ISOLATION AND CHARACTERIZATION OF ENZYME ACTIVITIES DEIODINATING THYROID HORMONE

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PROEFSCHRIFT

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PROF. DR. J. SPERNA WEILAND
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CO-REFERENTEN : PROF. DR. W.C. HÜLSMANN
PROF. DR. H.J. VAN DER MOLEN

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Voor Saskia, Sandra en Michel
Voor mijn ouders

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LIST OF ABBREVIATIONS

DIT	3,5-diiodotyrosine
DOC	deoxycholate
DTT	dithiothreitol
rT_3	reverse T_3; 3,3',5'-triiodothyronine
T_0	thyronine
3- or 3'-T_1	3- or 3'-monoiodothyronine
3,5-, 3,3'- or 3',5'-T_2	3,5-, 3,3'- or 3',5'-diiodothyronine
T_3	3,3',5-triiodothyronine
T_4	thyroxine; 3,3',5,5'-tetraiodothyronine
Tetrac	3,3',5,5'-tetraiodothyroacetic acid
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone

CHAPTER 1

INTRODUCTION

1.1. Historic aspects and sources of thyroid hormone

The thyroid is an endocrine gland the function of which is under the direct control of thyroid stimulating hormone (thyrotropin, TSH). The secretion of the latter by the anterior pituitary is subject to neuroendocrine regulation involving the stimulatory action of thyrotropin releasing hormone (TRH; Fig. 1.1.) and the inhibitory actions of dopamine and somatostatin.

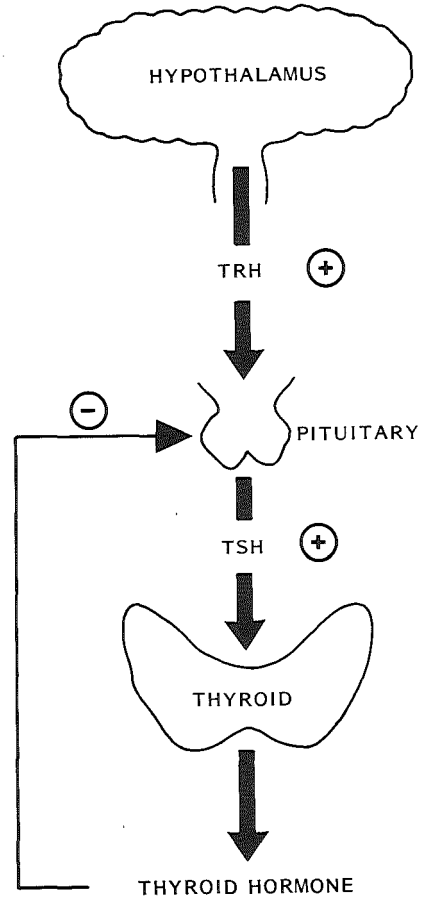

Fig. 1.1. Hypothalamus-pituitary-thyroid axis.

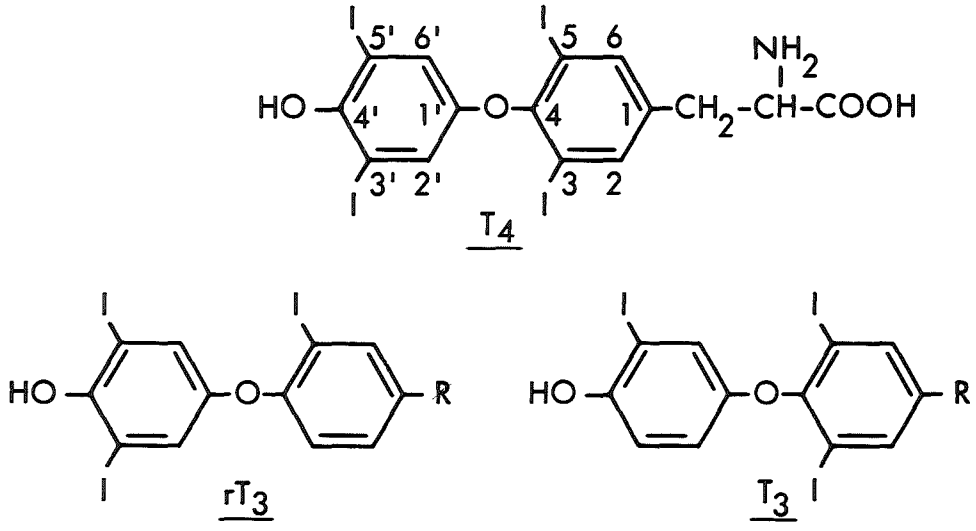

Fig. 1.2. Structures of T_4, T_3 and rT_3.

The thyroid "hormones" are iodinated amino acids (see Fig. 1.2.), which are synthesized by the follicular cells of the thyroid and influence many metabolic processes. These hormones also regulate TSH secretion through a direct action on the anterior pituitary (negative feedback control).

The thyroid hormone thyroxine (3,3',5,5'-tetraiodothyronine, T_4) has been crystallized by Kendall (1) in 1915 and the chemical structure of T_4 was elucidated by Harington (2) and Harington and Barger (3) in 1926 and 1927, respectively.

In 1952 Gross and Pitt-Rivers (4) and Roche et al (5) discovered 3,3',5-triiodothyronine (T_3), which proved to be physiologically more potent and more rapid in onset of action than T_4. Pitt-Rivers et al (6) showed that T_4 can be deiodinated in vivo to T_3 and this finding led to the proposal that T_4 is the form in which thyroid hormone is secreted, while T_3 is the form which is active in the tissues. However, conversion of T_4 into T_3 in man could not be demonstrated by Lassiter and Stanbury (7). In 1970 Braverman et al (8) provided new evidence for the peripheral (extrathyroidal) conversion of T_4 into T_3. These investigators were able to demonstrate formation of T_3 from T_4 in athyreotic human subjects, in whom substitution therapy with T_4 was the only possible source of T_3. Peripheral monodeiodination of T_4 in man has now been confirmed in many laboratories (9-14).

Another iodothyronine, 3,3',5'-triiodothyronine (reverse T_3, rT_3), has been identified in 1956 both in serum and thyroglobulin (an iodinated protein in the thyroid which stores thyroid hormones as peptide linked iodoamino acids) of the rat (15,16). It was only in 1974 that the natural existence of rT_3 in human serum could be established using a radioimmunoassay (17). This compound has little or no metabolic activity (18) and is therefore currently regarded to be biologically inactive. rT_3 can, comparable to T_3, be generated peripherally from T_4 in man (19-22).

It is generally believed that the thyroid gland does not contribute importantly to circulating T_3 and rT_3 in normal humans, but that monodeiodination of T_4 in extrathyroidal tissues is the major source of T_3 and rT_3 (19). The relative contributions of the peripheral deiodination to the daily production of T_3 and rT_3 are 80 and 94%, respectively (for reviews see 23-25). These figures have been calculated from the plasma concentrations, metabolic clearance rates and thyroidal secretion rates (which are assumed to be related to the proportion in which T_3 and rT_3 are present in thyroglobulin) of T_4, T_3 and rT_3. On the other hand, in the rat the contribution of the peripheral deiodination of T_4 to the total production of T_3 has been estimated at 20% (26) and 32% (27). However, the results in the latter study are contradictory, because in this study the fractional conversion rate of T_4 into T_3 was reported to be approximately 30%. From this value and the production rates of T_4 and T_3, which are for the former 2-3 times higher than for the latter, it can be calculated that at least 60% of circulating T_3 is derived from peripheral deiodination of T_4. Moreover, Abrams and Larsen (28) have calculated that two-thirds of the T_3 utilized per day by the iodine-sufficient rat arises from T_4. Finally, comparison of serum T_3 levels in T_4-substituted athyreotic rats with those in euthyroid animals yielded similar values (29). The above results point to a situation in the rat which is not different from the one in humans.

1.2. Metabolism of thyroid hormone in humans

The thyroid gland is, as far as presently known, the only source of T_4. Only a small amount of T_3 and a negligible amount of rT_3 are also of thyroidal origin. Upon entering the blood, the iodothyronines bind strongly but reversibly to three serum proteins. Of these proteins thyroxine-binding globulin (TBG) has the highest affinity for T_4 and T_3 and carries more than 70% of thyroid hormone in serum. The other two transport proteins are thyroxine-binding prealbumin (TBPA) and albumin (30). TBPA binds 15-20% of circulating T_4 and almost no T_3. Albumin binds 5-10% of serum T_4 and 25-30% of serum T_3. About 0.02% of serum T_4

and 0.2% of serum T_3 circulate in a free, not protein bound form. The free moiety of thyroid hormone is the only form which can enter tissue cells. Thyroid hormone is translocated over the plasma membrane by a specific, energy-dependent transport process as shown by *in vitro* studies (31,32). In the cell, thyroid hormone is bound to cytosolic protein but interaction with this protein is not a prerequisite for binding to the nucleus (33). The nucleus is supposed to be the main site of initiation of thyroid hormone action (34,35). Binding to a specific nuclear receptor leads to increased transcription rates and synthesis of proteins involved in the metabolic response to thyroid hormone. However, a mitochondrial route of thyroid hormone action cannot be ruled out (35,36). Recently, Müller and Seitz (37) provided evidence for a rapid action of T_3 on intermediairy metabolism independent of "de novo" protein synthesis. They found that T_3 at the supraphysiologic concentration of 10^{-6} M rapidly stimulates hepatic glucose production by increasing amino acid uptake and ATP regeneration. It still remains a question whether T_4 has intrinsic biological activity, because most, if not all, of its effect is brought about by transformation into T_3. Therefore, T_4 is regarded as a prohormone which becomes activated after conversion into T_3.

