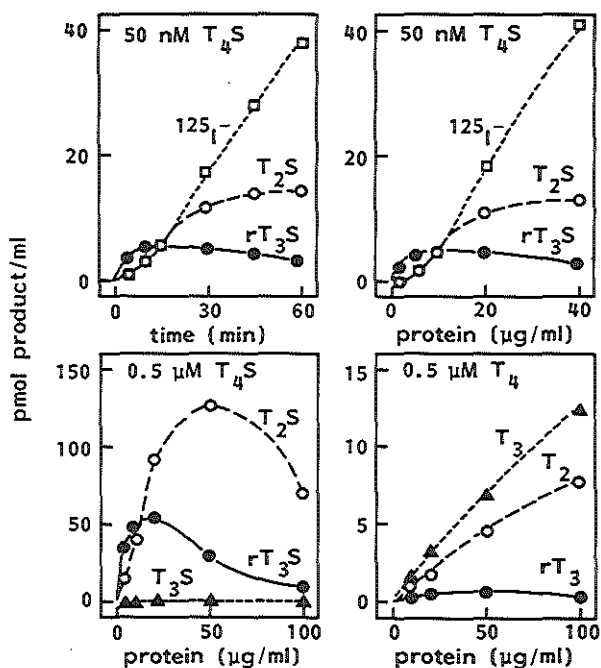[a] µM; [b] pmol/min/mg protein; [c] not detectable

Fig.1. *Sequential deiodination of unlabeled T_4 or T_4S as a function of reaction time or enzyme concentration. Products were measured by specific RIA except for the $^{125}I^-$ production which was quantitated by ion-exchange chromatography after reaction with labeled substrates. The results are the means of four closely agreeing experiments each performed in triplicate. Upper panels. Production of rT_3S (●), $3,3'-T_2S$ (○) and $^{125}I^-$ (□) from 0.05 μM T_4S as a function of time of incubation with 10 μg microsomal protein per ml (left), or as a function of microsomal protein concentration with an incubation time of 15 min (right). Lower panels. Production of rT_3S (●), T_3S (▲) and $3,3'-T_2S$ (○) from 0.5 μM T_4S (left), or production of rT_3 (●), T_3 (▲) and $3,3'-T_2$ (○) from 0.5 μM T_4 (right) as a function of microsomal protein concentration with an incubation time of 15 min.*

Not only the rate of T_4 metabolism but also the nature of the deiodination products is influenced dramatically by sulfation (Fig.1). Reaction of T_4S with rat liver deiodinase yields rT_3S as the initial product. Subsequently, $3,3'-T_2$ sulfate ($3,3'-T_2S$) and radioiodide (from

Deiodination products of T_4S were also measured by HPLC both before and after hydrolysis, using different conditions for the elution of free and sulfated iodothyronines. The same results were obtained with either method, supporting the validity of the measurements done by RIA following hydrolysis. Figure 2 shows the direct HPLC analysis of reaction mixtures of T_4S with increasing deiodinase concentrations. These results again clearly demonstrate the transient formation of rT_3S and $3,3'-T_2S$ with outer ring-derived iodide as the end product. Again, no formation of T_3S was observed.
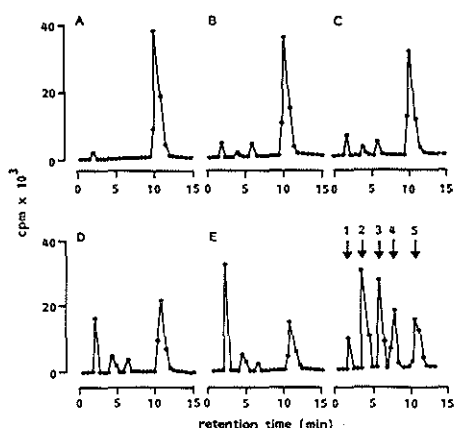

Fig.2. HPLC analysis of the sequential deiodination of 0.05 µM ^{125}I outer ring labeled T_4S as a function of microsomal protein concentration. Products were separated by HPLC without prior hydrolysis. Fractions of 0.5 min were collected and counted for radioactivity. Microsomal protein concentrations amounted to 0 (A), 5 (B), 10 (C), 20 (D) or 40 (E) µg/ml. The last panel shows the elution positions of the reference compounds I^- (1), $3,3'-T_2S$ (2), rT_3S (3), T_3S (4) and T_4S (5). Data are taken from one out of three closely agreeing experiments.

It appears, therefore, that T_4S undergoes the following sequence of deiodination reactions: 1) IRD to rT_3S (plus non-radioactive iodide); 2) ORD of rT_3S to $3,3'-T_2S$ plus $^{125}I^-$; 3) ORD of $3,3'-T_2S$ with liberation of the second $^{125}I^-$ (refs. 12 and 13).

The kinetics of the IRD of T_4S were studied under initial reaction rate conditions, i.e. short reaction times and low enzyme concentrations.

Under these conditions further deiodination of rT_3S proved to be negligible. In the presence of 5 mM DTT the apparent K_m for T_4S amounts to 0.29 ± 0.02 µM, with a V_{max} of 527 ± 91 pmol rT_3S formed/min/mg protein (mean $\pm$ S.E., n = 5). By comparison with the kinetic parameters of the conversion of T_4 to rT_3, the enhancement of the IRD of T_4 by sulfation appears to be due to a 30-fold increase in V_{max} together with a 7-fold decrease in apparent K_m (Table 1).

Deiodination of iodothyronines by rat liver deiodinase follows ping-pong type reaction kinetics with DTT as the cofactor, and is inhibited uncompetitively by PTU. This is demonstrated by the parallel lines in the Lineweaver-Burk plot if deiodination of varying iodothyronine concentrations is measured in the presence of different concentrations of DTT or PTU (15,17-19). There is also strong evidence that an essential sulfhydryl group of the deiodinase actively participates in the deiodination reaction. In consideration of the reactivity of thiouracil derivatives towards sulfenyl iodide (-SI) groups, these findings imply that in the catalytic cycle an enzyme-SI complex is generated which is subsequently reduced by DTT (15,18,19). Figure 3 demonstrates the stimulation of the IRD of T_4S by increasing DTT concentrations as tested at a single (0.5 µM) substrate concentration.
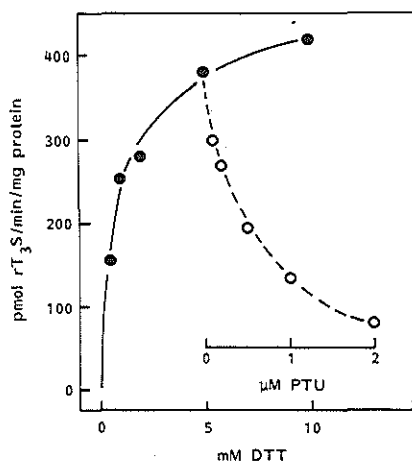

Fig.3. Effects of various concentrations DTT or PTU on the T4S IRD. 0.5 µM T4S was reacted with 0.2 µg microsomal protein at 37C and pH 7.2 in the presence of various DTT concentrations (●) or at 5 mM DTT with various concentrations PTU (○). Products from unlabeled substrate were measured after hydrolysis by a specific RIA. The results are the means of 2 experiments.

Under these conditions deodination rates approach a maximum at 10 mM DTT. In Fig. 3 is also demonstrated the dose-dependent inhibition by PTU in reaction mixtures with 0.5 µM T_4S and 5 mM DTT. Half-maximum inhibition is observed at 0.5 µM PTU. Figure 4 shows the parallel displacement of the Lineweaver-Burk plot of the IRD of T_4S by a decrease in the DTT concentration from 5 to 2 mM or by addition of 0.5 µM PTU. The Lineweaver-Burk plot of the ORD of rT_3S also undergoes a parallel displacement by variation of the DTT concentration from 2 to 10 mM as is depicted in Fig. 5. The parallel lines of the rT_3S ORD are also obtained in the presence of the competitive inhibition by 1 µM T_4, which is strong evidence for true ping-pong type reaction kinetics (20). Our results therefore indicate an identical mechanism for the deiodination of free and sulfated iodothyronines by rat liver deiodinase.
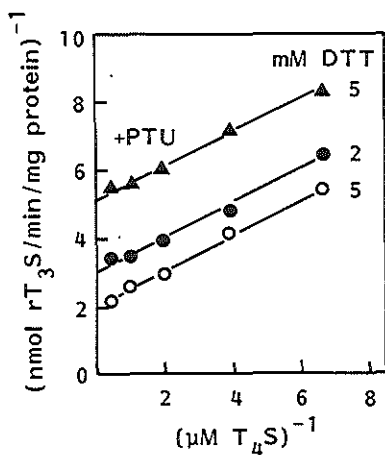

Fig.4. Double reciprocal plot of the rate of T4S IRD versus T4S concentration at 2 mM (●) or 5 mM (○) DTT, or at 5 mM DTT in the presence of 0.5 µM PTU (▲). Products from unlabeled substrate were measured after hydrolysis by a specific RIA. The results are the means of 4 experiments.

Previous studies have demonstrated that IRD and ORD of non-sulfated iodothyronines are catalyzed by a single rat liver enzyme. This was evidenced, among other things, by the identical effects on the IRD of T_3 and the ORD of rT_3 by a variety of competitive inhibitors (4). In a similar experiment the IRD of T_4S and the ORD of rT_3 were compared. Care was taken to avoid photodynamic degradation of substrate especially in the case of Rose Bengal by incubation in the dark (21). The results (Fig. 6)
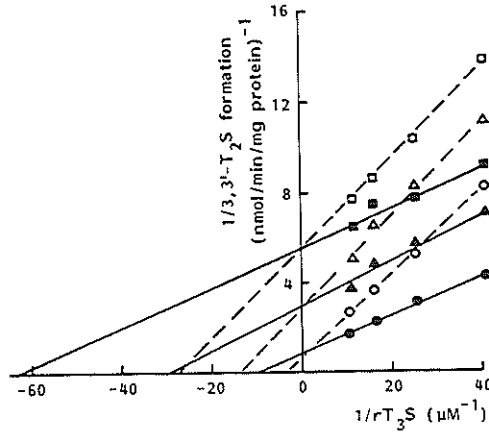
Fig.5. Double reciprocal plot of the rate of rT_3S ORD versus rT_3S concentrations at 2 mM (■), 5 mM (▲) or 10 mM (●) DTT in the absence or presence of 1 µM T_4 (open symbols). Radioiodide produced by ORD of ^{125}I-labeled substrate was quantitated by ion-exchange chromatography. The results are the means of two closely agreeing experiments, each performed in triplicate. Regression lines were calculated by the method of least squares applied to unweighted means.
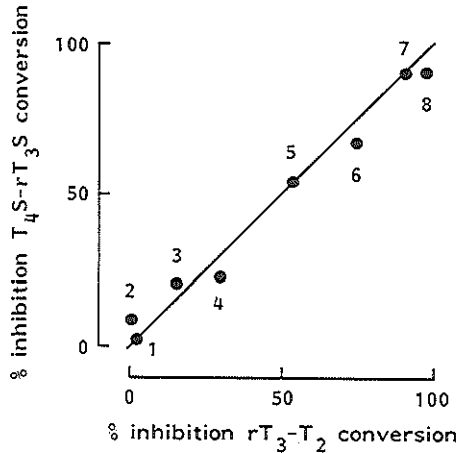
Fig.6. Correlation between the inhibition of rT_3 ORD or T_4S IRD by various substances. Reactions of 0.05 µM rT_3 or 0.15 µM T_4S with 0.4 µg microsomal protein were carried out in the absence or presence of 1 µM diiodotyrosine (1), 3,5-diiodothyronine (2), iopanoic acid (3), T_4 (4), diiodosalicylic acid (5), bromothymol blue (6), bromophenol blue (7) or rose bengal (8). ORD of rT_3 was quantitated by 3,3'-T_2 RIA and IRD of T_4S by summation of rT_3 and 3,3'-T_2 measured by RIA following hydrolysis.

show that various substances have equal inhibitory effects on these two reactions. The IRD of labeled T_4S is also inhibited 90% by 1 μM rT_3 as observed by HPLC analysis (not shown). These findings are compatible with the hypothesis that iodothyronines and their sulfate conjugates are substrates for IRD and ORD by a single liver enzyme (12,13).

DISCUSSION

Our findings demonstrate that sulfation prevents the ORD but facilitates the IRD of T_4. A similar facilitory effect of sulfation on the deiodination of iodothyronines has previously been observed for the IRD of T_3 and the ORD of $3,3'-T_2$ (12,13). As concluded from the V_{max}/K_m ratio's for the different reactions, the efficacy of the IRD of T_4 and that of T_3 are increased 200- and 40-fold, respectively (Table 1). To our knowledge, ORD of rT_3 is the most efficient reaction catalyzed by rat liver deiodinase (Table 1). The effect of sulfation on the IRD of T_4 may be owing to a closer approach of the inner ring to the catalytic sulfhydryl group of the enzyme in a way that resembles the presentation of the outer ring of rT_3. In this configuration the outer ring of T_4S may then be inaccessible for deiodination. For reasons yet unknown, sulfation does not influence the ORD of rT_3.

The physiological importance of the effects of sulfation on the deiodination of T_4 depends on the extent to which T_4 is sulfated in vivo. In normal rats, equilibrated with ^{125}I-labeled T_4, about 50% of the radioactivity appears in the urine as [125]I$^-$ and 50% is excreted with the bile mainly as glucuronic acid or sulfate conjugates (10,22). When in such rats deiodination is inhibited by administration of PTU or butyl 4-hydroxy-3,5-diiodobenzoate, there is a striking increase in biliary disposal concomitant with the decrease of radioactivity in the urine (11,23,24). However, biliary excretion of material with characteristics of T_4S is increased disproportionately in comparison with that of T_4 glucuronide, i.e. from 10 to 30% of the T_4 gluronide excretion, following the administration of these inhibitors (11,23). Formation of T_4 sulfate has also been observed with cultured rat hepatocytes, especially when deiodinase activity is saturated or inhibited with PTU (9). It is not

clear yet which sulfotransferases are responsible for the sulfation of T_4 (25).

The present paper is the first report on the use of pure synthetic T_4S in the investigation of its metabolism. The results indicate that if T_4S is produced in vivo it would be difficult to detect unless its deiodination is blocked, for instance with PTU. The availability of standard T_4S preparations should facilitate further investigations of the role of sulfation in the overall metabolism of T_4.

In conclusion, sulfation of the phenolic hydroxyl group of T_4 leads to a dramatic increase in the production of metabolites that are biologically inactive. At the same time, the activating pathway to T_3 is blocked. Together with our observation of a rapid degradation of T_3S (12,13), these results suggest that sulfation is an important step in the termination of thyroid hormone activity.

ACKNOWLEDGEMENTS

REFERENCES

1. Chopra IJ, Solomon DH, Chopra U, Wu SY, Fisher DA, Nakamura Y 1978 Pathways of metabolism of thyroid hormones. Recent Prog Horm Res 34: 521.
2. Larsen PR, Silva JE, Kaplan MM 1981 Relationship between circulating and intracellular thyroid hormones: physiological and clinical implications. Endocr Rev 2: 87.
3. Engler D, Burger AG 1984 The deiodination of the iodothyronines and their derivatives in man. Endocr Rev 5: 151.
4. Fekkes D, Hennemann G, Visser TJ 1982 Evidence for a single enzyme in rat liver catalysing the deiodination of the tyrosyl and the phenolic ring of iodothyronines. Biochem J 201: 673.
5. Chopra IJ, Chua Teco GN 1982 Characteristics of inner ring (3 or 5) monodeiodination of 3,5-diiodothyronine in ``` liver: evidence suggesting marked similarities of inner `nd ot `er ring deiodinases for iodothyronines. Endocrinology 110: `c
6. Visser TJ, Kaptein E, Krenning EF 1983 Deiodination of iodothyronines in human liver. Program of the 59th Meeting of the American Thyroid Association, New Orleans, Louisiana, T-16.
7. Visser TJ, Van der Does-Tobe I, Docter R, Hennemann G 1976 Subcellular localization of a rat liver enzyme converting thyroxine into triiodothyronine and possible involvement of essential thiol group. Biochem J 157: 479.
8. Visser TJ, Fekkes D, Docter R, Hennemann G 1979 Kinetics of enzyme reductive deiodination of iodothyronines. Biochem J 179: 489.
9. Sato K, Robbins J 1981 Thyroid hormone metabolism in primary cultured rat hepatocytes, effects of glucose, glucagon, and insulin. J Clin Invest 68: 475.
10. Morreale de Escobar G, Escobar del Rey F 1967 Extrathyroid effects of some antithyroid drugs and their metabolic consequences. Recent Prog Horm Res 23: 87.
11. Flock EV, Bollman JL 1962 The effect of thiouracil on the metabolism of L-thyroxine. Biochem J 84: 621.
12. Otten MH, Mol JA, Visser TJ 1983 Sulfation preceeding deiodination of iodothyronines in rat hepatocytes. Science 221: 81.
13. Visser TJ, Mol JA, Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112: 1547.
14. Mol JA, Visser TJ 1985 Synthesis and properties of sulfate esters and sulfamates of iodothryronines. Endocrinology
15. Leonard JL, Rosenberg IN 1980 Characteristics of essential enzyme sulfhydryl groups of thyroxine 5'-deiodinase from rat kidney. Endocrinology 106: 444.
16. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein ulitizing the principle of protein-dye binding. Anal Biochem 72: 248.
17. Chopra IJ 1977 A study of extrathyroidal conversion of thyroxine (T_4) to 3,3',5-triiodo-thyronine in vitro. Endocrinology 101: 453.
18. Visser TJ 1979 Mechanism of action of iodothyronine-5'-deiodinase. Biochim Biophys Acta 569: 302.
19. Leonard JL, Rosenberg IN 1978 Thyroxine-5'-deiodinase activity of rat kidney: observations on activation by thiols and inhibition

by propylthiouracil. Endocrinology 103: 2137.

20. Segel, IH 1975 In: Enzyme kinetics; behavior and analysis of rapid equilibrium and steady-state enzyme systems. John Wiley and Sons, New York.

21. Mol JA, Docter R, Hennemann G, Visser TJ 1984 Modification of rat liver iodothyronine 5'-deiodinase activity with diethylpyro-carbonate and Rose Bengal: evidence for an active site histidine residue. Biochem Biophys Res Commun 120: 28.

22. Földes O, Langer P, Straussova K, Brozmanova H, Gschwendtova K 1982 Direct quentitative estimation of several iodothyronines in rat bile by radioimmunoassay and basal data on their biliary excretion. Biochim Biophys Acta 716: 383.

23. Flock EV, Bollman JL 1964 Effect of butyl-4-hydroxy-3,5-diiodobenzoate on the metabolism of L-thyroxine and related compounds. Endocrinology 75: 721.

24. Takai NA, Rapoport B, Yamamoto M 1980 Biliary excretion of iodothyronines in rats as determined by high pressure liquid chromatograpy: effect of starvation. Endocrinology 107: 176.