There are several pathways of T_4 metabolism: a) deiodination, b) oxidative deamination, c) conjugation and d) ether bond cleavage (Fig. 1.3.). Deiodination is the most important pathway of metabolism, accounting for at least 60% of T_4 turnover in humans. Roughly half seems to undergo initial monodeiodination in the phenolic ring to yield T_3, while the remaining half is converted into rT_3. Although similar amounts of T_3 and rT_3 are generated, deiodination of T_4 is not a random process as has been suggested by Surks and Oppenheimer (38). In several situations (starvation, non-thyroidal disease) conversion of T_4 into T_3 is diminished, whereas conversion into rT_3 does not change (25,39). As rT_3 appears to be devoid of biological activity and T_3 is the most active iodothyronine, modulation of the alternate pathways of T_4 metabolism could provide an effective means for modifying the balance between activation and inactivation of T_4. Both T_3 and rT_3 are subject to further deiodination, which ultimately leads to the formation of thyronine (T_0) as illustrated in Figure 1.4. The presence of 3,3'-diiodothyronine (3,3'-T_2) in the thyroid gland and the serum of the rat was demonstrated originally in 1956 by Roche et al (16). Monodeiodination of T_3 and rT_3 into 3,3'-T_2 and of rT_3 into 3',5'-diiodothyronine (3',5'-T_2) in man has been demonstrated by several investigators (40-44). Recently, evidence was presented that 3,5-diiodothyronine (3,5-T_2) is derived from T_3 in normal humans (45). In vivo generation of 3'-monoiodothyronine (3'-T_1) from 3',5'-T_2 and rT_3 has also been found (46,47). Up to now, no evidence

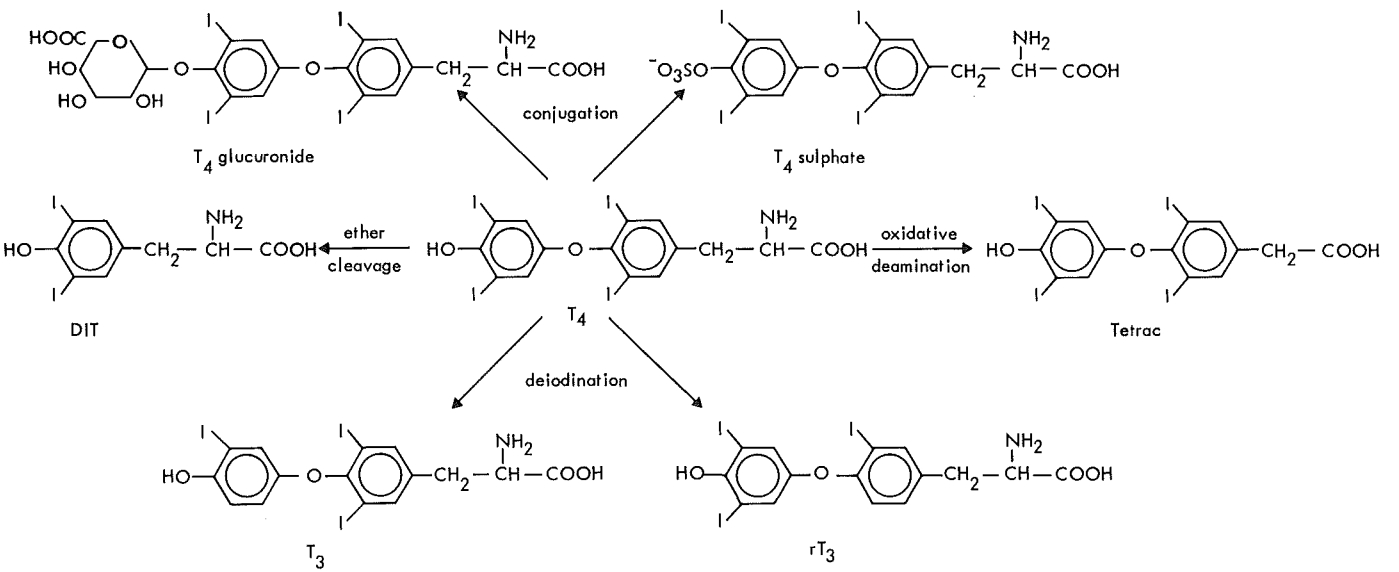

Fig. 1.3. Pathways of T_4 metabolism.

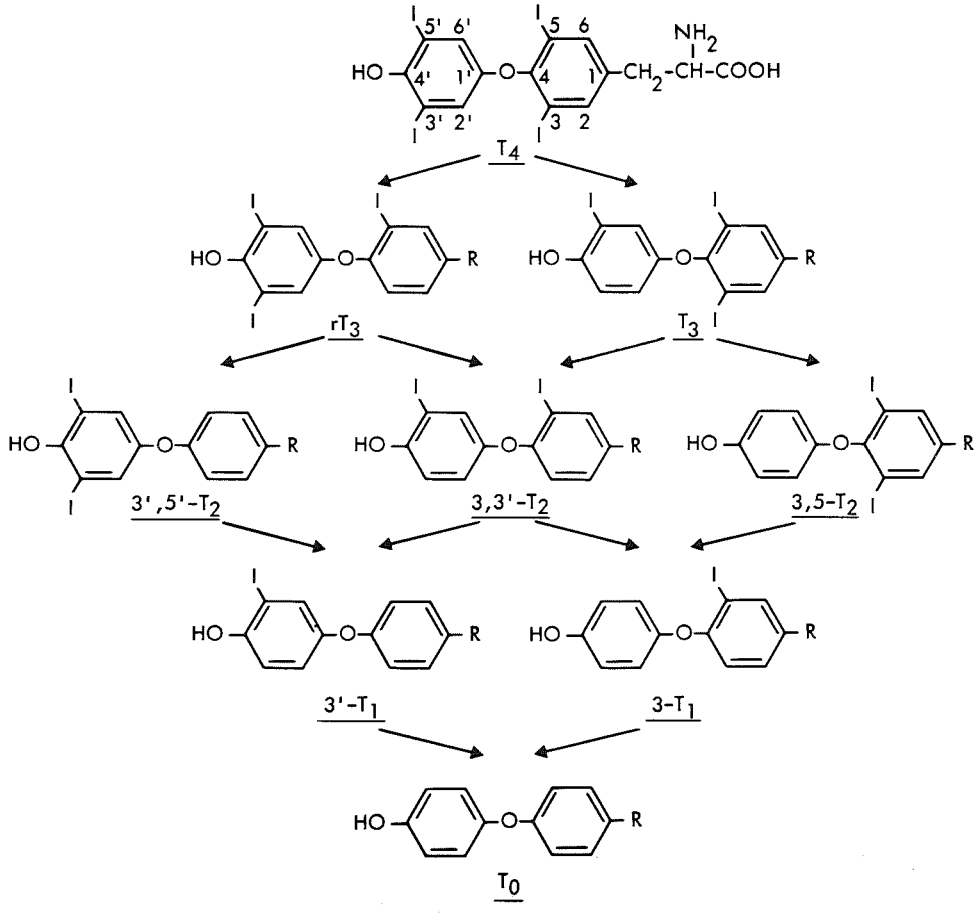

Fig. 1.4. Sequential 5-(↙) and 5'-(↘) deiodination of T_4.

has been presented for the production of 3-monoiodothyronine (3-T_1) from 3,5-T_2 or 3,3'-T_2 in humans. In vivo formation of T_0 from T_4 has been suggested by Pittman et al (48). Recently, excretion of T_0 in the urine has been measured by a combination of gas chromatography and mass-fragmentography (49). The sequential deiodination of T_4 in peripheral tissues is thought to be mediated by two enzyme activities, i.e. iodothyronine 5(or 3)*-deiodinase, which catalyzes deiodination of the tyrosyl ring and iodothyronine 5'(or 3')*-deiodinase, which catalyzes deiodination of the phenolic ring (50,51).

*Positions 3 and 5 are equivalent as are positions 3' and 5'.