25. Sekura RD, Sato K, Cahnmann HJ, Robbins J, Jakoby WB 1981 Sulfate transfer to thyroid hormones and their analogs by hepatic aryl sulfotransferases. Endocrinology 108: 54.

C H A P T E R   V

MODIFICATION OF RAT LIVER IODOTHYRONINE 5'-DEIODINASE ACTIVITY WITH
DIETHYLPYROCARBONATE AND ROSE BENGAL; EVIDENCE FOR AN ACTIVE SITE
HISTIDINE RESIDUE

Jan A. Mol, Roel Docter, George Hennemann and Theo J. Visser

Department of Internal Medicine III and Clinical Endocrinology,
Erasmus University Medical School, Rotterdam, The Netherlands

ABSTRACT.

Iodothyronine 5'-deiodinase activity of rat liver microsomes was
rapidly and completely lost by treatment with diethylpyrocarbonate (DEP)
and by photo-oxidation with Rose Bengal (RB). In both cases inactivation
followed pseudo first order reaction kinetics. Inactivation by DEP was
diminished in the presence of substrate or competitive inhibitors, and was
reversed by hydroxylamine treatment. In addition to photo-oxidation,
deiodinase activity was also inhibited by RB in the dark. This inhibition
was reversible and competitive with substrate (K_i 60 nM). These results
suggest the location of an essential histidine residue at or near the
active site of rat liver iodothyronine deiodinase.

Enzymatic 5'-deiodination of thyroxine (T_4) in peripheral tissues is
the major pathway for the production of the biologically active form of
thyroid hormone, 3,3',5-triiodothyronine (T_3) (1,2). The 5'-deiodinase
activity of the liver and the kidneys is associated with the microsomal
fractions of these tissues. It prefers 3,3',5'-triiodothyronine (reverse
T_3, rT_3) as substrate, and requires the reductive equivalents of thiols,

e.g. reduced glutathione, mercaptoethanol and dithiothreitol (DTT) (3-6). Deiodination is inhibited by sulfhydryl (SH) group-blocking agents such as N-ethylmaleimide, p-chloromercuriphenylsulfonic acid and especially iodoacetate, suggesting the participation of an enzyme SH group in the catalytic process (5). This is substantiated by the findings of potent and uncompetitive inhibition of deiodination by thiouracil derivatives, compounds known to react with sulfenyl iodide groups (7,8). Supposedly, the enzyme SH group acts as an acceptor of the 5'-iodine atom.

The nucleophilicity of catalytically active enzyme SH groups is often increased by hydrogen bond formation with the imidazole group of a neighbouring histidine (His) residue (9). In this study we investigated the possible presence of an essential His residue in the deiodinase of rat liver microsomes using two His-selective reagents. At neutral or near neutral pH, limited reaction of proteins with diethylpyrocarbonate (DEP) leads to the preferential modification of His residues forming the N^{im}-carbethoxy derivative (10). However, especially at alkaline pH DEP may also react with lysine and cysteine residues, but only carbethoxylation of His is reversible with hydroxylamine (10-15). Another approach to modification of His residues is photo-oxidation sensitized by Rose Bengal (9,12,15). Compared with other susceptible residues reaction with His is favoured by complex formation of the anionic dye with the protonated imidazole group. The results of the present study, employing both DEP and RB, indicate the presence of an essential His residue at or near the active site of the iodothyronine deiodinase of rat liver.

MATERIALS AND METHODS.

Reverse T_3 and 3,3'-diiodothyronine (3,3'-T_2) were obtained from Henning, Berlin, FRG; DEP from Aldrich; RB from Kodak-Eastman; iopanoic acid (IOP) from Sterling-Winthrop, Amsterdam, The Netherlands; and carrier-free Na ^{125}I from Amersham. [3',5'-^{125}I]rT$_3$ was prepared by radioiodination of 3.3'-T_2 with the chloramine-T method and purified on Sephadex LH-20 (16). All other reagents were of the highest quality commercially available. Rat liver microsomes were prepared in 0.15 M

sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT, essentially as previously described (17). Protein was measured according to Bradford using bovine serum albumin as the standard (18,19).

Enzyme modification with DEP.

DEP was dissolved and diluted to the desired concentrations in absolute ethanol. The actual DEP concentration was determined by reaction of 10 μl aliquots with 1 ml 0.1 M His in 0.1 M sodium phosphate (pH 6.0). From the increase in absorbance at 240 nM the DEP concentration was calculated using a molar extinction coefficient for N^{im}-carbethoxy-His of 3200 $M^{-1} \cdot cm^{-1}$ (20). Protein modification was carried out by reaction of 10 μl DEP in ethanol with 140 μg of microsomal protein in 0.5 ml 50 mM sodium phosphate (pH 6.5 unless stated otherwise), 1 mM EDTA and 1 mM DTT, at 21C. After various times the reaction was stopped by quenching 50 μl aliquots of the mixtures in 150 μl 0.1 M His in 0.1 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT. In control incubations DEP was added to microsomes diluted in quench buffer. At the concentration of 2 %, ethanol did not affect enzyme activity. Protection against carbethoxylation by substrate or competitive inhibitors was investigated by preincubation of microsomes with these substances for at least 5 min prior to addition of DEP. Reversibility of DEP inhibition was tested by incubation of carbethoxylated microsomes for 1 h at 21C with 0.5 M NH_2OH in 0.1 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT.

Photo-oxidation with RB.

Microsomal suspensions containing 1.4 mg protein per ml 0.15 M sodium phosphate (pH 7.2), 3mM EDTA and 3mM DTT, were irradiated in the presence of the indicated concentrations of RB. Irradiation was done for various times at 21C in polypropylene tubes placed at 10 cm distance from the lens of a slide projetor equipped with a 300 W lamp. To correct for reversible, competitive inhibition by RB, control incubations were carried out in the dark. Irradiation in the absence of RB did not affect enzyme activity.

Iodothyronine 5'-deiodination assay.

Appropriate dilutions of the inactivation mixtures (2-5 µg microsomal protein) were incubated for 10 min at 37C with 0.1 µCi ^{125}I-rT_3 and 0.5 µM unlabeled rT_3 in 0.25 ml 0.15 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT. The reaction was stopped by addition of 0.25 ml human serum, and the tubes were placed on ice. Serum protein-bound iodothyronines, i.e. substrate rT_3 and product 3,3'-T_2 were precipitated with 0.5 ml 10% trichloroacetic acid. Production of iodide was calculated from the radioactivity measured in 0.5 ml of the supernatant after centrifugation for 10 min at 1500 x g. The results were corrected for non-enzymatic deiodination as assessed in enzyme-free incubations, and multiplied by two to account for the equal distribution of the ^{125}I label among the products 3,3'-T_2 and I^-. Identical results were obtained if deiodinase activity was measured by quantitation of 3,3'-T_2 production from unlabeled rT_3 with a specific radioimmunoassay (2).
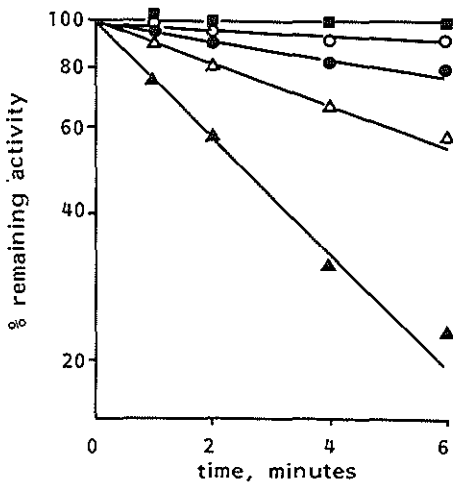

Fig.1 Inactivation of rT3 5'-deiodinase activity by reaction of rat liver microsomes for various times at pH 6.5 and 21C without (■) or with 0.3 (○), 0.6 (●), 1.2 (△) or 3 (▲) mM DEP.

RESULTS

Reaction with DEP.

Incubation of rat liver microsomes with millimolar concentrations of DEP at 21C resulted in a rapid loss of 5'-deiodinase activity. The time course of enzyme inactivation by different DEP concentrations at pH 6.5 is shown in Fig. 1. Inhibition by DEP followed pseudo first order kinetics during the first 6 min of reaction. The rate of inactivation was also determined over the pH range 6.5 – 8.5 (Fig. 2). Between pH 7.2 and 7.8 there was a sharp increase in the rate of enzyme inactivation by DEP to remain constant thereafter. Plots of inactivation rate constants as a function of DEP concentration were lineair (Fig. 3) and yielded second order rate constants for the simple bimolecular reaction of DEP with enzyme. Values for the second order k amounted to 40 $M^{-1}min^{-1}$ at pH 6.5 and 530 $M^{-1}min^{-1}$ at pH 8, corroborating the pH profile shown in Fig. 2.
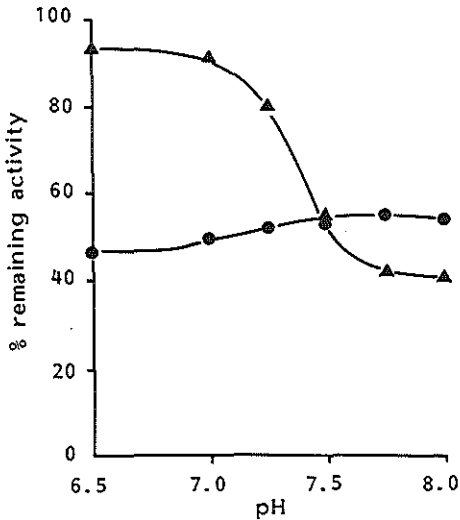
Fig.2 Inactivation of rT3 5'-deiodinase activity by DEP or RB-induced photo-oxidation as a function of pH. The percentage remaining activity was determined after reaction of rat liver microsomes for 2 min with 0.6 mM DEP (▲) or after photo-oxidation for 4 min with 1 µM RB (●).
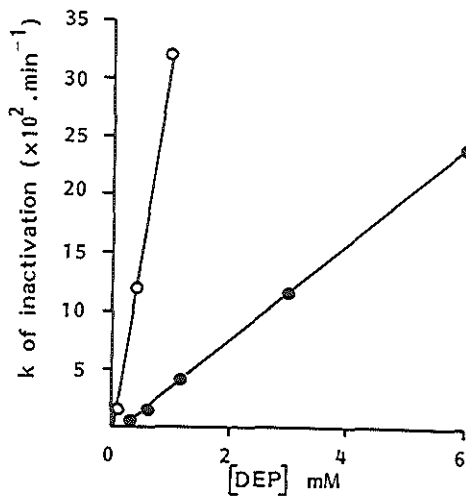
Fig.3 *First order rate constant (k) of inactivation of rT3 5'-deiodinase activity by DEP as a function of the DEP concentration at pH 6.5 (●) or pH 8.0 (○).*

TABLE I

Reversal of DEP inactivation of 5'-deiodinase activity by hydroxylamine

DEP concentration (mM)	% remaining activity before and after tratement with NH_2OH	
	BEFORE	AFTER
0.0	100	100
0.6	74	93
1.2	38	90
3.0	13	55
6.0	5	23

Fig.4 Inactivation of rT3 5'-deiodinase activity by reaction of rat liver microsomes for various times at pH 6.5 and 21C with 6 mM DEP in the absence (○) or the presence of 1 μM RB (●) (in the dark) 2 μM rT3 (△) or 2 μM IOP (▼).

Carbethoxylated microsomes were treated with 0.5 M NH_2OH to exclude the possibility that inactivation was due to reaction of DEP with lysine or cysteine residues. Table I demonstrates that, especially after limited reaction with DEP, NH_2OH treatment resulted in substantial recovery of deiodinase activity. To localize the residue(s) subject to carbethoxylation, reaction with DEP was carried out in the presence of 2 μM rT_3 or IOP or 1 μM RB. The concentration of these compounds carried over from the preincubation mixtures were insufficient to affect enzyme activity in the subsequent assay. This was substantiated in appropriate control experiments. Fig. 4 shows that the substrate and both competitive inhibitors provided partial protection against inactivation by DEP.

Reaction with RB.

Deiodinase activity was also strongly inhibited by photo—oxidation

sensitized by low concentrations of RB. Direct addition of RB to the deiodinase assay mixtures, incubated in the dark, also resulted in inhibition of enzyme activity. This inhibition was reversible by simple dilution, and Lineweaver-Burk analysis of the data demonstrated that RB was a competitive inhibitor with a K_i value of 60 nM (not shown). Care was taken, therefore, that following photo-oxidation mixtures were diluted sufficiently as to prevent "dark" inhibition by RB in the deiodinase assay. At the concentrations of RB used in the preincubations no effect on enzyme activity was observed without irradiation. The semilogarithmic plots of the percentage enzyme activity remaining as a function of time of irradiation at different RB concentrations were lineair (Fig. 5), showing that photo-oxidation follows pseudo first order kinetics.

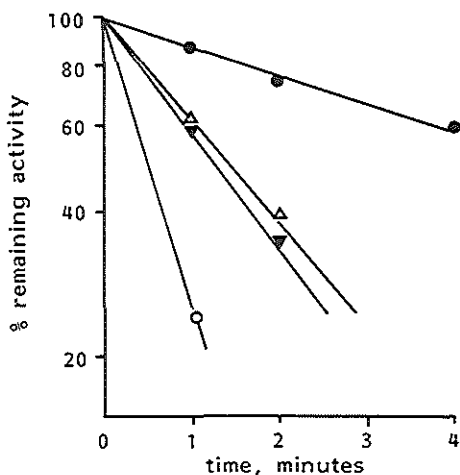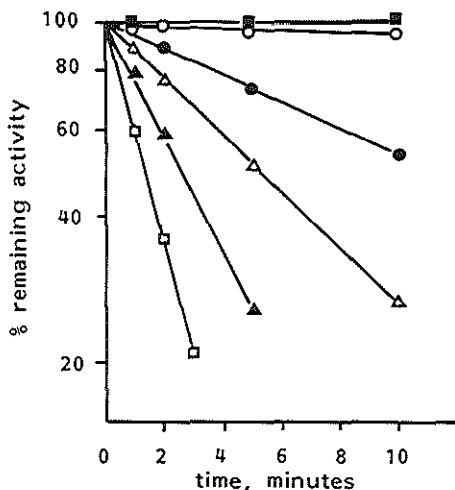*Fig.5 Inactivation of rT3 5'-deiodinase activity by irradiation of rat liver microsomes for various times at pH 7.2 and 21C in the absence (■) or the presence of 0.1 (○), 0.3 (●), 0.5 (△), 1 (▲) or 2 (□) μM RB.*

From the lineair replot of the inactivation constants versus RB concentration, the second order rate constant was calculated as 125 $mM^{-1}min^{-1}$. To prevent the possible modification of cysteine and methionine residues, 3 mM DTT was usually included in the reaction mixtures. It was found that 5 mM DTT and 5 mM sodium azide provided only 40-50% protection

rapid release of labeled iodide from the outer ring. Destruction of substrate did not occur in the dark. This phenomenon interfered with the test of the possible protective effect of substrate and other halogenated compounds against enzyme inactivation by RB. Only a high concentration of IOP gave partial protection, and degradation of IOP in the reaction mixtures seems likely.

b. In the dark controls we observed competitive inhibition of deiodinase activity by RB that reflects the structural relationship with iodothyronines as is the case with phenolphthalein dyes (24). Interestingly, the K_i for RB (0.06 µM) is less than the K_m for rT_3 (0.1 uM), making RB one of the most potent inhibitors of deiodination.

c. Photo-oxidation reactions can proceed by two distinct mechanisms. Type II mechanism is that in which the energy of the sensitizer is transferred to molecular oxygen producing singlet oxygen ($^1O_2$) (25,26). $^1O_2$ is the reactive species in enzyme inactivation by the type II mechanism. As compared to other dyes, RB and the frequently used dye methylene blue, give rise to high $^1O_2$ production rates (25), which may be quenched by scavengers such as azide and DTT (25,26). Since the present experiments were carried out in the presence of DTT, inactivation must have taken place by a mechanism other than via production of $^1O_2$.

d. In the type I mechanism there is a direct reaction of triplet sensitizer with susceptible residues in the enzyme, followed by reaction of these residues with molecular oxygen. Inactivation by RB was slightly faster below pH 7 possibly due to complex formation of the anionic dye with a protonated His residue. It is expected that such complex formation is a saturable reaction at concentrations above the K_i value for RB (60nM). Saturation of RB-induced photo-oxidation, however, was not observed. This may be caused by the use of high microsomal protein concentrations resulting in a lowering of the free RB concentration for interaction with the enzyme.

In conclusion, the present study suggests that there is an essential His residue in the active site of rat liver iodothyronine deiodinase. It is speculated that this residue forms a hydrogen bond with the catalytically important SH group, resulting in an increased nucleophilicity of the latter.

REFERENCES

1. Schimmel, M., and Utiger, R.D. (1977) Ann. Intern. Med. 87, 760-768
2. Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1978) Biochem. J. 174, 221-229.
3. Fekkes, D., Van Overmeeren-Kaptein, E., Docter, R., Hennemann, G. and Visser, T.J. (1979) Biochim. Biophys. Acta 587, 12-19.
4. Auf dem Brinke, D., Hesch, R.D. and Köhrle, J. (1979) Biochem. J. 180, 273-279.
5. Visser, T.J. (1978) Molec. Cell. Endocrinol. 10, 241-247.
6. Chopra, I.J. (1978) Science 199, 904-906.
7. Visser, T.J. (1979) Biochim. Biophys. Acta 569, 302-308.
8. Leonard, J.L. and Rosenberg, I.N. (1980) Endocrinology 107, 1376-1383.
9. Tsai, C.S., Wand, A.J., Godin, J.R.P. and Buchanan, G.W. (1982) Arch. Biochem. Biophys. 217. 721-729.
10. Miles, E.W. (1977) Methods in Enzymology, volume 47, p.p. 431-442, Academic Press, New York.
11. Schneider, F. (1978) Angew. Chem. 90, 616-625.
12. Roy, B.P. and Ng, A.Y. (1982) Biochem. Biophys. Res. Commun. 109, 518-526.
13. Pilch, P.F. (1982) Biochemistry 21, 5638-5644.
14. Saluja, A.K. and McFadden, B.A. (1982) Biochemistry 21, 89-95.
15. Bhagwat, A.S. and Ramakrishna, J. (1981) Biochim. Biophys. Acta 662, 181-189.
16. Visser, T.J., Docter, R. and Hennemann, G. (1977) J. Endocrinol. 73, 395-396.
17. Visser, T.J., Van der Does-Tobe, I., Docter, R. and Hennemann, G. (1976) Biochem. J. 157, 479-482.
18. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
19. Read, S.M. and Northcote, D.H. (1981) Anal. Biochem. 116, 53-64.
20. Roosemont, J.L. (1978) Anal. Biochem. 88, 314-320.
21. Little, C. (1977) Biochem. J. 167, 399-404.
22. Wijnands, R.A. and Müller, F. (1982) Biochemistry 21, 6639-6646.
23. Holbrook, J.J. and Ingram, V.A. (1973) Biochem. J. 131, 729-738.
24. Fekkes, D., Hennemann, G. and Visser, T.J. (1982) FEBS Lett. 137, 40-44.
25. Houba-Herin, N., Calberg-Bacq, C.M., Piette, J. and Van de vorst, A. (1982) Photochem. Photobiol. 36, 297-306.
26. Mäkinen, K.K., and Mäkinen, P.L., Wilkes, S.H., Bayliss, M.E. and Prescott, J.J. (1982) J. Biol. Chem. 257, 1765-1772.