About 5-10% of T_4 is metabolized via oxidative deamination of the alanine side chain to yield 3,3',5,5'-tetraiodothyroacetic acid (52). In normal humans 10-25% and in rats a somewhat greater fraction of the daily disposal of T_4 takes place through fecal excretion, mainly of glucuronyl- and sulfoconjugates (52). Hydrolysis of the ether bond is only a very minor pathway of T_4 degradation.

1.3. Scope of the present study

It is well established that in man peripheral deiodination of T_4 accounts for most of the biologically active T_3 and virtually all of the inactive rT_3. In several situations reciprocal changes in serum T_3 and rT_3 levels occur, which indicates that enzymatic deiodination is not an uncontrolled process. To get more insight into the regulation and mechanism of thyroid hormone metabolism, basic knowledge of the deiodinating enzyme(s) is necessary. Therefore, the aim of this study was to localize, purify and characterize the iodothyronine deiodinase(s). The availability of pure enzyme preparations will not only facilitate the elucidation of the molecular mechanism of thyroid hormone regulation, but also answer the question of whether one or two enzymes are involved in the deiodination of T_4.

In Chapter 2, in vitro observations of the enzymatic deiodination of iodothyronines are described. In addition to a review of the literature, results from my own work are also included. The results of this work are discussed in relation to the findings by other investigators. In the following chapters, which are papers already published, my investigations of the enzymatic deiodination of several iodothyronines in rat liver preparations are described in detail.

CHAPTER 2

MONODEIODINATION OF IODOTHYRONINES - IN VITRO STUDIES

2.1. Monodeiodination of thyroxine

Soon after the discovery of T_3 (4,5), conversion of T_4 into T_3 was demonstrated in rat kidney slices by Albright et al (53). This deiodination reaction has now been demonstrated in many tissues and cell preparations, including cultured human liver cells (54), isolated rat liver cells (55), rat liver homogenate (55-60) and microsomes (55,57), cultured human kidney cells (54), rat kidney homogenate (59,61,62) and isolated rat renal tubules (63), cultured human fibroblasts (64), human polymorphonuclear leukocytes (65,66), isolated perfused rat heart (67) and dog heart homogenate (68), homogenates of rat cerebellum and cerebral cortex (69,70), rat skeletal muscle homogenate (59,71) and perfused rat skeletal muscle (72), rat thyroid tissue (73,74), isolated human thyroid cells (75), human thyroid homogenate (76), rat thyroid particulate fraction (77), human and dog thyroid microsomes (78), rat pituitary homogenate (79,80) and human placenta and fetal membranes (81).

Although conversion of T_4 into rT_3 is difficult to study in vitro, because rT_3 is metabolized very rapidly to 3,3'-T_2, this reaction has been demonstrated in dog liver microsomes (78), cytosol (82), homogenate (83-85) and microsomes of rat liver (86), isolated rat renal tubules (63), human polymorphonuclear leukocytes (66), dog heart homogenate (68) and cultured chick-embryo heart cells (87), rat cerebellar and cerebral cortical homogenates (69,70), monkey hepatocarcinoma cells (88), rat thyroid particulate fraction (77), dog thyroid microsomes (78) and human placenta and fetal membranes (81,89).

2.2. Monodeiodination of tri- and diiodothyronines

The conversion of rT_3 into 3,3'-T_2 has been studied in rat pituitary tumour cells (90), rat liver homogenate (85,91-96) and microsomes (86), rat kidney homogenate (91,95), rat thyroid particulate fraction (77) and monkey hepatocarcinoma cells (97).

Monodeiodination of T_3 into 3,3'-T_2 was found to occur in rat liver homogenate (85,91-93) and microsomes (86), rat kidney homogenate (91), cultured chick-embryo heart cells (87), rat thyroid particulate fraction (77) and monkey hepatocarcinoma cells (88,97).

Very interesting are the monkey hepatocarcinoma cells which, besides the above mentioned deiodinations, can at the same time convert rT_3 into 3',5'-T_2, 3,3'-T_2 and 3',5'-T_2 into 3'-T_1 and 3,5-T_2 into 3-T_1 (97). The generation of 3'-T_1 from 3',5'-T_2 has also been found in rat liver microsomes (98-100) and cytosol (100) and in rat kidney microsomes and cytosol (100). Recently, the conversion of 3,3'-T_2 into 3-T_1 has been shown to occur in rat liver microsomes (99). Although production of 3,5-T_2 from T_3 seems to exist in normal humans (45), this reaction has not been demonstrated directly in vitro nor has metabolism to the completely deiodinated thyronine been observed.

2.3. Nature of the deiodination process

Factors modulating the deiodination reaction include the temperature and pH of incubation, and the concentration of substrate (25,56,59-61,66,83-86,92). The deiodination is stimulated by several thiols (mercapto compounds) such as dithiothreitol (DTT) and 2-mercaptoethanol (57,101), and by reduced glutathione (39, 96,101-103). However, several investigators found no or only small stimulatory effect of the latter compound (60,61,104 and D. Fekkes, unpublished observations). On the other hand, the sulphhydryl-oxidizing agent diamide, the sulphhydryl-binding reagents N-ethylmaleimide, iodoacetic acid and mercuric chloride, and oxidized glutathione are potent inhibitors of the monodeiodination (25,51). These characteristics suggest that the monodeiodinating activity is enzymatic in nature and that the presence of sulphhydryl groups is essential for the deiodination of the various iodothyronines. Probably, an essential cysteine residue is located in the active site of the enzyme. It has recently been established that thiols are cofactors in the deiodination reactions. These compounds act as the second substrate in the 5'-deiodination of T_4 (105) and of rT_3 (106).

2.4. Localization of deiodinase activity

The monodeiodinating activities have been observed in several tissues, such as liver, kidney, heart, leukocytes, fibroblasts, skeletal muscle, thyroid gland, pituitary, cerebral cortex, cerebellum and placenta (see Section 2.1. and 2.2.). Of these tissues, liver and kidney demonstrated the greatest deiodinating capacity.

However, because of its large mass, muscle cannot be excluded as an important site for the deiodination of thyroid hormone. Rat spleen and intestines showed very low deiodinase activity (59,91,100), while lungs were devoid of activity (59,100).

Subcellular fractionation studies have revealed different locations of the deiodinating activities in rat liver and kidney tissues (see Table 2.1.).

Table 2.1. SUBCELLULAR LOCALIZATION OF IODOTHYRONINE DEIODINATING ACTIVITIES IN RAT LIVER AND KIDNEY

Organ	Activity	Localization	Reference
liver	$T_4 \rightarrow T_3$	microsomes	Hesch et al, 1975 (55), Visser et al, 1976 (57), Cavalieri et al, 1977 (82)
liver	$T_4 \rightarrow rT_3$	cytosol	Cavalieri et al, 1977 (82)
kidney	$T_4 \rightarrow T_3$	plasma membrane and mitochondria	Chiraseveenuprapund et al 1978 (61)
kidney	$T_4 \rightarrow T_3$	plasma membrane	Leonard and Rosenberg,1978 (52)
liver	$T_4 \rightarrow T_3$, $rT_3 \rightarrow 3,3'\text{-}T_2$	plasma membrane	Maciel et al, 1979 (107)
liver	$T_4 \rightarrow T_3 + rT_3$, $rT_3 + T_3 \rightarrow 3,3'\text{-}T_2$	endoplasmic reticulum	Fekkes et al, 1979 (108)
liver	$T_4 \rightarrow T_3$	endoplasmic reticulum	Auf dem Brinke et al,1979 (109)
liver	$T_4 \rightarrow T_3$	microsomes	Takaishi et al, 1979 (110)
liver	$T_4 \rightarrow rT_3$	microsomes	Auf dem Brinke et al, 1980(111)
liver	$T_4 \rightarrow T_3$	endoplasmic reticulum	Saito et al, 1980 (112)
liver	$T_4 \rightarrow rT_3$, $rT_3 \rightarrow 3,3'\text{-}T_2$	mitochondria and microsomes	Sorimachi et al, 1980 (113)
liver and kidney	$3',5'\text{-}T_2 \rightarrow 3'\text{-}T_1$	microsomes and cytosol	Smallridge et al, 1981 (100)
liver	$3',5'\text{-}T_2 \rightarrow 3'\text{-}T_1$, $3,3'\text{-}T_2 \rightarrow 3\text{-}T_1$	plasma membrane	Chopra, 1981 (99)

Most investigators reported on a microsomal localization of the deiodinase activity. In some studies iodothyronine deiodinase activity was localized in the endoplasmic reticulum (108,109,112), whereas in others an association with the plasma membrane fraction was observed (99,107). However, close examination of the data in the latter reports do not support this conclusion. On the other hand, Chiraseveenuprapund et al (61) and Leonard and Rosenberg (62) showed that the kidney enzyme is associated with the plasma membrane. The above mentioned studies all show an association of the deiodinase activity with the particulate fraction of tissue homogenates. These results are disconcordant with those of Cavalieri et al (82) and Smallridge et al (100), who found deiodinating activity in cytosol. Probably, contamination of cytosol with microsomal components is responsible for this observation (111). Existence of phenolic- as well as nonphenolic ring deiodinase activity has also been shown in particulate fractions of cultured monkey hepatocarcinoma cells (113), human placenta and chorion (81), rat thyroid homogenate (77), dog liver and thyroid homogenate (78) and human thyroid homogenate (78).