Biochem. Biophys. Res. Commun. 120, 28-36, (1984).

CHAPTER VI

INACTIVATION AND AFFINITY-LABELING OF RAT LIVER IODOTHYRONINE
DEIODINASE WITH N-BROMOACETYL-3,3',5-TRIIODOTHYRONINE.

Jan A. Mol, Roel Docter, Ellen Kaptein, Godfried Jansen,
George Hennemann and Theo J. Visser.

Erasmus University Medical School, Rotterdam, The Netherlands.

ABSTRACT

The thyroid hormone derivative N-bromoacetyl-3,3',5 -triiodothyronine
($BrAcT_3$) acts as an active site-directed inhibitor of rat liver
iodothyronine deiodinase. Lineweaver Burk analysis of enzyme kinetic
measurements showed that $BrAcT_3$ is a competitive inhibitor of the
5'-deiodination of 3,3',5' -triiodothyronine (rT_3) with an apparent K_i
value of 0.1 nM. Preincubations of enzyme with $BrAcT_3$ indicated that
inhibition by this compound is irreversible. The inactivation rate obeyed
saturation kinetics with a limiting inactivation rate constant of 0.35
min^{-1}. Substrates and substrate analogs protected against inactivation by
$BrAcT_3$. Covalent incorporation of ^{125}I-labeled $BrAcT_3$ into
"substrate-protectable" sites was proportional to the loss of deiodinase
activity. The results suggest that $BrAcT_3$ is a very useful affinity label
for rat liver iodothyronine deiodinase.

Active site-directed reagents have provided information about the
catalytic mechanism of type I iodothyronine deiodinase that occurs in rat
liver and kidneys. Enzyme activity is associated with the microsomal

fraction of these tissues and catalyzes the 5'-deiodination of thyroxine (T_4) to the biologically active 3,3',5-triiodothyronine (T_3) as well as the 5-deiodination of T_4 to the inactive 3,3',5-triiodothyronine (reverse T_3, rT_3) (1-4). Both T_3 and rT_3 are further deiodinated to 3,3'-diiodothyronine (3,3'-T_2) by type I deiodinase. The enzyme requires the reductive equivalents of thiols such as dithiothreitol (DTT) (5,6), and it exhibits optimal activity in the 5'-deiodination of rT_3 (3,7). The 5-deiodination of T_4 and T_3 is greatly facilitated by sulfation of the substrates (8,9).

Studies with iodoacetate and N-ethylmaleimide indicate the presence of an essential SH group in the active site of type I deiodinase (10-12). Apparently, this SH group acts as an acceptor for the iodinium ion from the substrate as evidenced by the uncompetitive inhibition by 6-propyl-2-thiouracil (PTU), a selective reagent for sulfenyl iodide (SI) groups (5,6). Modification with diethylpyrocarbonate or Rose Bengal suggests the location of an essential histidine residue at or near the active site (13).

In the present study the use of N-bromoacetyl-T_3 (BrAcT_3) as an affinity label of type I deiodinase is investigated. In contrast with reports on the reaction of N-bromoacetyl derivatives of T_3 and T_4 with the rat liver nuclear receptor (14) or with human thyroxine binding globulin (15) and human thyroxine binding prealbumin (16), the reaction with the rat liver deiodinase is extremely fast, indicating a very close interaction of the N-bromoacetyl side chain with a nucleophilic amino acid residue in the enzyme.

MATERIALS AND METHODS.

T_4, rT_3, T_3 and 3,3'-T_2 were obtained from Henning, Berlin, FRG; bromoacetyl chloride and bromoacetic acid from Aldrich; acetyl chloride from Merck; bromothymol blue from BDH; 3,5-diiodosalicylic acid and DTT from Sigma; and iopanoic acid from Sterling Winthrop Laboratories (New York, NY). T_4 sulfate was prepared as described elsewhere (9). [3'-^{125}I]T_3 (> 1200 μCi/μg) was purchased from Amersham. Rat liver

microsomes were prepared as described previously (3). Protein was measured according to Bradford using bovine serum albumin (BSA) as the standard (17).

Synthesis of N-bromoacetyl-T$_3$.

BrAcT$_3$ was synthesized as described by Cheng et al, method B (16). In short, 100-500 nmol unlabeled plus ^{125}I-labeled T$_3$ were dissolved in 20 ml dry ethylacetate. After addition of 10 µl (12 µmol) bromo acetylchloride the mixture was refluxed for 45 min. The ethylacetate was evaporated under reduced pressure and the residue was dissolved in 20% ethanol in 0.1 N NaOH. The solution was acidified with 1 N HCl and subsequently applied to a small 1 ml Sephadex LH-20 column. After washing the column with water, BrAcT$_3$ was eluted with 100% ethanol and stored at -20°C. The purity was checked by thin layer chromatography according to Cheng (16) or by reversed-phase high performance liquid chromatography on a µBondapak C18 column using a mixture of methanol and 20 mM sodium acetate, pH4 (55/45,v/v), at a solvent flow of 1.5 ml/min. Under these conditions BrAcT$_3$ and T$_3$ eluted with retention times of 4.8 and 5.9 min, respectively. Yields were typically over 90%. N-acetyl-T$_3$ (AcT$_3$) was synthesized in the same way by the use of acetyl chloride.

Inhibition of enzyme activity with BrAcT$_3$, AcT$_3$ or bromoacetate.

Deiodinase (0.7 µg microsomal protein) was reacted for 10 min at 37°C with 0.1 µM rT$_3$ and increasing concentrations of the inhibitors in 200 µl 0.2 M sodium phosphate (pH 7.2), 4 mM EDTA and 5 mM DTT. The reaction was terminated by the addition of 1 ml 0.06 M barbitone buffer (pH 8.6) containing 0.1% (w/v) SDS and 0.1% (w/v) BSA. The amount of 3,3'-T$_2$ produced was measured in duplicate with a specific radioimmunoassay (3). For the determination of the inhibition constant Ki a similar experiment was done with varying (0.05-2 µM) rT$_3$ concentrations and a fixed inhibitor concentration. The inactivation rate was determined by preincubation of microsomes (7 µg protein/ml) at 37°C with several concentrations BrAcT$_3$ (0.1-2 nM) in 100 µl 0.2 M sodium phosphate (pH 7.2), 4 mM EDTA and 5 mM

DTT. At various times the remaining activity was determined by addition of 200 µl 10 µM rT_3 in the same buffer and further incubation for 10 min at $37^{\circ}C$. In control experiments microsomes were added to the mixture of 10 uM rT_3 and the appropriate dilution of $BrAcT_3$. Protection by substrate or analogous compounds against inactivation by $BrAcT_3$ was determined by preincubation of microsomes for 5 min at $21^{\circ}C$ with 0.2 nM $BrAcT_3$ in the absence or presence of 1 µM test substance. The remaining activity was determined at $37^{\circ}C$ as described above.

Binding of [125I]$BrAcT_3$ to microsomes.

The specific incorporation of $BrAcT_3$ was determined by incubation of microsomes (13 µg/ml) with 0.2 nM 125I-labeled $BrAcT_3$ (50.000 cpm) in 400 ul 0.2 M sodium phosphate (pH 7.2), 4 mM EDTA and 5 mM DTT at $21^{\circ}C$ with or without 10 µM diiodosalicylic acid. At various times the incubation was stopped by the addition of 0.5 ml 1% BSA in 150 mM NaCl immediately followed by 2 ml acetone. The difference in precipitated radioactivity between reactions in the absence or the presence of diiodosalicylic acid was assumed to represent specific incorporation of [125I]$BrAcT_3$ in the deiodinase. Under identical circumstances the decrease in enzyme activity was determined.

RESULTS.

Inhibition of rat liver deiodinase with $BrAcT_3$, AcT_3 and bromoacetate.

Addition of increasing concentrations of $BrAcT_3$, AcT_3 or bromoacetate resulted in a progressive inhibition of the deiodination of a non-saturating concentration of rT_3 as assayed at $37^{\circ}C$ and pH 7.2 (Fig.1). Half-maximum inhibition was obtained at 0.1 nM $BrAcT_3$, 0.1 µM AcT_3 and 1 µM bromoacetate. Lineweaver-Burk analysis demonstrates that in coincubations with substrate both $BrAcT_3$ and AcT_3 behave as competitive inhibitors with apparent K_i values of 0.1 nM and 0.08 µM, respectively (Fig.2). The K_m value of 0.09 µM with a V_{max} of 830 pmol $3,3'-T_2$ formed/min/mg protein for

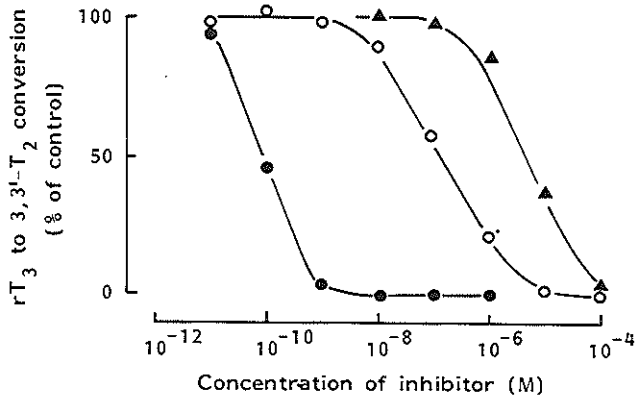Fig.1 *Inhibition of the conversion of rT3 into 3,3'-T2 by increasing concentrations of BrAcT3 (⊘), AcT3 (◯) or bromoacetate (▲). Results are the means of two closely agreeing experiments.*
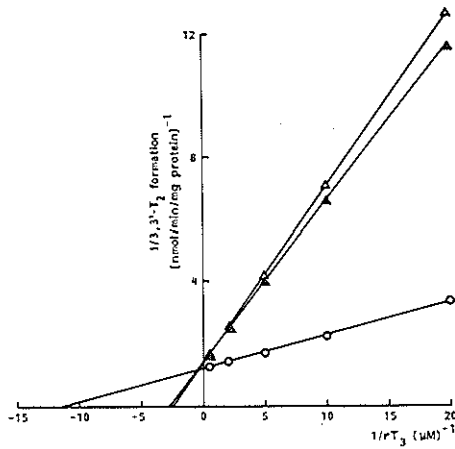

Fig.2 *Lineweaver-Burk plot of the conversion of rT3 into 3,3'-T2 at pH 7.2 in the absence (◯) or presence of 0.25 nM BrAcT3 (▲) or 0.25 µM AcT3 (△). Results are the means of 4 experiments performed in duplicate.*

rT$_3$ 5'-deiodination are in close agreement with values reported earlier (3). In Fig.3A the time course of enzyme inactivation is shown when microsomes are preincubated with different concentrations BrAcT$_3$. Only during the first two min the inactivation of the deiodinase by BrAcT$_3$ follows pseudo first order kinetics with rate constants that depend on the inhibitor concentration. If BrAcT$_3$ is an active site-directed inhibitor, it may be assumed that the non-covalent enzyme-inhibitor complex E·I is an intermediate in the formation of the covalent complex E-I that is accompanied by the irreversible loss of enzyme activity: E + I $\overset{Ki}{\rightleftharpoons}$ E·I $\overset{k3}{\longrightarrow}$ E-I. According to Kitz and Wilson the kinetics of inactivation are governed by the equation $1/k_{app} = K_i/(k_3 I) + 1/k_3$ (18). Figure 2B is a double-reciprocal plot of k_{app} versus the BrAcT$_3$ concentration. Obviously, inactivation by BrAcT$_3$ obeys saturation kinetics with values of 0.2 nM for the equilibrium dissociation constant K$_i$ and 0.35 min^{-1} for the limiting rate constant k$_3$.
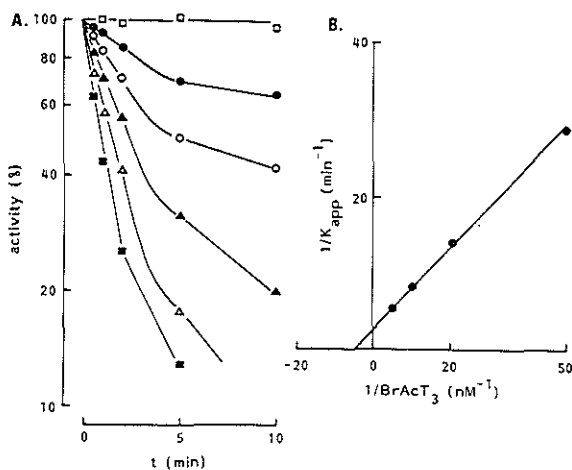
Fig.3A Inactivation of rT3 5'-deiodinase activity by reaction of rat liver microsomes for various times at pH 7.2 and 37°C without (□) or with 0.02 (●), 0.05 (○), 0.10 (▲), 0.20 (△) or 0.50 (■) nM BrAcT3.
*   3B Double-reciprocal plot of the first order rate constant (k$_{app}$) versus the concentration of BrAcT3.*
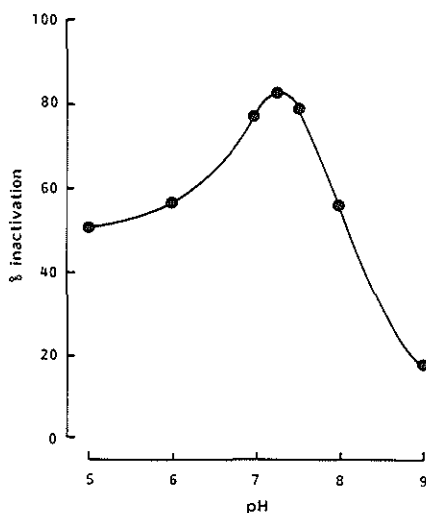
Fig.4 *Inactivation of rT3 5'-deiodinase activity by BrAcT3 as a function of pH. Preincubation with or without 0.2 nM BrAcT3 was carried out for 5 min at 21°C in 0.05 M sodium acetate (pH 5-6), sodium phosphate (pH 6-8) or sodium borate (pH 8-9) containing 4 mM EDTA and 5 mM DTT. The remaining activity was determined as described under Materials and Methods at pH 7.2.*

Figure 4 depicts the pH dependence of the rate of inactivation by BrAcT$_3$ over the pH range of 5 to 9. The inactivation is clearly optimal at pH 7.25 and rapidly diminishes at increasing pH values. A slower decline in the inactivation rate is observed with a decrease in pH. Evidence for the reaction of BrAcT$_3$ with an amino acid residue at or near the active site was obtained by investigation of the effects of various substrates or competitive inhibitors on the inactivation rate (Table I). The compounds with the lowest K$_i$ values gave the best protection against BrAcT$_3$-mediated enzyme inactivation.

Incorporation of [^{125}I]BrAcT$_3$.

In order to correlate the time course of enzyme inactivation with the covalent incorporation of BrAcT$_3$, microsomes were incubated with 0.2 nM

TABLE I

Protection by various substrates and analogous compounds against

BrAcT3 mediated deiodinase inactivation.[a]

Test agent (1μM)	K_i (μM)	% remaining activity	% protection
–	–	30	–
T_3	17.3[b]	32	2
T_4	2.7[b]	35	7
T_4 sulfate	0.3[c]	65	50
diiodotyrosine	191.0[b]	31	0
iopanoic acid	1.8[b]	61	44
bromothymol blue	1.4[d]	60	43
diiodosalicylic acid	0.3[b]	71	59

a. Deiodinase was reacted with BrAcT3 as described under Materials and Methods. The results are the means of 3 experiments each performed in duplicate.
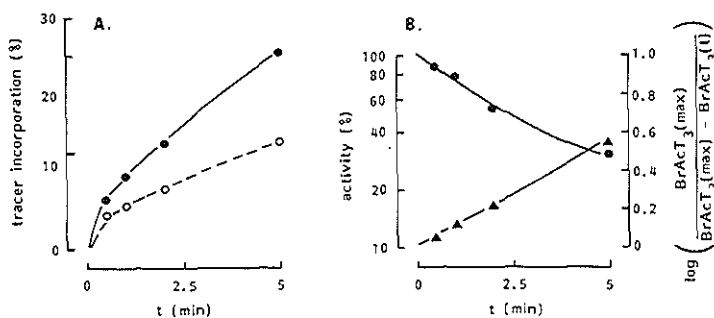b. ref 22; c. ref 9; d. ref 23.

Fig.5A Time course of [125I]BrAcT3 incorporation into rat liver microsomes. Incubations were done at 21°C in the presence (○) or absence (●) of 10 μM diiodosalicylic acid.

   5B Comparison of the incorporation rate of [125I]BrAcT3 into "protectable" sites (▲) with the inactivation rate (○) estimated under identical circumstances at 21°C.

^{125}I-labeled BrAcT$_3$ in the presence or absence of the competitive inhibitor diiodosalicylic acid. Figure 5 shows the results of this labeling experiment. At the concentration used, about 45% of the label is incorporated into "protectable" sites. The maximal incorporation of $[^{125}$I]BrAcT$_3$ into "protectable" sites amounted to 2.4 pmol/mg protein as revealed by reaction with increasing concentrations BrAcT$_3$ (not shown). Analysis of the time course of incorporation showed a rate constant of 0.11 min^{-1} which was slightly less than the inactivation rate of 0.135 min^{-1} measured under the same conditions. Identical inactivation rates were observed with the 5'-deiodination of rT$_3$ as with the 5-deiodination of T$_4$ sulfate (not shown).

DISCUSSION.

The T$_3$ analog BrAcT$_3$ has been found to be a very potent inhibitor of rat liver iodothyronine deiodinase through formation of an irreversible enzyme-inhibitor complex. The apparent K$_i$ as revealed by Lineweaver Burk analysis amounted to 0.1 nM which is extremely low in comparison with the K$_m$ for T$_3$ deiodination of 6 µM (3). The results with AcT$_3$ indicate that only introduction of the non-reactive acetyl group is sufficient to cause a decrease in the K$_i$ of T$_3$ to 0.08 µM. This is in keeping with the observed decrease in the K$_i$ of other iodothyronines by N-acylation (19). The inactivation of the deiodinase with different BrAcT$_3$ concentrations follows only during the first two min pseudo first order kinetics. The observed deviation is probably due to exhaustion of BrAcT$_3$ by reaction or hydrofobic interaction with microsomal proteins. The value of 0.35 min^{-1} for the limiting inactivation rate constant at 37°C is lower than that for iodoacetate-mediated inactivation of the renal deiodinating enzyme, i.e. 1.56 min^{-1} at 25°C (12). However, due to the high affinity of the BrAcT$_3$ for the enzyme the pseudo bimolecular rate constant (k$_3$/K$_i$) amounts to 2 10^9 M^{-1}min^{-1} compared with 3 10^5 M^{-1}min^{-1} for iodoacetate.