2.5. Characteristics of the enzymatic deiodination in rat liver

All deiodination reactions so far studied obey Michaelis-Menten kinetics. Many different K_m - and especially V_{max} values of the several deiodination reactions in rat liver have been reported. These discrepancies may be caused by the use of different crude enzyme preparations such as rat liver homogenate (containing soluble iodothyronine-binding proteins) or microsomes, and by studying the reactions at different pH values and cofactor concentrations. A summary of the apparent kinetic parameters of the deiodination reactions is shown in Table 2.2.

Table 2.2. RANGES OF APPARENT K_M AND V_{MAX} VALUES OF IODOTHYRONINE 5- AND 5'-DEIODINASE-CATALYZED REACTIONS IN RAT LIVER PREPARATIONS

Reaction	K_m (μM)	V_{max} (pmol/min per mg of protein)	Reference
$T_4 \rightarrow T_3$	1-10	0.1-0.5 and 10-70	59,60,83,86,95,109,112, 114,115
$T_4 \rightarrow rT_3$	0.1 and 2-5	13-24	82,86,114
$rT_3 \rightarrow 3,3'\text{-}T_2$	0.01-0.3	0.2-0.4 and 340-830	60,86,91-93
$T_3 \rightarrow 3,3'\text{-}T_2$	5-10	21-42	86,91
$3',5'\text{-}T_2 \rightarrow 3'\text{-}T_1$	0.01 and 0.2-0.8	0.1-0.8 and 150-170	99,100,Chapter 7
$3,3'\text{-}T_2 \rightarrow 3\text{-}T_1$	0.5	0.1	99

The apparent K_m values of the 5'-deiodination of T_4 and of the 5-deiodination of T_4 and T_3 vary between 1-10 µM. Only Höffken et al (114) found a different value for T_4 (0.1 µM) in the 5-deiodination to rT_3. The apparent K_m values for the 5'(3')-deiodinations of 3',5-T_2 and 3,3'-T_2 are, with the exception of one study (100), in the range of 0.1-1 µM. The lowest apparent K_m values have been determined for the 5'-deiodination of rT_3, viz. 0.01-0.3 µM. However, all estimated values are expressed as the total substrate concentration and, therefore, only represent *apparent* K_m values. Recently, Heinen et al (115) calculated the real K_m value for the 5'-deiodination of T_4 on the basis of free instead of total T_4 concentration to be 9.7 nM. This value is substantially lower than the apparent K_m of 2.1 µM, which was found by the same investigators after applying the total T_4 concentration. However, the significance of these findings remains unclear as reactions were carried out with large amounts of microsomes without added cofactor. The large discrepancies in the estimated V_{max} values, as shown in Table 2.2., are mainly caused by different cofactor concentrations in the incubation medium and not by a different ratio substrate/microsomes.

The pH-optima of the deiodination reactions have been determined by many investigators. The 5'-deiodination of T_4 was found to be optimal at pH 6-7 (59,82-86,92,109,116), whereas the 5-deiodination of T_4 is most effective around pH 8 (82,85,86). However, earlier studies revealed an optimal pH for this latter reaction at pH 9 (83) and 9.5 (84). The reason for this discrepancy is the rapid degradation of rT_3 into 3,3'-T_2 between pH 5-8 (85,91-93), which was not taken into consideration by these investigators. The 5-deiodination of T_3, which is analogous to the conversion of T_4 into rT_3 (both reactions are 5-deiodinations), has also been shown to be optimal at pH 8 (85,86,91). However, Höffken et al (93) and Gavin et al (116) found somewhat higher optimal values (pH 8.4). Optimal pH values for the 5'-deiodination of rT_3, which is a similar type of reaction as the conversion of T_4 into T_3, have been reported to amount to 6.5 (85), 7.3 (93), 7.7 (116) and 8.0-8.5 (86,91,92,104). It has been shown that the pH-optimum for this reaction depends on the concentration of the substrate (86,98). At low rT_3 concentrations reaction rate is highest at pH 6-7, whereas at high concentrations optimal pH value is shifted towards pH 8. A similar dependence of the optimal pH on the substrate concentration also holds for the 5'-deiodination of 3',5'-T_2 (98). A broad pH plateau (pH 4.9-7.6) and a narrower pH peak (pH 5.0-6.5) for this reaction have been found by Smallridge et al (100), whereas Chopra (99) reported on a maximal deiodination rate between pH 7.5-8.1 both for the 5'(3')-deiodination of 3',5'-T_2 and 3,3'-T_2.

Despite differences in pH-optima and kinetic parameters of the several

deiodination reactions, evidence has been obtained that the 5'-deiodinations of both T_4 and rT_3 and 3',5'-T_2 are catalyzed by a single enzyme, viz. iodothyronine 5'-deiodinase (60,85,86,95,96,99,107,117). Thus, rT_3 is a competitive inhibitor of the 5'-deiodination of T_4 (59,60,115) and T_4 is a competitive inhibitor of the production of 3,3'-T_3 from rT_3 (60,86,96). K_i and K_m values were found to be equal under various conditions. In only one study rT_3 was found to be a noncompetitive inhibitor of the 5'-deiodination of T_4 (83). In this study, degradation of rT_3 was not taken into account when inhibition of T_4 to T_3 conversion was investigated. 3',5'-T_2 has been shown to be a competitive inhibitor of the 5'-deiodination of T_4 (25) and of rT_3 (Chapter 7). The conversion of 3',5'-T_2 into 3'-T_1 is also inhibited competitively by rT_3 (Chapter 7). On the other hand, Auf dem Brinke et al (109) suggested that three enzymes are involved in the 5'-deiodination of T_4, while Smallridge et al (100) reported on a microsomal and a cytosolic enzyme mediating the conversion of 3',5'-T_2 into 3'-T_1.

The 5-deiodinations of T_4 and rT_3 were initially thought to be mediated by a separate enzyme, viz. iodothyronine 5-deiodinase. Both reactions exhibit similar pH profiles (85) and the kinetic parameters of these deiodinations demonstrate a similar dependence on pH (86). However, 3,5-T_2 also appears to be deiodinated by iodothyronine 5'-deiodinase (118, Chapters 7 and 9). In addition, T_3 was found to be a competitive inhibitor of the 5'-deiodination of both rT_3 and 3',5-T_2, while 3',5'-T_2 competitively inhibits the 5-deiodination of T_3. Values for K_i are identical with the respective K_m values (Chapter 9). These data point strongly to a single enzyme catalyzing both the 5- and 5'-deiodination of all iodothyronines. This hypothesis is sustained by the finding that all deiodinating activities, involved in the production of 3,3'-T_2 from T_4 via T_3 and rT_3, are located in the endoplasmic reticulum of the rat liver cell (108). Moreover, it has not been possible to separate 5- and 5'-deiodinase activity in detergent solubilized microsomes (119).

Several regulatory mechanisms in the deiodination process have been proposed, such as changes in intracellular pH (84,114), changes in cofactor concentration (or the glutathione sulphhydryl-disulphide ratio) (39), substrate-induction of the deiodinating enzymes (120), the concentration of the iodothyronines themselves, the concentration of naturally occurring inhibitors (including iodothyronines) and changes in cellular uptake of T_4 (32,121). It may be well that some of these mechanisms operate in concert resulting in the observed changes in thyroid hormone metabolism (decreased peripheral T_3 production and rT_3 degradation leading to low serum T_3 and high rT_3 concentrations) induced by drugs and disease states.

2.6. Inhibitors of the deiodination reactions

Many investigators have reported on dose-dependent inhibitions of the several deiodination reactions in rat liver homogenate or microsomes by a variety of agents. However, only in a few studies the type of inhibition has been described. Three types of inhibition may be distinguished: a) competitive, b) noncompetitive and c) uncompetitive inhibition. Competitive inhibition of the 5'-deiodination of T_4 has been found by the iodothyronines rT_3 (59,60,115) and 3',5'-T_2 (25), by the radiographic contrast agent ipodic acid (25) and by salicylic acid (122). The conversion of rT_3 into 3,3'-T_2 is inhibited competitively by T_4 (60,86,96), ipodic acid (25) and salicylic acid (122), while ipodic acid also inhibits the 5-deiodination of T_3 in a competitive fashion (25). In Chapter 7, competitive inhibition of the 5'-deiodination of both rT_3 and 3',5'-T_2 by several iodothyronines and iodothyronine analogues, radiographic contrast agents, 8-anilino-1-naphthalene sulphonic acid and salicylic acid derivates is described. Furthermore, in Chapter 8 the competitive inhibition of rT_3 5'-deiodinase by phenolphthalein dyes is discussed. Some of these derivatives may be regarded as substrate analogues (both types of compounds contain a 3,5-dihalogen-4-hydroxyphenyl group). In addition, Sorimachi and Robbins (123) reported on a competitive inhibition by T_4 of both the 5'-deiodination of rT_3 and the 5-deiodination of 3,5-T_2 in monkey hepatocarcinoma cells, though with widely different K_i values.