The reported data for the modification of type I deiodinase by iodoacetate strongly suggest that the inactivation is caused by carboxymethylation of a catalytically important SH group. Our study gives

no clue as to the identity of the amino acid residue(s) involved in the reaction with BrAcT$_3$. The attachment of the bromoacetyl group to the alanine side chain makes reaction with the catalytic SH group less probable since this SH group is thought to be positioned close to the leaving iodinium ion. It is also hazardous to interpret the pH profile of the inactivation in terms of the dissociation of a single amino acid residue.

The protection by various substrates and competitive inhibitors against inactivation by BrAcT$_3$ indicate the reaction of BrAcT$_3$ with an amino acid residue at or near the active site of the deiodinase. As is also seen in the case of iodoacetate-mediated inactivation, there is a good correlation between inactivation and incorporation of inhibitor into the deiodinase by the use of BrAcT$_3$ (12). The finding that the incorporation is somewhat slower in comparison with the rate of inactivation may be due to the uncertainty of the precise specific activity of the [^{125}I]BrAcT$_3$. SDS-PAGE of microsomes labeled with [^{125}I]BrAcT$_3$ shows that the label is mainly incorporated into two proteins with a molecular weight of 56 kD and 25 kD, respectively. Only incorporation in the latter was inhibited by 10 μM rT$_3$ (not shown). From the published molecular weights between 50-60 kD of deiodinase solubilized with cholate or desoxycholate it is suggested that the enzyme consists of two subunits (20,21).

In conclusion, the present paper demonstrates that BrAcT$_3$ acts as an affinity label for type I deiodinase and may provide a valuable tool in the purification of the enzyme.

REFERENCES

1. Cavalieri, R.R. and Pitt-Rivers, R. (1981) Pharmacol. Rev. 33, 55–80.
2. Larsen P.R., Silva, J.E. and Kaplan, M.M. (1981) Endocr. Rev. 2, 87–102.
3. Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1979) Biochem. J. 179, 489–495.
4. Fekkes, D., Hennemann, G. and Visser, T.J. (1982) Biochem. J. 201, 673–676.
5. Leonard, J.L. and Rosenberg, I.N. (1978) Endocrinology 103, 274–280.
6. Visser, T.J. (1979) Biochim. Biophys. Acta 579, 302–308.
7. Leonard, J.L. and Rosenberg, I.N. (1980) Endocrinology 107, 1376–1383.
8. Visser, T.J., Mol, J.A. and Otten, M.H. (1983) Endocrinology 112, 1547–1549.
9. Mol, J.A. and Visser, T.J. submitted for publication.
10. Chopra, I.J., Wu, S.Y., Nakamura, Y. and Solomon, D.H. (1978) Endocrinology 102, 1099–1105.
11. Leonard, J.L. and Rosenberg, I.N. (1980) Endocrinology 106, 444–451.
12. Leonard, J.L. and Visser, T.J. (1984) Biochim. Biophys. Acta, 787, 122–130.
13. Mol, J.A., Docter, R., Hennemann, G. and Visser, T.J. (1984) Biochem. Biophys. Res. Commun. 120, 28–36.
14. Anselmet, A. and Torresani, J. (1981) Biochem. Biophys. Res. Commun. 98, 685–692.
15. Erard, F., Cheng, S.Y. and Robbins, J. (1981) Arch. Biochem. Biophys. 206, 15–20.
16. Cheng, S.Y., Wilchek, M., Cahnmann, H.J. and Robbins, J. (1977) J. Biol. Chem. 252, 6076–6081.
17. Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
18. Kitz, R. and Wilson, I.B. (1962) J. Biol. Chem. 237, 3245–3249.
19. Köhrle, J. (1983) In: "Schilddrüsenhormonstoffwechsel". Ferdinand Enke Verlag, Stuttgart.
20. Fekkes, D., Van Overmeeren, E., Hennemann, G. and Visser, T.J. (1980) Biochim. Biophys. Acta 613, 41–51.
21. Leonard, J.L. and Rosenberg, I.N. (1981) Biochim. Biophys. Acta 659, 205–218.
22. Fekkes, D., Hennemann, G. and Visser, T.J. (1982) Biochem. Pharmacol. 31, 1705–1709.
23. Fekkes, D., Hennemann, G. and Visser, T.J. (1982) FEBS Lett. 137, 40–44.

Biochem. Biophys. Res. Commun. 124, 475–483 (1984)

C H A P T E R   V I I

ADVANCES IN THE PURIFICATION OF THE MICROSOMAL RAT LIVER
IODOTHYRONINE DEIODINASE.

I. Effect of detergents on solubilization and ion-exchange
chromatography.

Jan A. Mol, Tom P. van den Berg and Theo J. Visser

Department of Internal Medicine III and Clinical Endocrinology,
Erasmus University Medical School, Rotterdam, The Netherlands.

SUMMARY

Rat liver microsomal fraction was treated with several non-ionic,
anionic or zwitter-ionic detergents in order to investigate which is most
suitable for subsequent purification of the iodothyronine deiodinase.
Criteria for effective solubilization were a non-sedimentable activity by
centrifugation at 105,000 x g together with the least molecular weight
possible as determined by Sephacryl S-300 gelfiltration in the presence of
detergent. In addition, the inhibitory activity of several detergents on
the deiodinase activity was investigated.

The optimal solubilization procedure consisted of treatment of the
microsomes with cholate and subsequent precipitation with 30% ammonium
sulfate. In this way the enzyme was stripped of adhering phospholipids and
was redissolved best with the non-ionic detergents Brij 56 or Emulgen 911
in the presence of 0.5 M NaCl. This deiodinase preparation with an
isoelectric point at pH 9.3 was further purified by subsequent
chromatography on DEAE-Sephacel and CM-Sepharose. Only the
Emulgen-dispersed enzyme was bound to the CM-Sepharose. Further

purification was investigated by chromatofocusing. This resulted in a rapid inactivation of the Emulgen extract whereas with the Brij 56 extract the enzyme was ultimately purified 400 times after DEAE-Sephacel and chromatofusing.

In rat liver, iodothyronine inner ring and outer ring deiodinating activities are intrinsic to a single integral membrane protein (1,2). This enzyme, called type I deiodinase, is also found in kidney and catalyzes the conversion of thyroxine (T_4) into the biologically active thyroid hormone 3,3',5-triiodothyronine (T_3) or into the inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3) (3,4). It is distinct from the type II deiodinase found in pituitary, cerebral cortex or brown adipose tissue, which converts T_4 only into T_3, as well as from the type III deiodinase found in brain and placenta, which converts T_4 only into rT_3 (5-8). Under euthyroid conditions, the type I deiodinase is responsible for most of the circulating T_3 (9), but this enzyme is most active in the conversion of rT_3 into 3,3'-diiodothyronine (3,3'-T_2) (3,4). In order to characterize the deiodinase, attempts have been made to solubilize the enzyme (10,11). Further purification of detergent-dispersed deiodinase, however, has been proved to be difficult (11).

The present report concerns a reinvestigation of detergents useful in the solubilization and further purification of type I deiodinase.

MATERIALS AND METHODS

Materials.

3,3',5'-Triiodothyronine (rT_3) and 3,3'-diiodothyronine (3,3'-T_2) were obtained from Henning, Berlin, FRG; taurodeoxycholic acid, cholic acid, and dithiothreitol (DTT) from Sigma; 3-[(3-Cholamidopropyl) dimethylammonio] 1-propane sulfonate (CHAPS); Brij 35, 56 and 58, Myrj 52, and Tween 20, 40 and 60 were from Serva; Zwittergent 3-08, 3-10, 3-12 and

3-14 were from Calbiochem-Behring. Emulgen 910 and 911 were a generous gift from Kao Atlas, Tokyo, and Dobanol 91-8 was a generous gift from Shell, The Hague. Sephacryl S-300, DEAE-Sephacel, CM-Sepharose CL-6B, Sephadex LH-20, Sephadex G-25, and Pharmalyte pH 8-10.5 were purchased from Pharmacia, and carrier-free $Na^{125}I$ from Amersham. $[3',5'-^{125}I]rT_3$ was prepared by radioiodination of $3,3'-T_2$ with the chloramine-T method and purified on Sephadex LH-20. All other reagents were of the highest quality commercially available.

Iodothyronine deiodinase assay.

Enzyme activity was determined by incubation of appropriate amounts of microsomal protein for 10 min at $37^{\circ}C$ with 0.1 µCi ^{125}I-rT_3 and 0.5 µM unlabelled rT_3 in 0.25 ml 0.2 M sodium phosphate (pH 7.2), 4 mM EDTA and 5 mM DTT. The reaction was stopped by addition of 0.25 ml human serum. Serum protein-bound iodothyronines, i.e. substrate rT_3 and product $3,3'-T_2$, were precipitated with 0.5 ml 10% trichloroacetic acid. Production of iodide was calculated from the radioactivity in the supernatant after centrifugation for 10 min at 1500 x g. The results were corrected for non-enzymatic deiodination as assessed in enzyme-free incubations, and multiplied by two to account for the equal distribution of the ^{125}I label among the products $3,3'-T_2$ and I^-.

Preparation of microsomes.

The liver of male Wistar rats was perfused in situ through the portal vein with buffer A, 0.25 M sucrose, 50 mM Tris/HCl (pH 7.4) and 1 mM DTT, at $4^{\circ}C$. All further steps were carried out at $0-4^{\circ}C$. The liver was excised, minced, washed and homogenized using a Potter-Elvehjem homogenizer with Teflon pestle in 3 volumes of buffer A. The homogenate was centrifuged for 10 min at 8000 x g. To the supernatant was added a 30% (w/v) polyethyleneglycol (PEG) 6000 solution in water to a final concentration of 5% PEG, and after stirring for 10 min, the mixture was centrifuged for 10 min at 8000 x g. The pellet was resuspended using the Potter-Elvehjem homogenizer in buffer B, 50 mM Tris/HCl (pH 7.2), 3 mM EDTA

and 3 mM DTT. The resultant suspension was centrifuged for 1 h at 105,000 x g. The floating fat layer was carefully removed and the pellet resuspended in buffer B. This microsomal fraction was kept at -20°C until used for the solubilization experiments.

Protein was measured according to Bradford (15) with some modifications (16) using bovine serum albumin as the standard.

Solubilization experiments.

The solubilization of iodothyronine deiodinase activity was investigated essentially with two different microsomal preparations. Firstly, microsomes (10 mg protein/ml) were treated directly for 1 h at 0°C with an equal volume of 1% detergent in buffer B in the absence or presence of 0.5 M NaCl. After centrifugation for 1 h at 105,000 x g enzyme activity in the supernatant was determined. Secondly, microsomes were solubilized with cholate using the above conditions (see also ref.10). To the supernatant a solution of saturated ammonium sulfate in water, adjusted to pH 7 with diluted NaOH, was added slowly at 4°C under gentle stirring until 30% saturation was achieved. After 30 min the precipitate was spun down for 20 min at 20,000 x g, and the pellet was used for solubilization as described for the microsomal fraction.

Inhibition of the enzymatic deiodination of rT_3 by the various detergents was tested by addition of 0.01-1.0 % (w/v) detergent to deiodinase assay mixtures containing 2-5 µg/ml crude microsomal protein. Iodide production was estimated as described above.

Chromatography.

Gel filtration was carried out at 4°C with samples concentrated using a Minicon B-15 unit (Amicon). Solubilized proteins were applied to a Sephacryl S-300 column (1.6 x 40 cm) equilibrated with 10 mM Tris/HCl (pH 7.2), 3 mM EDTA, 3 mM DTT and 0.01% detergent (w/v). The column was eluted at a flow of 0.5 ml/min. The column was calibrated with thyroglobulin, ferritine, catalase, aldolase, bovine serum albumin and ovalbumin as standards in buffers containing the various detergents. Absorption at 280

TABLE I

Comparison of several detergents on the solubilization and activity of iodothyronine deiodinase.

Detergent	CMC [a] % (w/v)	% inhibition of enzyme activity at 0.1% (w/v) detergent	% solubilization [b] microsomes		0.5 M NaCl A.S.	
pellet			−	+	−	+
ANIONIC						
Cholate	0.6	30	92	98	10	8
Taurodeoxycholate	0.2	100	77	77	20	16
ZWITTERIONIC						
CHAPS	0.3	35	12	115	7	14
Zwittergent 3-08	large	0	−	63	1	0
" 3-10	1.2	20	−	54	0	0
" 3-12	0.12	100	−	29[c]	1	17
NON-IONIC						
Brij 35	0.008	75	14	41	23	29
" 56	0.0001	58	74	198	30	76
" 58	0.008	76	−	44	17	26
Tween 20	0.006	60	33	59	13	17
Myrj 52	−	2	−	4	−	6
Dobanol 91-8	−	78	36	123	33	51
Emulgen 911	−	10	−	210	52	82

[a] Critical micel concentration (Ref. 20, 26, 27).
[b] The activity in 105,000 x g supernatants was determined with an appropriate dilution in the presence of 0.01% detergent.
[c] Activity measured in the presence of 25 ug/ml detergent.
A.S. pellet= Ammonium sulfate pellet.

nm was monitored with a 8300 Uvicord 11 (LKB). Fractions of 2 ml were collected and assayed for protein and deiodinase activity.

Ion-exchange chromatography was mainly carried out with the Brij 56 and Emulgen 911 extracts of the ammonium sulfate precipitate. DEAE-Sephacel or CM-Sepharose columns (bed volume approximately 20 ml) were equilibrated with 10 mM sodium phosphate (pH 7.2), 3 mM EDTA, 3 mM DTT, 20% (v/v) glycerol and 0.1% Brij 56 or 0.1% Emulgen 911. Solubilized proteins were applied in the same buffer as used for equilibration, accomplished through a buffer change on Sephadex G-25. Desorption from the ion-exchange gels was carried out by increasing NaCl concentrations.

Chromatofocusing experiments were conducted (by fast protein liquid chromatography (FPLC)) on a Mono-P column (Pharmacia) using a Model 6000A solvent delivery system and monitoring absorbance at 280 nm with a Model 440 fixed wavelength detector (Waters). The column was equilibrated with 25 mM triethylamine/HCl (pH 10.5), 10% glycerol and 0.2% detergent. Samples were concentrated with a Minicon B-15 unit and brought into elution buffer using a PD-10 column (Sephadex G-25, Pharmacia). Protein was applied to the column after starting the pH gradient with 4 ml of the elution buffer. Fractions of 1 ml were collected and analyzed for deiodinase activity, protein content and pH. The elution buffer consisted of polybuffer 96 diluted 1:20 and ampholine 9-11 diluted 1:200, adjusted with HCl to pH 8, 10% glycerol, 1 mM DTT, 10 mM NaCl and 0.2% detergent.

RESULTS

Detergent effects on enzyme solubilization and activity.

A series of anionic, zwitter-ionic or non-ionic detergents were tested for their capacity to solubilize the deiodinase from microsomes or the ammonium sulfate pellet of the cholate extract as well as for their inhibitory activity in the deiodinase assay. As shown in table I the ionic detergents were very effective in the solubilization of enzyme activity from the microsomal fraction but failed to reactivate the ammonium sulfate pellet. In the case of the anionic detergents cholate and

102

taurodeoxycholate no improvement of the solubilization was detected after addition of 0.5 M NaCl whereas the zwitter-ionic detergents, especially CHAPS, required a high-salt buffer for effective solubilization. The zwittergents proved to become strong inhibitors of rT_3 5'-deiodination with a decrease of the critical micel concentration. In fact, zwittergent 3-14 and 3-16 completely inhibited enzyme activity at concentrations of 25 µg/ml detergent (w/v). The non-ionic detergents tested solubilized both the microsomes and the ammonium sulfate precipitate best in the presence of 0.5 M NaCl. The solubilization of microsomes with Dobanol 91-8, Brij 56 and Emulgen 911 resulted in a stimulation of the deiodinase activity of 1.2, 2.0 and 2.1 times, respectively. These detergents were also most effective in the solubilization of the ammonium sulfate pellet. Figure 1 shows the inhibition of the rT_3 5'-deiodination by increasing concentrations of several detergents in a direct incubation with microsomes. Again a stimulation of deiodinase activity is seen with the detergents Emulgen 911 and Brij 56, although to a lesser extent. At higher concentrations these detergents show a dose dependent inhibition of enzyme activity as is also shown for the ionic detergents CHAPS and cholate.
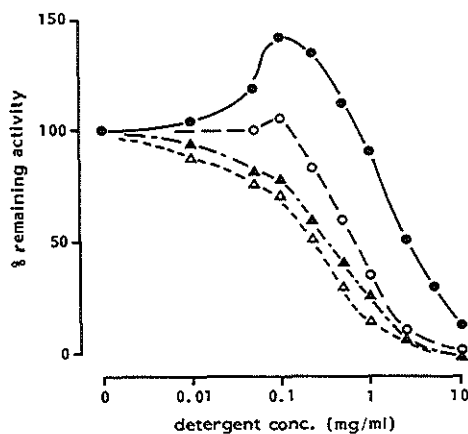
Fig.1. Alteration of rT_3 5'-deiodinase activity by incubation of rat liver microsomes for 10 min at $37°C$ with 0.5 µM rT_3 and 5 mM DTT in the presence of various concentrations Emulgen 911 (●), Brij 56 (○), Dobanol 91-8 (▲) or CHAPS (△).

Of several solubilized protein preparations the molecular weight of the deiodinase was determined in the presence of 0.01% detergent (w/v) by gelfiltration on Sephacryl S-300. The results are shown in Figure 2.
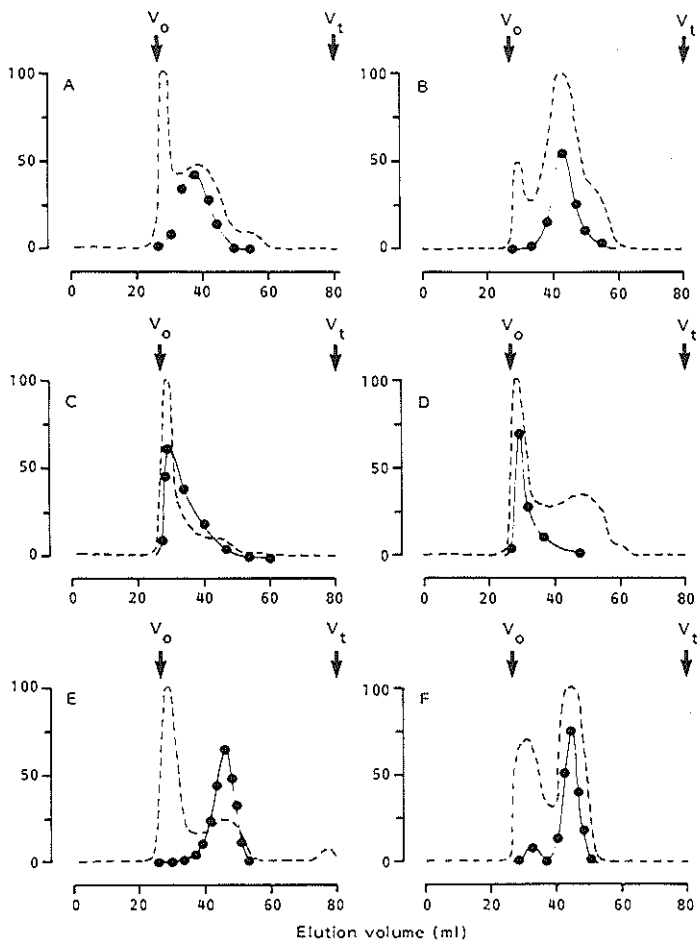
Fig.2. Sephacryl S-300 gel filtration of solubilized rat liver deiodinase. Depicted are chromatographies of deiodinase solubilized directly from the microsomal fraction by Brij 56 (A), Dobanol 91-8 (B), taurodeoxycholate (C) and CHAPS (D) or solubilization extracts of the ammonium sulfate pellet by Brij 56 (E) or Emulgen 911 (F). V_o and V_t indicate the void volume and the total volume of the column, respectively. Protein (- - -) is plotted on the vertical axis as a percentage of the maximal absorption at 280 nm in the peak fraction. Deiodinase activity (●) is depicted as percentage deiodination of rT_3 under standard conditions (See Materials and Methods). Recovery of activity ranged between 50 and 70%.

Solubilization of the microsomes with the non-ionic detergents Brij 56 or Dobanol 91-8 (Fig. 2A and 2B) yielded deiodinase preparations with apparent molecular weights of 320,000 and 240,000, respectively. Although the ionic detergents taurodeoxycholate and CHAPS (Fig. 2C and 2D) gave rise to deiodinase preparations which were non-sedimentable by centrifugation at 105,000 x g, the solubilized complexes eluted in the void volume of the column, indicating a molecular weight of over 1,000,000. The smallest complexes are obtained by solubilization of the ammonium sulfate pellet with the non-ionic detergents Brij 56 or Emulgen 911 (Fig. 2E and 2F) which yields molecular weights of 170,000 and 190,000, respectively. Addition of the various detergents to the mixture of calibration proteins did not change their elution positions. From the results of the molecular weight estimations it was concluded that for subsequent purification of the deiodinase the non-ionic detergents Brij 56, Dobanol 91-8 or Emulgen 911 were most suitable in substitution for the phospholipids that had been removed by ammonium sulfate precipitation.

Ion-exchange chromatography.

A. Phospholipid-containing extracts.

Previous work in our laboratory (12) has shown that direct solubilization of microsomes with W-1 ether yields a deiodinase preparation with an isoelectric point at approximately pH 6.4 while the enzyme recovered with W-1 ether from the ammonium sulfate pellet has a pI of 9.3. This increase in pI value is explained by the loss of acidic phospholipids. W-1 ether is a mixture of the detergents Brij 56 and Brij 58, and similar results have been obtained by preparative isoelectric focusing (IEF) of the respective Brij 56 extracts (data not shown). Attempts were made firstly to purify direct microsomal extracts in Brij 56, Dobanol or Emulgen further by ion-exchange chromatography on DEAE-Sephacel at pH 7.5. Despite an overall negative charge on the enzyme-lipid-detergent complex, only weak binding to the anion-exchange gel was observed when applied in 15 mM sodium phosphate buffer (pH 7.5), containing 1 mM EDTA, 1 mM DTT and 0.1% detergent. Between 10 and 30% of the enzyme activity combined with the column and could be eluted with 0.5 M NaCl in the same buffer. Most of the

remaining activity (40–60%) eluted in the void volume. Since during enzyme purification partial removal of acidic phospholipids may occur with a change in isoelectric point (D. Fekkes, personal communication), we decided to take the phospholipid depleted ammonium sulfate pellet as a starting point for further purification.

B. Delipidated extracts.

Ammonium sulfate precipitation of a cholate extract and subsequent solubilization with non-ionic detergents led to a 3-fold increase in specific enzyme activity. In the following experiments we have compared the ion-exchange chromatographies of the delipidated extracts obtained by solubilization of the ammonium sulfate pellet with 1% Dobanol 91–8, Brij 56 or Emulgen 911. After desalting by Sephadex G-25 filtration, the chromatography of these extracts on DEAE-Sephacel resulted in elution of over 60–80% of enzyme activity in the void volume together with 25–30% of applied protein, representing a purification of 2–3 times. Data for the Emulgen and Brij preparations are given in Table II but the same results were obtained with Dobanol. After elution over DEAE-Sephacel, the Brij extract was applied to a CM-Sepharose cation-exchange gel equilibrated in buffer B containing 0.05% Brij 56. However, almost all deiodinase activity passed through the column in the same buffer without enrichment in specific activity. Lowering of the buffer concentration or pH did not improve binding of the deiodinase to the CM-Sepharose whereas a decrease in detergent concentration resulted in a loss of enzyme recovery probably due to precipitation of protein.

The Dobanol and Emulgen extracts behaved quite differently on the cation-exchange gel. These deiodinase preparations bound almost completely to the CM-Sepharose and could be eluted with a lineair NaCl gradient. After DEAE-Sephacel, binding of the Emulgen preparation was clearly visible as a dark brown band on the top of the CM-Sepharose column. Since elution with 1 M NaCl gave rise to a broad elution peak, a search was made for optimal conditions. Application of the Emulgen extract was optimal in a low ionic strenght buffer containing 10 mM Tris/HCl (pH 7.2), 1 mM EDTA, 1 mM DTT, and supplemented with 20% glycerol (v/v), 0.2% (w/v) Emulgen 911, 0.05% (w/v) cholate and 15 mM NaCl. Each of the latter additions resulted

in an improvement of the CM-Sepharose chromatography by preventing loss of
deiodinase activity due to protein aggregation, and chromatography under
these optimal conditions is illustrated in Fig. 3. Addition of glycerol
to the Dobanol 91-8 preparations did not improve their binding to and
elution from the CM-Sepharose. Moreover, the deiodinase activity in the
Dobanol extracts appeared to be unstable when kept for longer time at $-20^{\circ}C$
and, therefore, Dobanol was inferior to the use of Emulgen. The elution of
the Emulgen extract from the CM-Sepharose was not or only slightly improved
using a NaCl gradient instead of an immediate change to 1 M NaCl, while the
latter has the advantage of eluting deiodinase activity as a narrow peak.
By these ion-exchange chromatographies we obtained a deiodinase preparation
in Brij 56 with a total activity of 40% of the microsomal fraction and a
protein content of 5% resulting in a purification of 8 times. The Emulgen
extract contained after CM-Sepharose chromatography 65% activity together
with 3% protein leading to a purification of approximately 20 times (Table
II).
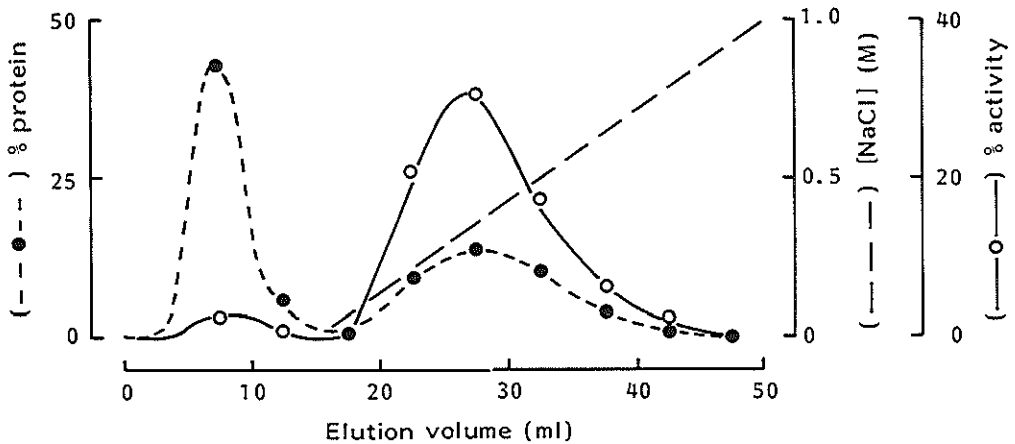
Fig.3. CM-Sepharose chromatography of an Emulgen extract of the ammonium
sulfate pellet after DEAE-Sepharose chromatography. Protein (●) and
5'-deiodinase activity (○) were determined (as described under Materials
and Methods), and depicted as a percentage of the total activity and
protein applied to the column. Proteins were eluted with a linear NaCl
gradient (---). The recovery of deiodinase activity amounted to a mean of
80% in 3 experiments.
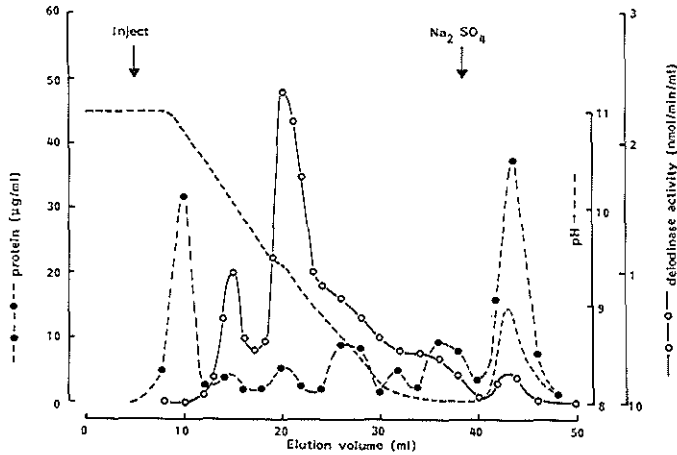
Fig.4. *High performance chromatofocusing of rat liver iodothyronine deiodinase solubilized with Brij 56 from the ammonium sulfate pellet and partially purified by ion-exchange chromatography. The Brij 56 extract (0.5 mg protein in 1 ml) was injected at min 5. At fraction 38, 0.5 ml 1M Na_2SO_4 was injected to elute proteins retained by the mono-P column. One ml fractions were collected and assayed for pH (---), protein (●) and 5'-deiodinase activity (○). The figure shows the results of a representative experiment.*

Chromatofocusing.

Due to the difficulties in purification of rat liver deiodinase by affinity-chromatography (12) we investigated the possibility of separation by means of chromatofocusing. As indicated by preparative isoelectric focusing (12) of the Brij 56 extract of the ammonium sulfate pellet, the pI of the delipidated enzyme is about 9.3 (not shown). In order to minimize deterioration of enzyme activity above this pH, protein was applied to the column in elution buffer after starting the pH gradient with one column volume of elution buffer (see Materials and Methods).

Attempts to purify the CM-Sepharose pool of the Emulgen preparation were completely unsuccessful. Apparently the deiodinase was inactivated on the chromatofocusing column since no deiodinase activity eluted within the formed pH gradient neither by a further decrease of pH or increase in ionic strength. Incubation of the Emulgen or Brij extracts for 1 hour at room temperature and different pH values followed by measurement of the activity

108

at pH 7.2 showed that the solubilized enzyme is stable between pH 6 and 8 but especially with Emulgen is rapidly and irreversible inactivated at pH 9. This may be due to protein aggregation which is often seen close to the isoelectric point (pI 9.3). In the case of the Brij 56 extract we were more successful. Figure 4 demonstrates a typical purification of a Brij extract by chromatofocusing. Optimal resolution with retention of enzyme activity was obtained by including 10 mM NaCl in the elution buffer, whereas the total activity found after omission of NaCl or by addition of 2M urea or 10% ethanol (v/v) to prevent hydrophobic interaction was below 30%. Often the deiodinase eluted in two activity peaks of pI 10.2 and pI 9.4. The peak at pH 9.4 contained 8.8% of the initial activity of the microsomes together with 0.022% protein representing a purification of 400 times (Table II).

TABLE II

Initial purification of rat liver iodothyronine deiodinase.

Step	%protein	%activity	purification
1. Microsomes	100.0	100.0	1.00
2. Cholate extract	92.9	90.5	0.98
3. Emulgen extract of A.S. pellet	28.6	82.0	2.87
— DEAE-Sepharose	8.0	71.4	8.93
— CM-Sepharose	3.2	65.3	20.41
4. Brij 56 extract of A.S. pellet	18.8	70.2	3.73
— DEAE-Sepharose	5.0	40.6	8.12
— Chromatofocusing	0.022	8.8	400.00

A.S. pellet= ammonium sulfate pellet.

DISCUSSION

The present report concerns an extension of other studies on the
solubilization of rat liver or kidney type I deiodinase (10-12). Our study
was initiated by the disappointing results of purification of W-1
ether-solubilized microsomes by ion-exchange chromatography or by various
"affinity" gels (11). As summarized in Table I ionic detergents were
effective in the solubilization of deiodinase activity only from the
microsomal membranes. The thus obtained detergent-phospholipid-protein
complexes formed, however, large aggregates as shown by Sephacryl S-300 gel
filtration. This is in agreement with the findings of Fekkes et al (10)
and Leonard et al (11) who found also large complexes using cholate or
deoxycholate, respectively. Only at high detergent concentrations and 0.1
M NaCl small deiodinase complexes were found in case of cholate (10). Gel
filtration in the presence of deoxycholate resulted in substantial losses
of deiodinase activity due to lipid depletion. Addition of phospholipids
was needed to reactivate the enzyme (11). Attempts to create a small
detergent-deiodinase complex in the case of CHAPS by addition of 10 mM
CHAPS, 0.5 M NaCl and 20% glycerol to the gel filtration buffer were
unsuccessful (not shown). Together with our observation that
solubilization of phospholipid- depleted enzyme with ionic detergents is
nearly impossible, this leads to the conclusion that ionic or zwitterionic
detergents are less useful in the replacement of membrane phospholipids and
further purification of rat liver iodothyronine deiodinase. Also the
sedimentation analysis of the renal iodothyronine deiodinase on a sucrose
gradient with deoxycholate as detergent suggests that very little of the
ionic detergent binds to the deiodinase (11).

Solubilization with non-ionic and zwitterionic detergents was optimal
in the presence of 0.5 M NaCl. This is also seen in the solubilization of
other membrane proteins with non-ionic detergents (17,18), and surprisingly
also with the zwitterionic detergent CHAPS in the solubilization of Vitamin
K-dependent carboxylase (19). This salt effect may be caused by an
increase in hydrophobic interactions through which detergents are more
tightly bound to the hydrophobic sites of the enzyme. In this way
non-ionic detergents seem to be better in replacing phospholipids in

comparison with ionic detergents.

The inhibition of enzyme activity with the zwittergents 3-08 up to 3-16 appears to be related to the critical micel concentrations suggesting that sequestration of hydrophobic iodothyronines by a detergent micel may take place (11). This does not hold in case of the non-ionic detergents which possess in general lower critical micel concentrations. The detergent effects may, however, be more complicated as seen in the stimulation of enzyme activity when the non-ionic detergents Brij 56 and Emulgen 911 are used in the direct solubilization of the microsomes. Due to binding to functional sites on the deiodinase, hydrophobic detergents may inhibit at higher concentrations (see ref. 20).

Solubilization of the ammonium sulfate pellet with non-ionic detergents results in apparent molecular weights for the deiodinase activity of 170,000 - 190,000 as determined by gel filtration on Sephacryl S-300. The higher molecular weights in case of direct solubilization of microsomes are probably caused by adhering phospholipids. The apparent molecular weights for the deiodinase in non-ionic detergents are in close agreement with those found for other microsomal enzymes for instance 230,000 for UDP-glucuronyltransferase (21), 280,000 for arylsulfatase (22), 220,000 for cAMP phosphodiesterase (23) and 160,000 for cytochrome P-450 (24). In our view, these relatively high values in comparison with the molecular weights determined by gel electrophoresis in the presence of SDS are caused by the micellar form of the protein-detergent complex rather than by formation of polymeric protein complexes (21,22). Addition of cholate or CHAPS to the solubilization mixture of Emulgen and the ammonium sulfate pellet caused no further decrease of the molecular weight of the deiodinase as determined by gel filtration.

The choice of the correct detergent for solubilization and subsequent purification, although a critical step, is largely a trial-and-error process. This is also shown in the attempts to purify the deiodinase by ion-exchange chromatography. The binding of the Brij 56 extract of delipidated enzyme on the CM-Sepharose is frequently impaired. This may be caused by detergent binding to the carboxymethyl group of the gel or more likely binding to functional sites on the deiodinase. Lowering of the detergent concentration, especially in low ionic-strength buffers resulted

in considerable loss of activity recovered from the column. The presence of at least 15 mM NaCl and, in the case of Emulgen, 20% glycerol appeared to be essential for protein stability and in the case of Emulgen also 20% glycerol. Addition of cholate to the Emulgen buffer clearly improved the salt elution of the deiodinase from the CM-Sepharose probably by prevention of hydrophobic interaction with the gel matrix.

In some chromatofocusing experiments deiodinase activity eluted from the column at pH 10.2 before the main activity peak at pH 9.3. This may be deiodinase which is less tightly bound to the column due to the high detergent concentration used or due to the 10 mM NaCl which is added to optimize recovery of enzyme activity. Using Brij as detergent activity recoveries ranged between 35-50%. It is yet unclear why the solubilized enzyme is unstable at high pH in contrast with membrane-associated deiodinase. Besides irreversible unfolding close to the isoelectric point, the catalytic important SH group is more prone to irreversible oxidation at high pH, and also dissociation of subunits may occur. The excistence of subunits has been suggested by affinity-labeling experiments (25). The availability of a pure enzyme preparation should give an answer if in analytical isoelectric focusing fully delipidated rat liver deiodinase shows a microheterogeneity in isoelectric points.

In conclusion, the iodothyronine deiodinase of rat liver is optimally solubilized in a lipid-free form by the non-ionic detergents Brij 56 or Emulgen 911. The thus obtained, probably monomeric form can be purified some 20 times by ion-exchange chromatography in a mixture of Emulgen and cholate. Further purification by chromatofocusing is only possible with Brij 56 preparations and results in a purification of about 400 times. The recovery of activity is lowered due to deiodinase lability at high pH. The enzyme preparations purified by several ion-exchange chromatographies serve as a good starting point for subsequent affinity chromatography (28).