Noncompetitive inhibition of the conversion of T_4 into T_3 has been found by compounds which react more or less specifically with sulphhydryl groups, such as iodoacetic acid, diamide, N-ethylmaleimide and mercuric chloride (25). Iodoacetic acid has also been shown to inhibit production of 3,3'-T_2 from both T_3 and rT_3 in a noncompetitive manner (91).

The uncompetitive type of inhibition is achieved by reaction of compounds with an oxidized form of the essential sulphhydryl group of the deiodinase, which is generated upon reaction with substrate. An example of such an uncompetitive inhibitor is 6-propyl-2-thiouracil, a derivative of 2-thiouracil (124), which inhibits not only the 5'-deiodination of T_4 (59,60,105) and rT_3 (60,91,106), but also the 5-deiodination of T_3 (91). A similar mode of inhibition is exerted by sulfite (125).

2.7. Mechanism of the reductive deiodination of iodothyronines

Recent observations by Leonard and Rosenberg (105) and Visser (106) have given insight into the mechanism of action of iodothyronine 5'-deiodinase.

Thiols, such as DTT, stimulate 5'-deiodination of both T_4 and rT_3 (25,57,62,85, 101), while 2-thiouracil inhibits these reactions uncompetitively with respect to substrate (59,60,91,105,106), but competitively with respect to DTT (105,106). A change in the cofactor (DTT) concentration results in a parallel displacement of the double reciprocal (Lineweaver-Burk) plots of the deiodination rate as a function of the substrate concentration (105,106).In presence of DTT, oxidized glutathione inhibits 5'-deiodination of rT_3 uncompetitively with respect to substrate and competitively with respect to DTT (D. Fekkes and E.v.Overmeeren, unpublished observations). Based on the kinetics of stimulation by thiols and inhibition by 2-thiouracil, a ping-pong mechanism has been implied. According to the proposed model, the reductive deiodination of iodothyronines is envisaged as a transfer of an iodinium ion from the substrate to a sulphhydryl group of the enzyme leading to the formation of an enzyme-sulphenyl iodide (E-SI) complex. The E-SI intermediate is subsequently reduced by thiol compounds (cofactor) to free enzyme. Both phenolic and tyrosyl ring deiodination may occur via such a mechanism. The reaction pathway may, therefore, be visualized as consisting of two half-reactions:

$$TI_n \text{ (substrate)} + \text{E-SH (enzyme)} \rightarrow TI_{n-1} \text{ (product)} + \text{E-SI (intermediate)}$$
$$\text{E-SI} + 2\ \text{R-SH (cofactor)} \rightarrow \text{E-SH} + \text{R-S-S-R} + \text{HI}.$$

The deiodinase is inhibited by derivatives of 2-thiouracil (X-SH) as these compounds react with the ESI intermediate forming an enzyme-thiouracil mixed disulphide:

$$\text{E-SI} + \text{X-SH} \rightarrow \text{E-S-S-X} + \text{HI}$$

A prediction of this model is that 2-thiouracil would only react with the deiodinase in the presence of substrate. This is true since binding of radioactive 6-propyl-2-thiouracil to rat liver microsomes is specifically induced by iodothyronines (126,127). Moreover, persistent and reversible inactivation of the enzyme by thiouracils requires the presence of substrate (105,118,127,128).

2.8. Solubilization, characterization and partial purification of iodothyronine deiodinases

Enzymatic activity catalyzing the reductive 5'-deiodination of T_4 and rT_3 has been solubilized from rat liver and kidney microsomes by treatment with 1% sodium cholate (119,129), 0.25% of the polyoxyethylene ether W-1 (119) or 0.1-0.33% sodium deoxycholate (103,127,130,131). The structures of two of these

detergents are shown in figure 2.1.. The 5-deiodinating activity has also been solubilized with these detergents from rat (119) as well as from beef liver microsomes (131). A review of published data concerning solubilization of iodothyronine deiodinase activity is given in Table 2.3. In all studies, the concentration of detergent necessary for effective solubilization inhibits deiodinase activity. Removal of detergent from the solubilized fraction results in restoration of activity and formation of enzymatically active aggregates (119, 127,130,131).

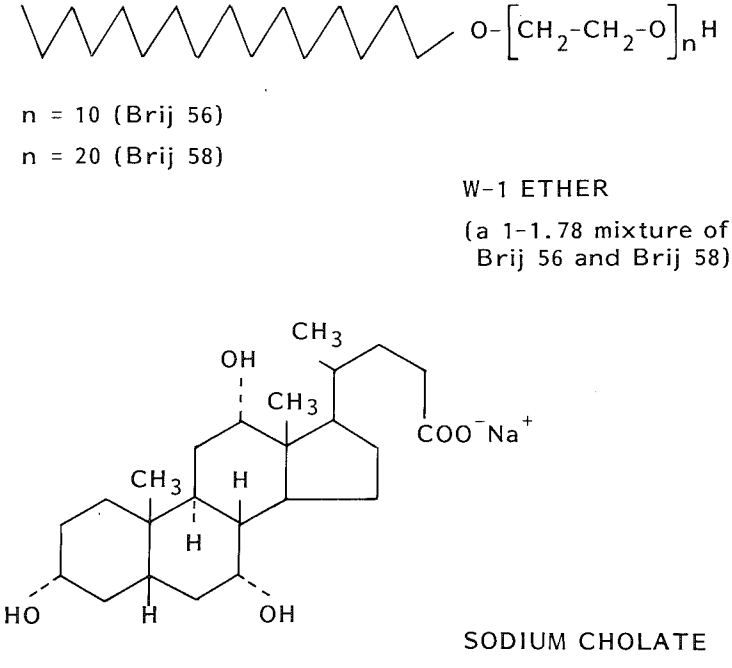

Fig. 2.1. Structures of W-1 ether and sodium cholate.

Low concentration of deoxycholate such as 0.05%, which is too low for solubilization of phospholipid of biological membranes but sufficient to induce reversible formation of membrane pores or openings in liver microsomes, releases protein from the lumen of microsomal vesicles (132). When 0.05% deoxycholate is applied to microsomes, no solubilization of iodothyronine deiodinase activity is observed (110,131). Treatment of microsomes with the chelating agent EDTA, 150 mM Tris buffer pH 8.0 or various salt (KCl) concentrations also

Table 2.3. IODOTHYRONINE DEIODINASE ACTIVITY AFTER SOLUBILIZATION OF MICROSOMES

origin of microsomes	activity investigated	detergent	recovery (%)	reference
rat liver	$T_4 \rightarrow T_3$	1% cholate	70	Köhrle et al, 1978 (129)
rat liver	$T_4 \rightarrow T_3$	0.125% DOC	41	Takaishi et al, 1979 (110)
rat liver	$T_4 \rightarrow T_3 + rT_3$, $rT_3 \rightarrow 3,3'\text{-}T_2$	1% cholate and 0.25% W-1 ether	50-60	Fekkes et al, 1980 (119)
rat kidney	$T_4 \rightarrow T_3$	0.2% DOC	60-70	Leonard and Rosenberg, 1980 (127)
rat liver	$T_4 \rightarrow T_3$	0.1% DOC	30	Imai et al, 1980 (103)
beef liver	$T_4 \rightarrow T_3 + rT_3$	0.33% DOC	78	Köhrle et al, 1980 (131)
rat kidney	$T_4 \rightarrow T_3$, $rT_3 \rightarrow 3,3'\text{-}T_2$	0.2% DOC	55-75	Leonard and Rosenberg, 1981, (130)

DOC = deoxycholate

does not solubilize any deiodinase activity (119,131). These findings strongly suggest that the enzyme is neither enclosed in the luminal cavity nor adsorbed to the outer side of the microsomal vesicles. It is therefore concluded that the iodothyronine deiodinating enzyme in both rat and beef liver microsomes is an integral (intrinsic) membrane protein. The same conclusion has been drawn for the renal enzyme (131).Deiodinases are accessible to the attack of proteolytic enzymes without making microsomal vesicles permeable by 0.05% deoxycholate (119,131).This is a further indication that the enzyme is a cytosolic orientated, membrane integrated protein of the endoplasmic reticulum.