REFERENCES

1. Fekkes, D., Hennemann, G. and Visser, T.J. (1982) Biochem. J. 201, 673-676.
2. Chopra, I.J. and Chua Teco, E.N. (1982) Endocrinology 110, 89-97.
3. Visser, T.J., Fekkes, D., Docter R. and Hennemann, G. (1979) Biochem. J. 179, 489-495.
4. Leonard, J.L. and Rosenberg, I.N. (1980) Endocrinology 106, 444-451.
5. Visser, T.J., Kaplan, M.M., Leonard, J.L. and Larsen, P.R. (1983) J. Clin. Invest. 71, 992-1002.
6. Leonard, J.L., Mellen, S.M. and Larsen, P.R. (1983) Endocrinology 112: 1153-1155.
7. Kaplan, M.M., Visser, T.J., Yaskoski, K.A. and Leonard, J.L. (1983) Endocrinology 112, 35-42.
8. Roti, E., Fang, S., Green, K., Braverman, L.E. and Emerson, C.H. (1983) Am. J. Obstet. · Gynecol. 147, 788-792.
9. Silva, J.E., Gordon, M.B., Crantz, F.R., Leonard, J.L. and Larsen, P.R. (1984) J. Clin. Invest. 73, 898-907.
10. Fekkes, D., Van Overmeeren, E., Henneman, G. and Visser, T.J. (1980) Biochim. Biophys. Acta 613, 41-51.
11. Leonard, J.L. and Rosenberg, I.N. (1981) Biochim. Biophys. Acta 659, 205-218.
12. Fekkes, D., Hennemann, G. and Visser, T.J. (1983) Biochim. Biophys. Acta 742, 324-333.
13. Visser, T.J., Docter, R. and Hennemann, G. (1977) J. Endocrinol. 73, 395-396.
14. Van der Hoeven, T.A. (1981) Anal. Biochem. 115, 398-402.
15. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
16. Read, S.M. and Northcote, D.H. (1981) Anal. Biochem. 116, 53-64.
17. Guillon, G., Roy, C. and Jard, S. (1978) Eur. J. Biochem. 92, 341-348.
18. Moss, J., Osborne, J.C. and Stanley, S.J. (1984) Biochemistry 23, 1353-1357.
19. Girardot, J.M. and Johnson, B.C. (1982) Anal. Biochem. 121, 315-320.
20. Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29-79.
21. Tukey, R.H. and Tephly, T.R. (1980) Arch. Biochem. Biophys. 209, 565-578.
22. Moriyasu, M., Ito, A. and Omura, T. (1982) J. Biochem. 92, 1189-1195.
23. Loten, E.G. (1983) Int. J. Biochem. 15, 923-928.
24. Mackenzie, P.I., Lang, M.A. and Owens, I.S. (1984) Membrane Biochemistry 5, 193-207.
25. Mol, J.A., Docter, R., Kaptein, E., Jansen, G., Hennemann, G. and Visser, T.J. (1984) Biochem. Biophys. Res. Commun.124, 475-483.
26. Hjelmeland, L.M., Nebert, D.W. and Osborne, J.C. (1983) Anal. Biochem. 130, 72-82.
27. Gonenne, A. and Ernst, R. (1978) Anal. Biochem. 87, 28-38.

28. Mol, J.A. and Visser, T.J. (1985) Submitted for publication.

C H A P T E R   V I I I

ADVANCES IN THE PURIFICATION OF THE MICROSOMAL RAT LIVER IODOTHYRONINE DEIODINASE.

II. Affinity chromatography

Jan A. Mol, Tom P. van den Berg and Theo J. Visser.

Department of Internal medicine III and Clinical Endocrinology,
Erasmus University Medical School, Rotterdam, The Netherlands.

ABSTRACT

The rat liver iodothyronine deiodinase has been solubilized and purified approximately 2400 times from liver microsomal fraction of male Wistar rats pretreated with thyroxine. The deiodinase was solubilized with 1% cholate, and stripped of adhering phospholipids by ammonium sulfate precipitation followed by solubilization with the non-ionic detergent Emulgen 911. The enzyme was further purified by successive ion-exchange chromatography on DEAE-Sephacel and Cellex-P, and affinity chromatography on a matrix of $3,3',5$-triiodothyronine (T_3)-Sepharose. At last, the iodothyronine deiodinase was further purified by covalent attachment onto a column of the mechanism-based inhibitor propylthiouracil (PTU) coupled to Sepharose 4B. Binding was achieved only in the presence of substrate which is in agreement with the proposed ping-pong mechanism. The covalently bound deiodinase was eluted by reduction of the formed enzyme-PTU mixed disulfide with 50 mM DTT. The enzyme exhibits a subunit molecular weight of 25,000 and is approximately 50% pure as judged by SDS-PAGE. The partially purified enzyme preparation is equally enriched in both outer ring and inner ring deiodinase activities in keeping with the concept that

both activities are intrinsic to a single, type I deiodinase in rat liver and perhaps also in kidney.

Thyroxine (T_4), the principal secretory product of the thyroid gland, is mainly metabolized in peripheral tissues by sequential mono-deiodinations (1,2). Outer ring deiodination of T_4 leads to the formation of the active thyroid hormone 3,3',5-triiodothyronine (T_3) whereas inner ring deiodination irreversibly inactivates the pro-hormone T_4 to 3,3',5'-triiodothyronine (rT_3) (1,2). Both triiodothyronines are further metabolized by deiodination mainly to 3,3'-diiodothyronine (3,3'-T_2). In rat liver and kidney both outer ring and inner ring deiodination are catalyzed by a microsomal enzyme (3-5). Using the crude microsomes as enzyme source the catalytic mechanism has been studied. The deiodinase contains an active site -SH group which can be blocked by iodoacetate (6,7). There is probably also an essential histidine residue in close proximity of the catalytic center which is modified by diethylpyrocarbonate with resultant loss of enzyme activity (8). The deiodinase requires the reductive equivalents of thiols for activity, dithiothreitol (DTT) and dihydrolipoamide being the most effective in vitro (9-11). It has been proposed that the enzyme deiodinates iodothyronines via a ping-pong mechanism (9,10). During deiodination of an iodothyronine molecule an oxidized enzyme intermediate is formed, probably an enzyme-sulfenyl iodide (E-SI), which is subsequently reduced by the thiol cofactor to regenerate the reduced enzyme. Inhibition of enzyme activity by propylthiouracil (PTU) is uncompetitive towards substrate and competitive towards cofactor DTT. It is suggested that PTU reacts readily with the E-SI under formation of an inactive disulfide complex between PTU and deiodinase. Such a reaction mechanism is consistent with the finding that covalent binding of labeled PTU to the deiodinase is stimulated by substrate (12). In the present report we make use of this property in the affinity chromatography on propionylthiouracil (PTU)-Sepharose.

More knowledge of the contribution of liver and kidney to production and elimination of the triiodothyronines is needed for a better

understanding of the regulation of their serum concentration in health and disease. Purified enzyme preparations would then also enable investigations of possible regulatory factors that determine how much T_4 is converted either to T_3 or to rT_3 through the action of rat liver deiodinase. In the preceeding paper solubilization of rat liver deiodinase and its partial purification by ion-exchange chromatographies has been described (13). The present paper reports on advances in the purification of this enzyme by affinity chromatography.

MATERIALS AND METHODS

Materials.

T_4, T_3, rT_3 and $3,3'-T_2$ were obtained from Henning, Berlin, FRG; cholic acid, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and dithiothreitol (DTT) from Sigma; 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS) from Serva. Emulgen 911 was a gift from Kao Atlas, Tokyo. Erythrosine (tetraiodofluorescein) was obtained from Eastman Kodak Co., and 2-thiouracil-6-propionic acid was a generous gift from Drs. D.S. Cooper and E.C. Ridgeway, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA. Cellex-P and all chemicals for polyacrylamide gel electrophoresis (PAGE) including high molecular weight standards were from Bio-Rad. All other chromatography media were products from Pharmacia. N-bromo acetyl-3,3',5-triiodothyronine (BrAcT$_3$) was prepared according to Cheng, method B (14). Iodothyronine sulfate esters were prepared by a recently developed method (15) using chlorosulfonic acid in dimethylformamide. All other reagents were of the highest quality commercially available.

Affinity column preparation.

T_3 and T_3 sulfate were coupled to activated CH-Sepharose 4B (which contains a six-carbon spacer group) or to CNBr-activated Sepharose 4B

(without a spacer) in 0.1 M $NaHCO_3$ buffer (pH 8.3) as published by Pharmacia . (16). The concentrations of coupled ligand were estimated by inclusion of a tracer quantity ^{125}I-labeled T_3 or T_3 sulfate. These concentrations amounted to 8-10 µmol T_3 or T_3 sulfate/ml settled gel for activated CH-Sepharose and approximately 6 µmol T_3 or T_3 sulfate/ml settled gel for CNBr-activated Sepharose.

Erythrosine-Sepharose and PTU-Sepharose were prepared by coupling of erythrosine and 2-thiouracil-6-propionic acid, respectively, to AH-Sepharose 4B in 50% dioxane using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) essentially as described by Tucker et al (17). The concentrations of coupled ligand were estimated by measurement of the absorbance of the erythrosine or PTU in the wash fluids and amounted to approximately 10 µmol/ml settled gel (17,27).

Before use the affinity gels were mixed with Sepharose 4B until a final concentration of approximately 2 µmol ligand/ml gel was obtained.

Solubilization and purification experiments.

Male Wistar rats ($\pm$ 250 g body weight) were made hyperthyroid by daily i.p. injections with 10 µg T_4 in 1 ml 0.01 M NaOH in saline. On day 10 the liver was perfused in situ and subsequently microsomes were prepared as described previously (13). All steps were carried out at 0-4°C unless stated otherwise. If necessary, complete fractions of the various purification steps were stored at -80°C before they could be applied to the next column. Microsomes from 10 pooled livers (10 mg protein/ml) were solubilized in buffer B, 50 mM Tris/HCl (pH 7.2), 3 mM EDTA and 3 mM DTT, using sodium cholate (1% w/v). Iodothyronine deiodinase activity was precipitated through addition of a saturated $(NH_4)_2SO_4$ solution in water until 30% saturation was achieved. The precipitate was spun down by centrifugation for 20 min at 20,000 x g and subsequently the pellet was solubilized with 1% (w/v) Emulgen 911 in 50 mM Tris/HCl, 3 mM EDTA, 3 mM DTT, 0.5 M NaCl and 2C glycerol in an equal volume as the original microsomal fraction (appro: ately 4 mg protein/ml). After centrifugation for 1 h at 105,000 x g, a buffer change and desalting was obtained by gel filtration on a Sephadex G-25 column (2.6 x 30 cm) equilibrated with buffer

C: 10 mM sodium phosphate (pH 7.2 at 4°C), 3 mM EDTA, 1 mM DTT, 15 mM
NaCl, 20% glycerol, 0.2 % (w/v) Emulgen 911 and 0.05% (w/v) sodium cholate
at a solvent flow of 2 ml/min. Subsequently solubilized proteins were
applied to a DEAE-Sephacel column (1.6 x 25 cm), equilibrated in buffer C,
at a flow rate of 1 ml/min. The deiodinase activity did not bind to this
column. Fractions of 10 ml were collected until the absorbance at 280 nm
returned to the baseline of buffer C as monitored by a 8300 Uvicord 11
(LKB). Retained proteins were eluted with 1 M NaCl in buffer C.   The
fractions containing deiodinase activity were pooled and applied to an in
buffer C equilibrated Cellex-P column (2.6 x 10 cm) which is a
cation-exchange gel on cellulose basis containing phosphate groups
(Bio-Rad). The column was washed with buffer C until the absorbance at 280
nm was low.   Elution of protein was achieved by a change to 1 M NaCl in
buffer C. Fractions of 10 ml were collected and assayed for deiodinase
activity.   After desalting on Sephadex G-25 this material was used as
starting material for the affinity chromatographies.

Affinity chromatographies were investigated on 10 ml bed volume columns
(1.6 x 5 cm) at flow rates of approximately 0.5 ml/min in buffer C. Only
binding to PTU-Sepharose was performed batchwise. The exact circumstances
of buffers used for elution are reported under Results.

Assays.

Aliqouts of fractions obtained after the different purification steps
were kept at -80°C until they were assayed for deiodinase activity and
protein in a single run.   Outer ring deiodinase activity was assayed
essentially as described previously by quantitation of $^{125}I^{-}$ released from
0.1 µCi ^{125}I-rT$_3$ in the presence of unlabeled rT$_3$ and 5 mM DTT at pH 7.2
and 37°C (preceeding paper) or by measurement of the production of 3,3'-T$_2$
from unlabeled rT$_3$ under the same standard assay conditions with a specific
radioimmunoassay (18). Therefore, 50 µl of enzyme diluted in buffer C was
incubated with 200 µl 0.2 M phosphate (pH 7.2) containing 3 mM EDTA, 5 mM
DTT and 5 µM rT$_3$.   In this way identical detergent concentrations were
obtained in all assays. The reactions were linear with respect to an
incubation time of 20 min. One unit of activity corresponds to the amount

Table I

Purification of rat liver iodothyronine deiodinase from rat liver microsomes.

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)	Purification
Microsomes	1310	6.7	8,856	100	1
Cholate extract	1074	7.4	7,894	89	1.1
Emulgen extract of $(NH_4)_2SO_4$ precipitate	375	19.4	7,265	82	2.9
DEAE-Sephacel	104	61.1	6,352	72	9.0
Cellex-P	55	113.0	6,215	70	16.7
T_3-Sepharose	7,45	514.0	3,827	43	76
PTU-Sepharose	$\sim$ 0,026	$\sim$ 16,074.0	434	4.9	$\sim$ 2400

of deiodinase which produces 1 nmol $3,3'-T_2$/min from 5 µM rT_3 under standard conditions. Inner ring deiodinase activity was determined equally using 5 µM T_4 sulfate as substrate. Production of rT_3 sulfate was determined after hydrolysis of the reaction mixtures for 1 h at 80°C in 1 N HCl, with a specific radioimmunoassay for rT_3 as published (19).

Protein concentrations were measured by the method of Bradford (20) as modified (21), using bovine serum albumin as the standard. When samples were too diluted or the detergent concentration was too high, causing high blank values, protein was precipitated with 10% TCA or with acetone (80% v/v). The precipitated protein was dissolved in 0.1 M NaOH before protein determination.

PAGE in the presence of SDS was performed on protein samples after precipitation with acetone and subsequent boiling in sample buffer containing 0.2% 2-mercaptoethanol according to Weber and Osborn (21). Electrophoresis of the proteins was performed on 8 cm gel rods of 7.5% acrylamide at 3 mA/tube for 6 h. Afterwards the gels were fixed and stained with 0.25% Coomassie brilliant blue R250 according to Weber and Osborn (21) and subsequently scanned at 595 nm. In addition, microsomal protein was labeled by reaction with ^{125}I-$BrAcT_3$ in the absence or presence of 10 µM rT_3. In this way, $BrAcT_3$ serves as an affinity label as is shown by Mol et al (23) and can be used to localize the deiodinase after gel electrophoresis. For this purpose gel rods were cut in 2 mm slices and counted for radioactivity using a Nuclear Enterprise NE 1600 gamma spectrometer.

RESULTS

Initial purification.

Following treatment of male Wistar rats with T_4 for 10 days, there was a marked induction of rat liver iodothyronine deiodinase as has been demonstrated previously (24). The apparent V_{max} value of the 5'-deiodination of rT_3 increased from 0.88 to 2.35 nmol $3,3'-T_2$ produced/min/mg microsomal protein as measured under standard conditions at

pH 7.2 and 37°C in the absence of detergent (not shown). Dilution of the microsomal fraction in buffer C resulted in a dramatical change of enzyme activity as is shown in Fig. 1. The apparent V_{max} increased almost 3 times to 6.76 nmol 3,3'-T_2 produced/min/mg protein with a concomitant increase of the apparent K_m from 0.17 to 1.67 μM. The purification of the hepatic iodothyronine deiodinase from the microsomal fraction of animals pretreated with T_4 is shown in Table I.
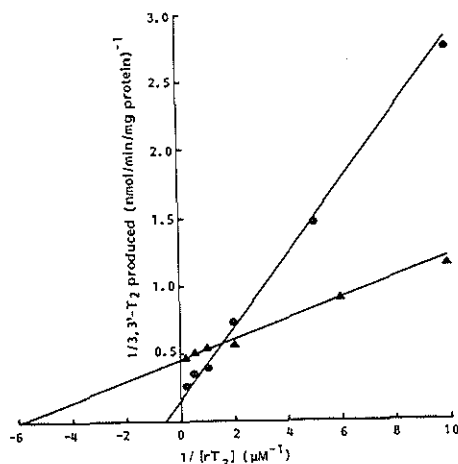
Fig.1. Lineweaver-Burk analysis of the deiodination rate of rT_3 into 3,3'-T_2 by rat liver microsomes diluted in buffer C in the absence (▲) or presence (●) of 0.04% Emulgen and 0.01% cholate. The microsomes were prepared from livers of thyrotoxic animals. Shown are the means of two experiments each performed in triplicate.

Microsomes were solubilized with 1% (w/v) sodium cholate and delipidated by precipitation with ammonium sulfate at 30% saturation (13). The resulting pellet was again solubilized with 1% (w/v) Emulgen 911 under the conditions described under Materials and Methods. This delipidated extract in non-ionic detergent was enriched almost three times in specific activity with a recovery of 82% (Table I). After ultracentrifugation and desalting on Sephadex G-25, the clear extract was applied to a column of DEAE-Sepharose. The deiodinase eluted without binding to the column and

was further purified approximately 3 times. Subsequently, the enzyme was bound to a Cellex-P cation exchange gel instead of the CM-Sepharose used previously. The advantage of the Cellex-P cation exchange gel is its greater capacity and a markedly sharper elution of enzyme activity from the column. The latter was achieved with 1 M NaCl in buffer C. After Cellex-P, deiodinase activity was enriched 17 times in comparison with the activity found in microsomes determined in the presence of detergent. After desalting on Sephadex G-25 it served as starting material for the affinity chromatography.

Affinity chromatography.

Deiodinase activity was fully retained by hydrophobic interaction chromatography on both octyl-Sepharose and phenyl-Sepharose when applied in buffer C. As much as 80% of the applied protein was adsorbed to the column. Enzyme activity could only be eluted by reduction of the hydrophobic interaction with a high detergent concentration of 2-5% Emulgen in buffer C. Application of larger volumes of the Cellex-P fraction (i.e. 50 mg protein in 40 ml buffer C) to the octyl-Sepharose column equilibrated in buffer C without detergent showed an initial visible binding of protein limited to the top of the column. Further application of protein resulted in the slow migration of the protein band over the column, probably due to saturation of the matrix with detergent. Elution of protein with high detergent concentrations gives rise to considerable tailing of eluted deiodinase activity. The recovery of enzyme activity amounted to 85% in the case of octyl-Sepharose with a minor increase in specific activity. Less than 40% of the enzyme activity that was bound to phenyl-Sepharose could be eluted by high detergent, indicating a stronger interaction with this matrix. Again, hardly a change in specific activity was obtained.

The possibility was tested to purify the type I deiodinase by affinity chromatography using immobilized substrates or substrate analogues. For this purpose affinity matrices were tested having T_3, T_3 sulfate or the competitive inhibitor erythrosine coupled covalently to Sepharose. Chromatography on erythrosine-Sepharose was conducted in the dark since erythrosine may give rise to photo-oxidation reactions (25) which lead to

the destruction of enzyme activity as has also been observed with the analogous dye Rose Bengal (8). Erythrosine is a potent inhibitor of deiodinase activity with 50% inhibition at a concentration of 10 nM (J.A. Mol and T.J. Visser, unpublished results). The erythrosine-Sepharose column fully retained both protein and deiodinase activity. The activity could not be eluted from the column with 2% Emulgen or a high detergent concentration in the presence of 1 M NaCl. When the column was equilibrated with 2% Emulgen prior to the application of the Cellex fraction, binding of protein and enzyme activity was completely prevented, probably due to encapsulation of the dye molecules by detergent.