Leonard and Rosenberg (131) showed an absolute requirement of the renal iodothyronine 5'-deiodinase for lipid. On the other hand, these investigators found that added phospholipid decreases deiodinating activity.This is in accordance with our findings on the liver enzyme (see Chapter 6). Therefore, phospholipids may activate the (delipidated) deiodinase via lipid reconstitution as well as lead to an apparent inhibition of the enzyme via sequestration of the substrate. Iodothyronine 5- and 5'-deiodinase activity, solubilized with cholate or deoxycholate from both rat and beef liver, were found to have an apparent molecular weight of 60-70 000 (119,131). This is in reasonably good agreement with the value (49 900) obtained for the enzyme in rat kidney (131). However, the deiodinating activities solubilized with W-1 ether, which is a

mixture of the nonionic detergents Brij 56 and 58 (see Fig. 2.1), were shown to have an apparent molecular weight of about 200 000 (119). Apparently, the calculated molecular weight depends on the type of detergent used for solubilization. The sedimentation coefficient for iodothyronine 5'-deiodinase activity of rat liver is 4.3 S (119) and for the renal enzyme 3.5 S (130). The Stokes' radii for the liver and kidney enzyme are also similar, viz. 35-37 Å (119) and 32 Å (130), respectively. Isoelectric focusing of the W-1 ether extract gives a major activity peak around pH 6.4 and a minor peak at pH 5.2 (see Chapter 6 and Fig. 2.2.).

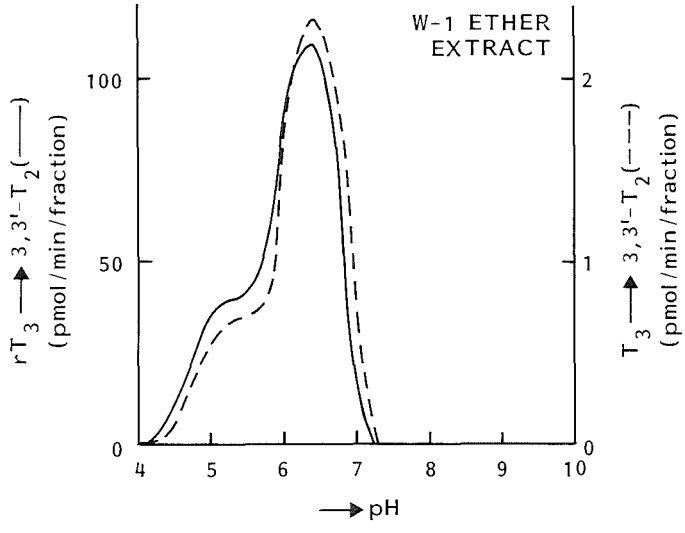

Fig. 2.2. Isoelectric focusing of the W-1 ether extract.

Ion exchange chromatography of solubilized liver deiodinase activity yields several activity peaks without remarkable increase in specific activity (119, 131). Affinity chromatography with T_4-substituted matrices also proofs useless in the purification of enzyme activity (131, Chapter 6).Several other chromatography media have been tested, but none of these affords a substantial purification (Chapter 6).The deiodinase activity in the cholate extract is precipitated completely with 35% ammonium sulphate. Solubilization of the precipitate with W-1 ether yields a preparation (ammonium sulphate extract) with a ten times higher specific enzyme activity compared to the cholate extract. It was found that ammonium sulphate treatment nearly completely removes phospholipids from the deiodinase. This in turn results in increased apparent K_m and V_{max} values

for the enzyme. Moreover, the isoelectric point of iodothyronine deiodinase is shifted to a much higher value (pH 9.3) after this treatment (see Chapter 6 and compare Fig. 2.3. with Fig. 2.2.). The enzymes catalyzing the 5- and 5'-deiodination of the iodothyronines could not be resolved by sedimentation, column chromatography or isoelectric focusing and showed similar behaviour toward solubilization. Furthermore, the several purification steps employed may have caused partial inactivation of the deiodinase activity, which could be a reason for the very slight increase in the specific enzyme activities obtained (119,130,131, Chapter 6).

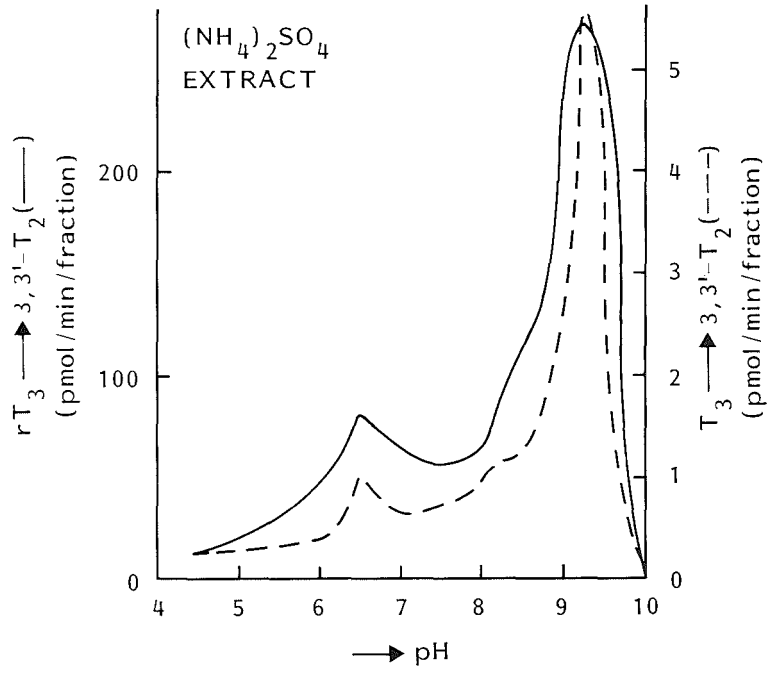

Fig. 2.3. Isoelectric focusing of the ammonium sulphate extract.

Colquhoun et al (133) reported on the partial purification of a soluble "T_4 5'-deiodinase" from rat kidney. These investigators used standard protein fractionation techniques combined with affinity chromatography using T_4 as the bound ligand, yielding a large, cold labile and soluble aggregate with a molecular weight of approximately 1-4 x 10^6. However, no evidence was presented that this preparation catalyzed T_3 formation, and I^- production via a non-enzymatic, degradative pathway seems more likely.

2.9. Concluding remarks

Our findings with rat liver microsomes and partially purified enzyme preparations lead to the conclusion that both 5- and 5'-deiodinations of iodothyronines are catalyzed by a single enzyme. Recently, the same conclusion was reached by another group of investigators (134). However, these findings have to be interpreted with caution, because both studies were performed with rat liver preparations. In rat brain (69,135) and pituitary (79,80) as well as in human (89) and rat placenta (136), really specific 5- and 5'-deiodinase activities have been shown. These latter findings suggest the existence of deiodinating enzymes in other tissues with properties being different from the deiodinase of rat liver.

Therefore, our results obtained with rat liver fractions leading to the conclusion that a single enzyme is responsible for the 5- as well as the 5'-deiodination of various iodothyronines, may not simply be extrapolated to the situation existing in other tissues or species.

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CHAPTER 3

Sequential Deiodination of Thyroxine in Rat Liver Homogenate

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Rat liver homogenate was incubated at 37°C with thyroxine, 3,3′,5-tri-iodothyronine, 3,3′,5′-tri-iodothyronine or 3,3′-di-iodothyronine. The degradation or accumulation of these compounds was measured by specific radioimmunoassays. (1) Production of 3,3′,5-tri-iodothyronine from thyroxine was highest at pH 6.0–6.5 and was markedly stimulated by the addition of dithiothreitol and effectively inhibited in the presence of 6-propyl-2-thiouracil. (2) Accumulation of 3,3′,5′-tri-iodothyronine on incubation of thyroxine with homogenate was only observed above pH 8.5. Otherwise the product was converted into 3,3′-di-iodothyronine too rapidly to allow its measurement. By measuring 3,3′-di-iodothyronine it was deduced that 5-deiodination of thyroxine was most effective at approx. pH 8.0. Dithiothreitol powerfully stimulated this reaction and 6-propyl-2-thiouracil strongly inhibited. (3) Monodeiodination of the tyrosine ring of 3,3′,5-tri-iodothyronine was the slowest reaction, was optimal at pH 8.0 and was less affected by dithiothreitol and 6-propyl-2-thiouracil than the above reactions. (4) 5′-Deiodination of 3,3′,5′-tri-iodothyronine was extremely rapid, with a pH optimum probably at about 6.5. Owing to the high reaction rate under the conditions used it was not possible to assess the effects of dithiothreitol and 6-propyl-2-thiouracil.

In recent years several findings have aroused new interest in the metabolism of thyroid hormones, especially the deiodinative pathways. It has become evident that under normal conditions the products of the thyroid, 3,3′,5,5′-tetraiodothyronine (thyroxine) and 3,3′,5-tri-iodothyronine (tri-iodothyronine), are secreted in a ratio that far exceeds the ratio of their turnover rates (Abrams & Larsen, 1973; Chopra & Solomon, 1973). This means that most of the circulating tri-iodothyronine is of extrathyroidal origin, which is in line with previous observations on plasma tri-iodothyronine concentrations in athyreotic subjects treated with thyroxine (Braverman *et al.*, 1970). Extrapolating from studies on thyroxine-supplemented individuals, Surks *et al.* (1973) calculated that in healthy humans with a sufficient iodine intake over 80% of the tri-iodothyronine turnover is derived from peripheral monodeiodination of thyroxine.

The rediscovery of 3,3′,5′-tri-iodothyronine (reverse tri-iodothyronine) by Chopra (1974) has also contributed considerably to our understanding of the mechanism of regulation of thyroid-hormone activity by the peripheral tissues. It is now generally considered that the metabolism of thyroxine, at least in man, proceeds primarily by means of deiodination (for a review see Cavalieri & Rapoport, 1977). Monodeiodination yields either the principal active form of thyroid hormone, tri-iodothyronine (by deiodination of the phenolic ring), or the inactive metabolite, reverse tri-iodothyronine (by elimination of an iodine atom from the tyrosine ring). Both products are probably subject to further degradation into lower substituted iodothyronines (Wu *et al.*, 1976; Rudolph *et al.*, 1977). It has been shown that plasma tri-iodothyronine and reverse tri-iodothyronine vary in a reciprocal fashion in several clinical situations: in starvation (Vagenakis *et al.*, 1975), systemic illness (Burger *et al.*, 1976), after surgical operation (Burr *et al.*, 1975) or on administration of the glucocorticosteroid analogue dexamethasone (Chopra *et al.*, 1975), of the β-adrenergic blocking agent propranolol (Verhoeven *et al.*, 1977) or of the goitrogenic compound 6-propyl-2-thiouracil (Westgren *et al.*, 1977). The time course of the changes during prolonged caloric restriction in the diet seems to indicate that monodeiodination of thyroxine involves separate processes (Visser *et al.*, 1978).

The development of specific radioimmunoassays for the measurement of iodothyronines provides us with valuable tools to conduct studies *in vitro* of the monodeiodinative pathways. We previously reported on the enzymic production of tri-iodothyronine from thyroxine in rat liver homogenate (Visser *et al.*, 1975*a*) and in the microsomal fraction (Visser *et al.*, 1976). We now demonstrate that conversion of thyroxine into reverse tri-iodothyronine also occurs in this system. This reaction, however, cannot be observed directly, owing to the very efficient

breakdown of reverse tri-iodothyronine into 3,3′-di-iodothyronine (di-iodothyronine). Similar findings have been reported by Hüfner & Grussendorf (1977). Consequently, monodeiodination of thyroxine to reverse tri-iodothyronine was determined by measuring the production of di-iodothyronine, since in this sequence the first reaction is the rate-limiting step and the contribution of the pathway via tri-iodothyronine was found to be negligible.

Experimental

Materials

L-Thyroxine, 3,3′,5-tri-iodo-L-thyronine and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 3,3′,5′-Tri-iodo-L-thyronine and 3,3′-di-iodo-L-thyronine were obtained by courtesy of Dr. E. Scheiffele, Henning G.m.b.H., Berlin, Germany, and 3-iodo-L-thyronine was kindly provided by Dr. P. Block, Jr., University of Toledo, Toledo, OH, U.S.A. [3′,5′-^{125}I]Thyroxine (specific radioactivity over 200μCi/μg), tri-[3′-^{125}I]-iodothyronine (specific radioactivity over 1200μCi/μg) and Na^{125}I (approx. 14mCi/μg) were from The Radiochemical Centre, Amersham, Bucks., U.K. Reverse tri-[3′,5′-^{125}I]iodothyronine (approx. 2700μCi/μg) was prepared as previously described (Visser *et al.*, 1977) and di-[3′-^{125}I]iodothyronine (approx. 3300μCi/μg) was prepared similarly starting from 3-iodothyronine. Goat anti-(rabbit γ-globulin) serum was from Antibodies Inc., Davis, CA, U.S.A.

Preparation of liver homogenate and conversion studies

The postnuclear supernatant of rat (Wistar) liver homogenate was prepared in 0.25M-sucrose in 5mM-Tris/HCl, pH7.4, as previously described (Visser *et al.*, 1975*a*). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The iodothyronines were dissolved in 0.1M-NaOH and diluted in 0.25M-sucrose in 0.025M-Tris/HCl, pH7.4, and added in 200μl quantities for incubation with 1800μl of homogenate. In the experiments dealing with possible effects of dithiothreitol, EDTA and 6-propyl-2-thiouracil (propylthiouracil), 200μl samples of homogenate were added to mixtures of 100μl of iodothyronine solution and 700μl of 0.25M-sucrose in 0.025M-Tris/HCl, pH7.4, containing 2.8mM-dithiothreitol, 2.8mM-EDTA or 0.1mM-propylthiouracil. In the experiments in which the dependence of the several reactions on pH was studied, 100μl samples of homogenate were added to mixtures composed of 100μl of the iodothyronine solution and 800μl of 0.15M-sodium acetate, 0.15M-sodium phosphate or 0.15M-sodium borate buffer, containing 4mM-dithiothreitol, with a pH range of 4.0–10.0. Control experiments were conducted in which homogenate was replaced by 0.25M-sucrose in 5mM-Tris/HCl, pH7.4. This yielded values for recovery and spontaneous deiodination. All experiments were carried out in duplicate at 37°C. After various time intervals, 250μl portions of the reaction mixture were added to 750μl of 95% (v/v) ethanol kept at 0°C. The precipitates were spun down (1500*g*, 10min) and the iodothyronine concentrations were measured in duplicate directly in 100μl portions of the supernatant.

Measurement of thyroxine, tri-iodothyronine, reverse tri-iodothyronine and di-iodothyronine

The iodothyronines were determined by specific radioimmunoassays. Thyroxine (Visser *et al.*, 1975*b*), tri-iodothyronine (Visser *et al.*, 1975*a*) and reverse tri-iodothyronine (Visser *et al.*, 1977) were measured as previously reported. Di-iodothyronine was assayed by the method of T. J. Visser, L. M. Krieger-Quist, R. Docter & G. Hennemann (unpublished work) as briefly described in the following. Di-[^{125}I]iodothyronine (about 12000c.p.m.) was made to react with appropriately diluted (1:200000) antiserum in the absence or presence of unlabelled di-iodothyronine or other materials to be tested (final volume 1ml). All reagents were dissolved in 0.06M-sodium barbitone/0.15M-NaCl containing bovine serum albumin, pH8.6. After incubation for 2–4 days at 8°C antibody-bound di-[^{125}I]iodothyronine was precipitated by addition of goat anti-(rabbit immunoglobulin G) antiserum. The lower limit of detection is 1pg of di-iodothyronine per assay tube. The relative affinity of thyroxine for the antiserum is less than 0.004 (di-iodothyronine = 100), that of tri-iodothyronine 0.6 and that of reverse tri-iodothyronine 0.06.

The composition of the assay mixtures is shown in Table 1.

Results

Fig. 1 shows the accumulation of the various iodothyronines when rat liver homogenate was incubated with 0.15μM-thyroxine, 0.14μM-tri-iodothyronine or 0.013μM-reverse tri-iodothyronine. Production of tri-iodothyronine and di-iodothyronine from thyroxine increased with time. Di-iodothyronine production from tri-iodothyronine appeared to slow down after 20min. In contrast, formation of reverse tri-iodothyronine from thyroxine was not measurable, owing to the very high conversion rate of reverse tri-iodothyronine into di-iodothyronine. Within 3min of incubation all reverse tri-iodothyronine added was converted into di-iodothyronine.

Table 1. *Composition of radioimmunoassay mixtures*
Standards were made in 0.06M-sodium barbitone/0.15M-NaCl, pH 8.6, containing 1% bovine serum albumin (buffer A). Antiserum was dissolved in the same buffer containing 0.1% bovine serum albumin (buffer B) and 2.5% normal rabbit serum. Extracts of reaction mixtures were made as described in the Experimental section. Extracts of homogenate for incorporation in the standard curve were prepared similarly.