In order to prevent hydrophobic interaction on the column as much as possible, the water-soluble T_3 sulfate which is a suitable substrate for the deiodinase (26), was coupled to AH-Sepharose 4B. After equilibration with buffer C almost all deiodinase activity of the Cellex pool was retained on the T_3 sulfate-Sepharose column. However, as much as 70% of applied protein was bound as well by this column, indicating a low degree of specificity. Enzyme activity could not be eluted with a high Emulgen concentration or 1 M NaCl in buffer C. Only a combination of Emulgen and 1 M NaCl afforded the elution of the deiodinase which emerged in a broad peak with only a twofold increase in specific activity at best. Hydrophobic interaction was further minimized by omission of the six-carbon spacer between the Sepharose and the T_3 sulfate. This was achieved by coupling of T_3 sulfate via its amino group to cyanogen bromide-activated Sepharose. This affinity gel retained over 80% of the deiodinase activity. The omission of the six-carbon spacer resulted also in a sharper elution of the deiodinase with 2% Emulgen and 1 M of the chaotropic salt KSCN in buffer C. However, in spite of the reduced hydrophobic interaction, the degree of enzyme purification remained disappointing and varied between 2 to 3 times. In subsequent experiments we compared the purification on T_3-sulfate Sepharose with an affinity column of T_3 coupled, without a hydrophobic spacer, directly to CNBr-activated Sepharose. As expected and already shown by Fekkes et al (27), the deiodinase was strongly bound to the T_3-Sepharose column. In this case it was possible to elute protein, which was bound on the basis of hydrophobic interaction with, a high detergent concentration. This was optimal with 2% (w/v) of the zwitterionic

124

detergent CHAPS in buffer C. This was not possible for the proteins retained by the T_3 sulfate-Sepharose. Deiodinase retained by T_3-Sepharose was eluted with 2% Emulgen and 1 M KSCN. A typical purification on T_3 Sepharose is shown in Fig. 2. This fraction was purified some 4.5-fold with respect to the Cellex-P fraction and about 75-fold with regard to the microsomal fraction.
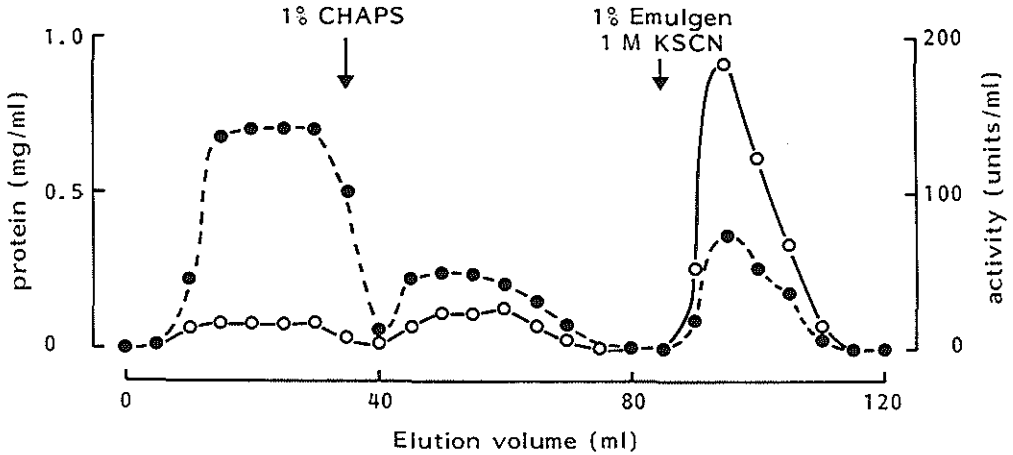

Fig.2. Affinity chromatography on a column of T_3-Sepharose. The Cellex-P fraction (33 mg protein in 22 ml buffer C) was applied to the T3-Sepharose after desalting on Sephadex G-25. Proteins bound due to hydrophobic interaction were removed by elution with 1% CHAPS in buffer C. The deiodinating activity was eluted with 1% Emulgen and 1 M KSCN in buffer C. Fractions of 5 ml were collected and assayed for deiodinase activity ($\bigcirc$) and protein concentration ($\bullet$). Shown is a representative chromatogram.

PTU-Sepharose chromatography.

PTU acts as a mechanism-based inhibitor of the deiodinase. It will only react with the deiodinase when the enzyme is converted by substrate into the E-SI form. Preliminary experiments concerning binding of the deiodinase by column chromatography onto PTU-Sepharose in the presence of rT_3 as the substrate in buffer C were disappointing. Very little deiodinase was retained by the column. Repetition of the same experiment without addition of rT_3 showed that the native deiodinase has no affinity

125

for the PTU column since all enzyme activity appeared in the void volume in contrast with a third of the applied protein which tightly bound to the PTU-Sepharose. Table II shows the result of this experiment. After column chromatography of the T_3-Sepharose fraction on PTU-Sepharose without the addition of substrate (rT_3), the subsequent attachment of the deiodinase in the presence of 5 μM rT_3 was greatly improved (Table II). The fraction obtained after column chromatography in the absence of substrate was diluted 1 : 1 with buffer C containing 10 μM rT_3 and 5 ml settled PTU-Sepharose without detergent. The final detergent concentrations of 0.1% (w/v) Emulgen and 0.025% (w/v) cholate inhibited the deiodinase activity for less than 10% (13,26). Coupling to the PTU-Sepharose was maximal when the gel was protected against oxidation by storing the gel in the presence of 50 mM DTT at 4 °C. Omission of these steps resulted in low binding to the gel. For optimal contact between deiodinase and PTU-Sepharose these experiments were performed batchwise under continious stirring for 1 hr at room temperature. The PTU-Sepharose was then extensively washed with buffer C to remove non-bound protein and rT_3, and covalently bound deiodinase was subsequently desorbed by batchwise

Table II

Purification of rat liver iodothyronine deiodinase on PTU-Sepharose.

Chromatography step	Total protein (mg)	Total activity (units)	Specific activity
T_3-Sepharose	9.0	4680	520
PTU-Sepharose			
1. without rT_3, non-bound fraction	5.9	3260	550
2. incubation with 5 μM rT_3, non-bound fraction	5.06	1063	210
3. elution with 50 mM DTT	$\sim$ 0.039	530	$\sim$ 13.600
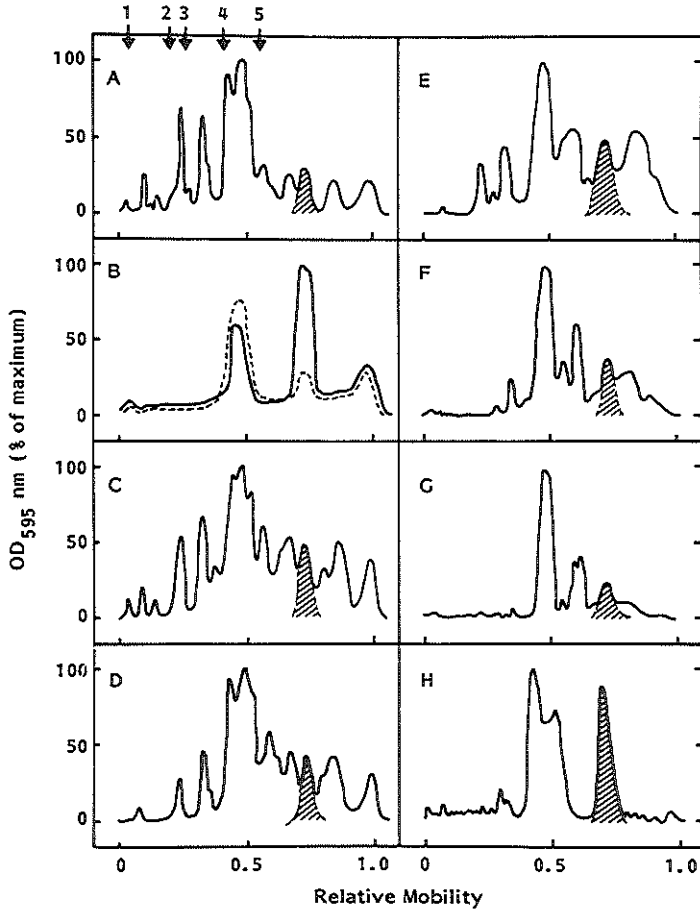
Fig.3. Densitometric scan of gel rods obtained after electrophoresis of different fractions of the purification steps and subsequent staining with Coomassie Blue R-250. A, and B - H show the absorbance at 595 nm plotted in the vertical axis as a percentage of the maximal absorption.
Panel B shows the incorporation of ^{125}I-BrAcT$_3$ in rat liver microsomes as a percentage of the maximal incorporation in the absence (——) or presence (- - -) of 10 μM rT$_2$. Shown are representative chromatograms from 2 experiments each performed in duplicate.
A= microsomes, B= microsomes labeled with BrAcT$_3$, C= microsomes from thyrotoxic animals, D= cholate extract, E= Emulgen extract after (NH$_4$)$_2$SO$_4$ precipitation, F= Cellex-P fraction, G= T$_3$-Sepharose fraction and H= PTU-Sepharose fraction. The shaded area shows the position of the deiodinating enzyme.

incubation with 50 mM DTT in buffer C at room temperature for 1 hr. This fraction contained approximately 11% of the activity of the T_3-Sepharose fraction, as determined directly after elution with DTT, and 0.4% of the protein. At this stage, deiodinase activity appeared to be labile, suffering a 50% loss by freezing at -20°C and subsequent thawing. In this way a deiodinase preparation was obtained which was enriched approximately 2400 times in 5'-deiodinase activity in comparison with the activity of the microsomal fraction. At the same time the 5-deiodinating activity of this fraction was determined using T_4 sulfate as the substrate. The increase in specific activity was also over 2000 times increased (not shown).

Electrophoresis.

Figure 3 shows the electrophoretic patterns of the fractions obtained after the various purification steps in 7.5% polyacrylamide gels in the presence of SDS (21). Panel B shows the relative incorporation of the affinity label ^{125}I-BrAcT$_3$ in the absence or presence of 10 µM rT$_3$. Substrate-protectable incorporation of radioactive BrAcT$_3$ is found at a relative migration of 0.73, corresponding to a molecular weight of 25,000, which is indicated by the shaded area in Fig. 3. The final enzyme preparation is still contaminated with proteins having a relative migration of about 0.5.

DISCUSSION

Solubilization of the rat liver iodothyronine deiodinase results in a marked change in kinetic parameters. The augmentation in V_{max} has also been observed after solubilization of other membrane proteins (28-30). The degree of activation varies between 3-10 times. If this effect is not taken into consideration, falsly high purification factors are obtained (31). The cause of this increase remains obscure although removal of inhibiting phospholipids has been suggested to play a role (27). In addition, enzyme may exist in a latent form with the active site on the luminal surface of the vesicles which becomes available after dysruption of

the vesicle structure by detergents. The change in K_m may be partially caused by inhibition of enzyme activity with detergent, either directly or by sequestration of the hydrophobic substrate. The K_m remained constant during further purification suggesting that the enzyme-detergent complex did not undergo major alterations by the chromatographic procedures. The results of the purification steps by ion-exchange chromatography were very similar to those reported previously and yielded an average purification of 17 times. This preparation was used for subsequent affinity chromatography.

The chromatographies on octyl-Sepharose or phenyl-Sepharose showed that the majority of membrane proteins bind strongly to these matrices, especially to the phenyl group. Proteins retained by the octyl sepharose could be eluted at high Emulgen concentrations although without enrichment. The affinity chromatogrphy on erythrosine-Sepharose was not specific since all protein was retained possibly through strong hydrophobic interaction. When the column was saturated with detergent no binding occurd. The addition of an anionic detergent such as deoxycholate in the buffer used as suggested by Robinson et al (32) for affinity chromatography in non-ionic detergent solutions did no improve the deiodinase binding at high Emulgen concentrations.

The major part of applied proteins did also bind to the more hydrophilic T_3 sulfate-Sepharose. Omission of the hydrophobic six-carbon spacer resulted in some improvement of the ultimate purification factor on this column. Removal of protein that was bound by non-specific hydrophobic interaction with 2% of the zwitterionic detergent CHAPS was not possible in contrast with the chromatography on the more hydrophobic T_3-Sepharose. It is conceivable that by the use of T_3 sulfate-Sepharose proteins are also bound by ion-pair formation involving the dissociated sulfate group. The advantage of the non-dissociated phenolic hydroxyl group in T_3 is that when proteins are non-specifically bound only by hydrophobic interaction, they may be eluted with high detergent concentrations. The purification by chromatography on T_3-Sepharose remains, however, still meager for an affinity column.

It is possible that also drug metabolizing enzymes have affinity for T_3 as appeared from the labeling experiments with ^{125}I-BrAcT$_3$. The

electrophoresis experiments show that also a 56,000 dalton protein is labeled with a high capacity, which is not inhibited by 10 uM rT_3. The major peak in the electrophoretic profile of the T_3-Sepharose eluate is also a 56,000 dalton protein. Worth mentioning is the induction by T_3 pretreatment of the microsomal UDP-glucuronyltransferase activity for 4-nitrophenol by 400% (33) which may be active in the glucuronidation of T_3 (34).

The purification of the deiodinase was also investigated by covalent binding of the deiodinase to PTU-Sepharose after rT_3-induced conversion of the enzyme into the E-SI form. In order to improve the binding of the deiodinase to the column, interfering proteins were removed by a chromatography step on PTU-Sepharose without addition of rT_3. Some reports mention the generation of radicals by non-ionic detergents (35) which will be quenched by DTT (36). These radicals may lead to oxidation of the catalytically important SH group and result in the irreversible loss of deiodinase activity. Therefore, a DTT concentration of 1 mM is necessary to keep the deiodinase SH in a reduced form (37). Also the irreversible oxidation of the PTU coupled to Sepharose is prevented by inclusion of DTT. In spite of the high PTU concentration on the gel, binding of deiodinase was slow and far from complete. Reasons for this may be that during synthesis of this affinity gel oxidation of PTU has occurred which diminished the actual concentration on the gel. Furthermore, steric hindrance of the carbon spacer which connects the PTU to the gel may interfere with a close interaction of PTU with the essential SH group of the deiodinase. The purified fraction of the PTU-Sepharose is still contaminated with proteins with molecular weights of approximately 55,000 dalton. Further research leading to the ultimate purification of rat liver iodothyronine deiodinase is necessary.

In conclusion, a useful strategy has been developed for the purification of rat liver iodothyronine deiodinase which appears to be only a minor component of the rat liver microsomal fraction as judged by SDS-PAGE and affinity labeling with $BrACT_3$ (23). Also calculations based on the specific incorporation of iodo[^{3}H]acetate into renal microsomes with roughly equal specific deiodinase activity shows that the deiodinase is only about 0.013% of the total renal membrane protein. This was based on

the assumption of an equimolar incorporation of the iodoacetate and a molecular weight of 50,000 for the deiodinase (7). In that case only one subunit of 25,000 should react with the affinity label $BrAcT_3$. The equal enrichment of both 5'-deiodinase and 5-deiodinase activities as measured by rT_3 and T_4 sulfate conversion, respectively, in the PTU-Sepharose fraction supports the hypothesis of a single liver enzyme which is able to catalyze both deiodinations. This is in contrast with the specific 5'-deiodinase activities found in pituitary and cerebral cortex and the specific 5-deiodinase activities of cerebral cortex and placenta of the rat (38-40). The exact underlying catalytic mechanism of the liver enzyme has yet to be elucidated.

REFERENCES

1. Schimmel, M. and Utiger, R.D. (1977) Ann. Intern. Med. 87, 760–768.
2. Larsen, P.R., Silva, J.E. and Kaplan, M.M. (1981) Endocr. Rev. 2, 87–102.
3. Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1979) Biochem. J. 179, 489–495.
4. Leonard, J.L. and Rosenberg, I.N. (1978) Endocrinology 103, 274–280.
5. Fekkes, D., Van Overmeeren-Kaptein, E., Docter, R., Hennemann, G. and Visser, T.J. (1979) Biochim. Biophys. Acta 587, 12–19.
6. Leonard, J.L. and Rosenberg, I.N. (1980) Endocrinology 107, 1376–1383.
7. Leonard, J.L. and Visser, T.J. (1984) Biochim. Biophys. Acta 787, 122–130.
8. Mol, J.A., Docter, R., Hennemann, G. and Visser, T.J. (1984) Biochim. Biophys. Res. Commun. 120, 28–36.
9. Visser, T.J. (1980) Biochim. Biophys. Acta 569, 302–308.
10. Leonard, J.L. and Rosenberg, I.N. (1978) Endocrinology 103, 2137–2144.
11. Visser, T.J. and Van Overmeeren-Kaptein, E. (1979) Biochem. J. 183, 167–169.
12. Goswami, A. and Rosenberg, I.N. (1983) Endocrinology 112, 1180–1187.
13. Mol, J.A., Van den Berg, T.P. and Visser, T.J. (1985) submitted for publication.
14. Cheng, S.Y., Wilchek, M., Cahnmann, H.J. and Robbins, J. (1977) J. Biol. Chem. 252, 6076–6081.
15. Mol, J.A. and Visser, T.J. (1985) Endocrinology, accepted for publication.
16. Pharmacia Fine Chemicals Publication (1979) Affinity Chromatography, Principles and Methods, Uppsala, Sweden.
17. Tucker, R.F. and Stellwagen, E. (1981) J. Biol. Chem. 256, 10993–10998.
18. Visser, T.J., Fekkes, D., Docter, R. and Hennemann, G. (1978) Biochem. J. 174, 221–229.
19. Mol, J.A. and Visser, T.J. (1985) Endocrinology, accepted for publication in conjunction with ref. 14.
20. Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
21. Spector, T. (1978) Anal. Biochem. 86, 142–146.
22. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
23. Mol, J.A., Docter, R., Kaptein, E., Jansen, G., Hennemann, G. and Visser, T.J. (1984) Biochim. Biophys. Res. Commun. 124, 475–483.
24. Kaplan, M.M. (1979) Endocrinology 105: 548–554.
25. Ruiz, M. and Ingbar, S.H. (1982) Endocrinology 110, 1613–1617.
26. Visser, T.J., Mol, J.A. and Otten, M.H. (1983) Endocrinology 112, 1547–1549.
27. Fekkes, D., Hennemann, G. and Visser, T.J. (1983) Biochim. Biophys. Acta 742, 324–333.
28. Moss, J., Osborne, J.C. and Stanley, S.J. (1984) Biochemistry 23, 1353–1357.

29. Freedman, R.B., Newell, A. and Walklin, C.M. (1978) FEBS Letters 88, 49–52.
30. Girardot, J.M. and Johnson, B.C. (1982) Anal. Biochem. 121, 315–320.
31. Mumford, R.A., Pierzchala, P.A., Strauss, A.W. and Zimmerman, M. (1981) Proc. Natl. Acad. Sci. USA 78, 6623–6627.
32. Robinson, J.B., Strottmann, J.M., Wick, D.E. and Stellwagen, E. (1980) Proc. Natl. Acad. Sci. USA 77, 5847–5851.
33. Chowdurry, J.R., Chowdurry, N.R., Mościoni, A.D., Tukey, R., Tephly, T. and Arias, I.M. (1983) Biochim. Biophys. Acta 761, 58-5.
34. Roche, J., Michel, R. and Tata, J. (1953) Biochim. Biophys. Acta 11, 543–547.
35. Chang, H.W. and Bock, E. (1980) Anal. Biochem. 104, 112–117.
36. Houba–Herin, N., Carlberg–Bacq, C.M., Piette, J. and Van de Vorst, A. (1982) Photochem. Photobiol. 36, 297–306.
37. Leonard, J.L. and Rosenberg, I.N. (1980) Endocrinology 106, 444–451.
38. Visser, T.J., Kaplan, M.M., Leonard, J.L. and Larsen, P.R. (1983) J. Clin. Invest. 71, 992–1002.
39. Kaplan, M.M., Visser, T.J., Yaskoski, K.A. and Leonard, J.L. (1983) Endocrinology 112, 35–42.
40. Roti, E., Fang, S., Green, K., Braverman, L.E. and Emerson, C.H. (1983) Am. J. Obstet. Gynecol. 147, 788–792.

SUMMARY

Thyroid hormone plays an essential role in the maintenance of a basal
metabolic rate and promotion of growth and development. The ultimate
effects of thyroid hormone are mediated by the biologically active
3,3',5-triiodothyronine (T_3) which is produced mainly by enzymatic
deiodination of thyroxine (T_4) in extrathyroidal tissues. The liver and
kidneys are thought to be essential for maintaining plasma T_3 levels and
thereby regulate all metabolic processes in tissues that depend
predominantly on plasma T_3. The aim of this thesis is to contribute to the
knowledge about the thyroid hormone deiodinating enzyme of rat liver in
order to get more insight in the processes that are involved with changes
in the plasma thyroid hormone levels.

Chapter I provides a general introduction to synthesis and metabolic
pathways of thyroid hormone. Under physiological conditions T_4 is
converted at roughly equal proportions either by 5'-deiodination to T_3 or
by 5-deiodination to 3,3',5'-triiodothyronine (rT_3). Although deiodination
is the major pathway for T_4, some 20% is metabolized by conjugation, ether
link cleavage or oxidative deamination.

In chapter II recent literature on the metabolism of iodothyronines by
the liver is reviewed. Increasing evidence becomes available indicating a
regulatory role for the plasma membrane in the cellular uptake of
iodothyronines. Nowadays consensus is achieved that a single rat liver
enzyme converts iodothyronines by either 5'- or 5-deiodination. This
enzyme is a normal constituent of the endoplasmic reticulum and needs the
reductive equivalents of thiol-containing compounds for catalytic activity.
It is concluded from kinetic measurements that the enzymatic deiodination
follows a ping-pong mechanism with an essential sulfhydryl group in the
enzyme as the acceptor of the leaving iodine atom. The liver enzyme,
called type I deiodinase, is distinct from the type II deiodinase and the
type III deiodinase, a specific 5'-deiodinase and a 5-deiodinase,
respectively. At last, factors that may control the deiodinase activity in
vivo are discussed.

Chapter III deals with the chemical synthesis of sulfate esters and
sulfamates of iodothyronines. A convenient method is developed for both

sulfation and purification. The conjugates are further characterized by
'H-NMR, HPLC and hydrolysis by acid or sulfatase activity. The sulfate
esters exhibited low cross-rectivities in the radioimmunoassays for the
native compounds. The so obtained pure sulfate esters are more prone to
deiodination than the native iodothyronines as is described in chapter IV
for rT_3 and T_4 sulfate. The efficiency of the 5-deiodination of T_4 sulfate
is increased 200-fold whereas 5'-deiodination is undetectable. Evidence is
presented that T_4 sulfate is deiodinated by the same type I deiodinase that
mediates the deiodination of native iodothyronines.

In chapter V the presence of an active site histidine residue is
described using chemical modification of the deiodinase with
diethylpyrocarbonate or rose bengal. This histidine residue nay be
important in the substrate binding or may increase the nucleophilicity of
the catalitically important sulfhydryl group by hydrogen bond formation.

Derivatization of T_3 by introduction of a bromoacetyl group in the
alanine side chain yields an affinity label that binds to the active site
of the enzyme as described in chapter VI. Analysis of $^{125}I-BrAcT_3$ labeled
microsomes by gel electrophoresis strongly suggests that the deiodinase is
composed of two subunits with a molecular weight of approximately 25,000
dalton.

Finally, chapter VII and VIII contain studies to the solubilization and
purification of rat liver iodothyronine deiodinase. Using solubilization
with cholate, delipidation by ammonium sulfate precipitation, renewed
solubilization with Emulgen, and subsequent ion-exchange chromatography on
DEAE-Sephacel and Cellex-P, followed by affinity chromatography on
T_3-Sepharose and PTU-Sepharose, a 2400 times purified deiodinase
preparation is obtained. This preparation, which is approximately 50%
pure, is both enriched in 5'- and 5-deiodinating activity, providing
further evidence for a single rat liver iodothyronine deiodinase.

In conclusion, progress is made in the purification of rat liver
iodothyronine deiodinase which is an enzyme that is capable to activate the
prohormone T_4 to T_3, but is also involved in degradative pathways of
thyroid hormone. Especially sulfation increases the formation of
iodothyronines that are biologically inactive. The ultimate answer how one
enzyme is capable to deiodinate T_4 alternately to T_3 or rT_3 awaits the

final purification of the enzyme and more information on substrate binding
to the enzyme.

SAMENVATTING (VOOR NIET INGEWIJDEN)

Dit hoofdstuk is bedoeld voor hen die zich niet dagelijks met schildklierhormoon bezighouden, en wil inzicht verschaffen in het doel en de resultaten van het onderzoek zoals dat in dit proefschrift te vinden is. Allereerst zal er worden ingegaan op de vraag wat schildklierhormoon is. Daarna zal het verband worden gelegd tussen de schildklier en het dejoderende enzym uit de rattelever, gevolgd door het beoogde doel van het onderzoek. Vervolgens zullen eigen waarnemingen wat betreft de samenhang tussen conjugatie en dejodering van schildklierhormoon besproken worden. Tot besluit zal de werking van het dejoderende enzym en de zuivering ervan worden samengevat.

Schildklierhormoon

Het schildklierhormoon speelt een essentiele rol in de handhaving van een basis niveau in de stofwisseling en de stimulering van groei en ontwikkeling. Vrijwel alle effekten worden gemedieerd door de biologisch aktieve vorm, het 3,3',5-triiodothyronine of kortweg T_3. Ongeveer 20% van het in het plasma voorkomende T_3 komt direkt uit de schildklier. De resterende 80% van het circulerende T_3 is afkomstig van een enzymatische dejoderings stap van het prohormoon thyroxine, kortweg T_4 genoemd, dat alleen door de schildklier wordt gemaakt. T_4 is een molekuul dat gekenmerkt wordt door een dubbele ring structuur die vier jodium atomen bevat (zie figuur). Afsplitsing van een jodium atoom uit de "buiten" ring geeft het biologisch aktieve T_3, terwijl dejodering van de "binnen" ring het omgekeerde of "reverse" T_3 oplevert.

139
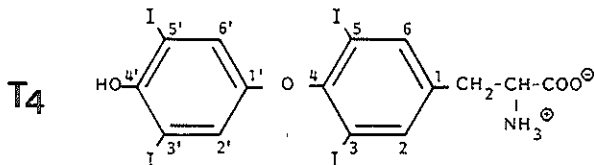
Het schildklierhormoon dejoderende enzym uit de rattelever

Op vele plaatsen in het lichaam kan de omzetting van T_4 naar T_3 plaatsvinden. Gezien echter de hoge concentratie van dejoderend enzym in de lever en nieren worden deze organen verantwoordelijk geacht voor een groot deel van de produktie van het plasma T_3. Daarbij is de lever een van de grootste organen uit het menselijk lichaam en is verantwoordelijk voor tal van stofwisselings processen. Hiertoe behoort het uit het bloed opnemen van slecht in water oplosbare stoffen en de omzetting ervan in meer water oplosbare, beter uit te scheiden verbindingen door middel van o.a. sulfatering, glucuronidering of hydroxylering. Voor dit doel beschikt de lever over een uitgebreid "biotransformatie" systeem van katalytisch aktieve eiwitten (enzymen) die zich voor een deel in membranen binnenin de cel bevinden. Hiertussen bevindt zich ook het enzym dat T_4 kan omzetten in zowel T_3 als reverse T_3. Uit onderzoek is gebleken dat de rattelever een enzym bevat met gelijke karakteristieken als het dejoderend enzym uit de humane lever. Hierdoor lijkt de rat een goed model te zijn om de enzymatische omzetting van T_4 te bestuderen.

Doel van het onderzoek

Uit vooronderzoek was gebleken dat de rattelever een enzym bevat dat T_4 zowel kan aktiveren (T_3) als inaktiveren (reverse T_3). Dit onderzoek werd geëntameerd om de manier waarop het enzym werkt verder te bestuderen en om door scheiding van het dejoderend enzym van andere eiwitten te komen tot een gezuiverd enzym preparaat. Hiervoor is steeds gebruik gemaakt van een gedeeltelijk gezuiverde membranen fraktie die werd gewonnen uit fijngemaakte ratte levers. Deze ruwe fraktie is gebruikt voor het onderzoek naar de relatie tussen sulfatering en dejodering, beschrijving van het enzym door chemisch het enzym te veranderen en diende tevens als uitgangsmateriaal voor de zuivering.

Conjugatie en dejodering van schildklierhormoon

Via onderzoek is aangetoond dat sulfatering van T_3 of van $3,3'-T_2$ de

molekulen op zo'n manier veranderde dat deze opeens veel beter gedejodeerd werden. Voor verder onderzoek hebben we een chemische methode ontwikkeld om grote hoeveelheden zuiver gesulfateerd schildklierhormoon te vervaardigen. Met geavanceerde chemische technieken zijn deze verbindingen nader gekarakteriseerd. Het synthetische T_3 sulfaat is gelijk aan het door levercellen in vitro gevormde T_3 sulfaat. Het bleek dat deze sulfaat verbindingen niet storend werken in de bepaling van schildklierhormoon zoals dat routinematig in vele laboratoria plaatsvindt.

Dejoderings experimenten met synthetisch T_4 sulfaat lieten zien dat ook hier sulfatering stimulerend werkt op de snelheid van dejodering in de binnenring. Tegelijkertijd werd echter de dejoderings snelheid van de buitenring vertraagd tot niet waar te nemen omzettingen. Duidelijk werd dat in dit geval door sulfatering de dejoderings reactie specifiek is geworden. Gepostuleerd is dat deze specificiteit wordt veroorzaakt door een veranderde binding aan het enzym waardoor de ene reaktie wordt bevoordeeld ten koste van de andere. Het gevormde reverse T_3 sulfaat bleek een even goed substraat voor buitenring dejodering te zijn als het niet geconjugeerde reverse T_3.

Beschrijving van het dejoderend enzym

Door middel van specifieke chemische veranderingen in het enzym is onderzoek gedaan naar aminozuren die van belang kunnen zijn voor de binding van schildklierhormoon aan het enzym, of betrokken zijn bij de katalyse van het dejoderings proces. Zo is gebleken dat het aminozuur histidine een essentiele rol speelt in dit enzym. Uit ander onderzoek was aangetoond dat het enzym tevens een uiterst belangrijke sulfhydryl groep bevat. Met gegevens uit de literatuur over enzym mechanismen is voorgesteld dat dit histidine residu het overnemen van een jodium atoom van het hormoon molekuul door de sulfhydrylgroep van het enzym kan bevorderen.

Door het T_3 molekuul in de zijketen met een reaktieve broomacetylgroep te verbinden, werd een stof gesynthetiseerd die tijdens incubatie met enzym hiermee een niet los te maken complex vormde. Hierdoor werd de aktiviteit van het enzym volledig geremd. Omdat een van de jodium atomen van het T_3 door een radioaktief jodium atoom was vervangen, kon de plaats van het

dejoderend enzym gedurende zuiverings experimenten worden vervolgd. Het is daardoor gebleken dat het dejoderende enzym uit ten minste 2 kleinere eiwitten is opgebouwd.

Zuivering van het dejodase

Het dejodase is ingebed met tal van andere enzymen in de membranen van het endoplasmatisch reticulum. Om het dejodase te kunnen scheiden van andere enzymen moet het eerst uit deze membranen worden gehaald met speciale detergenten. Onderzocht is welk detergent het meest geschikt is voor deze extractie. Het enzym dat zich door de solubilisatie niet meer in zijn natuurlijke omgeving bevindt kan daardoor nogal instabiel worden. Uit onderzoek zijn de juiste omstandigheden gevonden om het dejodase aktief te houden. Het detergent Emulgen (vgl. emulgeren) is hiervoor het meest geschikt. Met behulp van meer en minder specifieke zuiverings methodieken is het dejodase tot een redelijke mate van zuiverheid gebracht. De voor ongeveer 50% zuivere fraktie was nog steeds in staat om zowel buitenring als binnenring dejoderingen te katalyseren. Verder onderzoek zal moeten leren of selektieve manipulatie van deze dejoderingen tot de mogelijkheid zal kunnen behoren.

VERKLARENDE WOORDENLIJST

Aminozuur   Element waaruit een eiwit is opgebouwd

Conjugatie   Het door enzymatische omzetting beter
                                       water oplosbaar maken van verbindingen

Dejodase   Enzym dat de afsplitsing van een
                                       jodium atoom versneld

Detergent   Stof die de oppervlaktespanning
                                       verlaagt; zeep

Endoplasmatisch reticulum   Netvormig stelsel van membranen
                                       binnenin de cel

Enzym   Een eiwit dat ervoor zorgt dat een
                                       chemische reaktie die uit zichzelf
                                       traag verloopt, versneld wordt

Membraan   Vlies van vettige stoffen die o.a. de
                                       cel omsluit, maar ook binnenin de cel
                                       afgesloten compartimenten vormt

Stofwisseling   Omzetting van voedingsmiddelen in voor
                                       het lichaam bruikbare verbindingen
                                       waarbij tevens warmte vrijkomt

Solubilisatie   Het in oplossing brengen van het enzym

CURRICULUM VITAE

De auteur van dit proefschrift is geboren op 16 april 1954 te
Dordrecht. Na aan het Develstein College te Zwijndrecht het diploma
HBS-B te hebben behaald werd de studie Werktuigbouwkunde aan de
Technische Hogeschool te Delft aangevangen. De studie werd in 1975
vervolgd in de richting Moleculaire Wetenschappen aan de Landbouw
Hogeschool te Wageningen, waar   juni 1981 het ingenieurs examen
werd afgelegd met als hoofdrichtingen de Biochemie en de Toxicologie
gecombineerd met een stage aan het Nederlands Kanker Instituut te
Amsterdam en een bijvak in de Dierfysiologie. Van juli 1981 tot juli
1984 was de auteur in dienst van de Nederlandse Organisatie voor
Zuiver-Wetenschappelijk Onderzoek via een door FUNGO gesubsidieerd
onderzoek dat werd verricht op de afdeling Interne Geneeskunde III
van het Academisch Ziekenhuis te Rotterdam. Sinds november 1984 is
hij verbonden aan de Kliniek voor Kleine Huisdieren van de fakulteit
Diergeneeskunde van de Rijksuniversiteit Utrecht.

NAWOORD

Graag wil ik tot besluit de vele personen bedanken die betrokken zijn geweest bij de tot standkoming van dit proefschrift.

Allereerst dank ik mijn ouders voor de stimulans en mogelijkheid die zij mij geboden hebben om een academische opleiding te volgen. Het feit dat vader de slotfase van het promotie onderzoek niet meer in zo'n goede gezondheid kon meemaken, wordt door mij als een gemis ervaren. Niet zonder reden heb ik dit proefschrift aan mijn beide ouders opgedragen.

Lieve Ellen, jouw bijdrage was als van "een hulp die bij mij past". Op onnavolgbare wijze ben jij een stimulans geweest voor mij. Jou bedanken hoeft zich gelukkig niet te beperken tot het nawoord van het proefschrift.

Bijzondere dank ben ik verschuldigd aan mijn promotor Jorg Hennemann, die met grote belangstelling de ontwikkeling van het onderzoek heeft gevolgd. Aan de vele "vrijdagochtend" besprekingen onder zijn leiding bewaar ik fijne herinneringen.

Theo Visser heeft door zijn enthousiaste en kundige begeleiding van het onderzoek en de korrektie van het manuscript bijzonder inspirerend op mij gewerkt. Deze woorden kunnen slechts vaag aanduiden hoeveel dank ik hem verschuldigd ben.

De aandacht van Roel Docter en de opvang bij de afwezigheid van Theo gedurende de eerste periode van het onderzoek zijn door mij zeer gewaardeerd.

Een speciale vermelding verdient Tom van den Berg, die "ons" projekt op uitstekende wijze analytisch heeft ondersteund. Hier wil ik het accuraat uitvoeren van de talloze zuiverings experimenten en de nauwkeurige verslaggeving memoreren.

Zeer erkentelijk ben ik Yvonne van Dodewaard die mij heeft ingewijd in het gebruik van de tekstverwerker en veel van het typewerk voor haar rekening heeft genomen.

De medewerkers van het laboratorium hebben alle door het scheppen van een prettig werkklimaat hun bijdrage geleverd aan de voortgang van het onderzoek. Een woord van dank gaat uit naar Ellen Kaptein en

stagiaire Godfried Janssen die daadwerkelijke steun hebben verleend
aan de experimenten.

Het "uptake werk" van Eric Krenning was de basis voor het vervaar-
digen van een monoklonaal antilichaam tegen het opname eiwit in de
hepatocyt. Alhoewel dit onderzoek niet is opgenomen in het proefschrift
was het mij een genoegen om met hem samen te werken. Onmisbaar hierbij
was de analytische hulp van Bert Bernard, en de hybridoma experts
Jan Rozing en Len Vaessen van Interne Geneeskunge I.

Tenslotte wil ik al diegenen bedanken die op welke wijze dan ook
anderszins hun bijdrage hebben geleverd. Hierbij denk ik aan de
collegiale samenwerking met Marten Otten en Huib Pols, de vele kopjes
koffie van de balansafdeling, de steun van Marco Roede en Henk van Beek
bij de tekstverwerking en de medewerkers van de audiovisuele dienst voor
het tekenen en reproduceren van de figuren.