	Volume for standard curve (μl)	Volume for samples (μl)
Standard	100	—
Extract of reaction mixture	—	100
Extract of homogenate	100	—
Buffer A	—	100
[^{125}I]Iodothyronine in buffer A	100	100
Antiserum	100	100
Buffer B	600	600

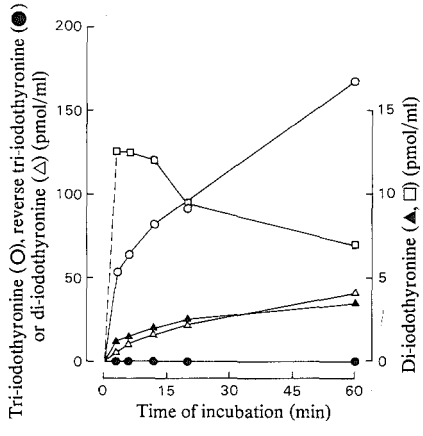


Fig. 1. *Deiodination of several iodothyronines in rat liver homogenate at pH 7.4 as function of time of incubation*
Accumulation of tri-iodothyronine (○), reverse tri-iodothyronine (●) and di-iodothyronine (△) from 1.15 μM-thyroxine, of di-iodothyronine from 0.14 μM-tri-iodothyronine (▲) and of di-iodothyronine from 0.013 μM-reverse tri-iodothyronine (□) was studied. The reaction mixtures contained, in addition to the iodothyronines, 2.7mM-dithiothreitol and 26.8mg of protein/ml.

The stability of the iodothyronines in rat liver homogenate is shown in Fig. 2. Thyroxine and tri-iodothyronine were stable under the conditions used. Di-iodothyronine was degraded more rapidly; during 60min of incubation its concentration decreased by over 50%. Reverse tri-iodothyronine was broken down very effectively in the homogenate;

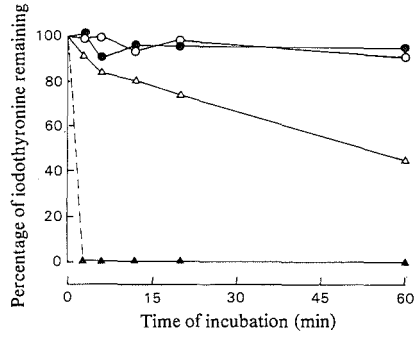


Fig. 2. *Fate of the several iodothyronines during incubation with rat liver homogenate at pH 7.4*
Rat liver homogenate was incubated with 1.15 μM-thyroxine (○), 0.14 μM-tri-iodothyronine (●), 0.013 μM-reverse tri-iodothyronine (▲) and 0.01 μM-di-iodothyronine (△). After several time intervals the concentration of iodothyronine remaining was determined and expressed in terms of the concentration added. For incubation conditions see the legend to Fig. 1.

after 3min all added reverse tri-iodothyronine had disappeared. Fig. 1 shows that this rapid disappearance reflects conversion into di-iodothyronine.

Experiments were conducted in which the effect of the addition of dithiothreitol, EDTA and propylthiouracil was studied (Table 2). The presence of 2.8mM-dithiothreitol resulted in approx. 8-fold enhancement of the deiodination of thyroxine into tri-iodothyronine and into di-iodothyronine after incubation for 60min. The enhancement of the conversion of tri-iodothyronine into di-iodothyronine and the degradation of added di-iodothyronine was approx. 2-fold. The effect of dithiothreitol was more pronounced after 60min than after 5min. Addition of EDTA further enhanced the conversion rates during incubation for 60min. Again, tri-iodothyronine was stable; even in the presence of dithiothreitol and EDTA, loss of tri-iodothyronine was less than 20%, as with thyroxine (results not shown). Reverse tri-iodothyronine was again broken down extremely rapidly. Even in the control experiment (without any addition) only 9% of added reverse tri-iodothyronine was left after incubation for 5min.

Propylthiouracil inhibited the conversion of thyroxine into tri-iodothyronine and di-iodothyronine markedly (80–90% inhibition). However, the inhibition of the conversion of tri-iodothyronine into di-iodothyronine was only approx. 10%. Propylthiouracil seemed to enhance the production of di-iodothyronine from reverse tri-iodothyronine.

Table 2. *Effect of addition of dithiothreitol, EDTA and propylthiouracil on the deiodination of the several iodothyronines in rat liver homogenate at pH* 7.4
Incubations were performed as outlined in the Experimental section. Liver protein concentration was 5.8 mg/ml.

	Iodothyronine measured (nM)					
	Tri-iodothyronine		Reverse tri-iodothyronine		Di-iodothyronine	
Additions Incubation time (min) ...	5	60	5	60	5	60
0.4 μM-Thyroxine (control)	3.3	6.4	0.0	0.0	1.1	2.0
Control+2.8 mM-dithiothreitol	6.3	57.8	0.0	0.0	2.0	14.3
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA	5.4	80.2	0.0	0.0	2.1	19.1
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA+0.1 mM-propylthiouracil	4.3	9.2	0.0	0.0	1.8	3.8
0.1 μM-Tri-iodothyronine (control)	100.0	99.9			0.1	0.8
Control+2.8 mM-dithiothreitol	100.0	89.6			0.1	1.6
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA	100.0	84.4			0.3	1.9
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA+0.1 mM-propylthiouracil	100.0	87.1			0.3	1.7
0.01 μM-Reverse tri-iodothyronine (control)			0.9	0.1	11.1	10.1
Control+2.8 mM-dithiothreitol			0.0	0.0	10.7	4.7
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA			0.0	0.0	8.7	3.4
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA+0.1 mM-propylthiouracil			0.0	0.0	10.1	7.9
0.01 μM-Di-iodothyronine (control)					8.6	6.2
Control+2.8 mM-dithiothreitol					9.0	3.0
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA					9.4	4.1
Control+2.8 mM-dithiothreitol+2.8 mM-EDTA+0.1 mM-propylthiouracil					9.5	5.1

This is probably due to the inhibitory effect of propylthiouracil on the degradation of di-iodothyronine, as shown in Table 2. All effects of propylthiouracil were, as with dithiothreitol, more pronounced during prolonged incubation.

Table 3. *Effect of addition of propylthiouracil on the deiodination of the various iodothyronines in rat liver homogenate at pH* 7.4 *in the absence and presence of dithiothreitol*

For details see legend to Table 2. Incubation time was 60 min; liver protein concentration was 6.4 mg/ml.

Reactions	Concn. of dithiothreitol (mM) ...	Inhibition by propylthiouracil (%) 0	2.8
Thyroxine → tri-iodothyronine		60–80	90
Thyroxine → di-iodothyronine		60–70	90
Tri-iodothyronine → di-iodothyronine		Not detectable	25
Reverse tri-iodothyronine → di-iodothyronine		20–40*	Not detectable
Di-iodothyronine → ?†		20–30	35

* Representing an approximate value, since initial reaction rates were not measured.

† The product of this reaction has not been identified.

Next, the effects of propylthiouracil on the various reactions were studied in the absence as well as in the presence of dithiothreitol (Table 3). Owing to the slow conversion rate of tri-iodothyronine into di-iodothyronine in the absence of dithiothreitol, the inhibition by propylthiouracil of this reaction was not detectable under these conditions. Since for the generation of di-iodothyronine from reverse tri-iodothyronine initial reaction rates were too high for determination, the influence of the addition of propylthiouracil could not properly be measured. Addition of dithiothreitol did not alter the inhibitory effect of propylthiouracil. In all instances the reaction rates in the presence of both dithiothreitol and propylthiouracil were higher than without any addition.

Fig. 3 shows the pH profile of the various deiodination reactions in rat liver homogenate. The conversion of thyroxine into tri-iodothyronine was optimal at pH 6.0–6.5, which has also been found by other investigators (Hüfner *et al.*, 1977; Höffken *et al.*, 1977). Production of di-iodothyronine from thyroxine or from tri-iodothyronine was optimal at pH 8.0. In all circumstances less than 20% of added thyroxine and tri-iodothyronine was degraded after incubation for 60 min (results not shown).

Since the pH-dependence of the conversion of reverse tri-iodothyronine into di-iodothyronine and

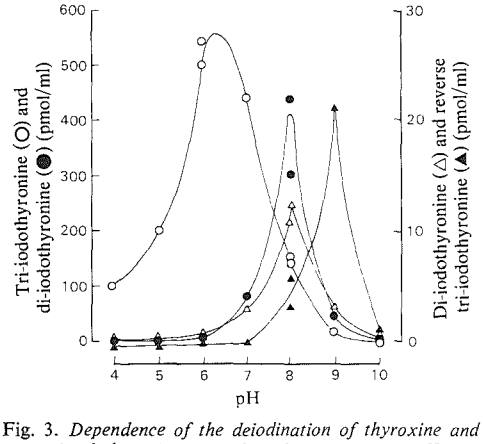


Fig. 3. *Dependence of the deiodination of thyroxine and tri-iodothyronine in rat liver homogenate on pH*

The incubation mixture contained 3.2 mM-dithiothreitol and 3.6 mg of protein/ml. Incubation time was 60 min. Accumulation of tri-iodothyronine (○), di-iodothyronine (●) and reverse tri-iodothyronine (▲) from 1.0 μM-thyroxine and of di-iodothyronine (△) from 0.1 μM-tri-iodothyronine was studied. The buffers used were acetate (pH 4–6), phosphate (6–8) and borate (8–10). For details see the Experimental section.

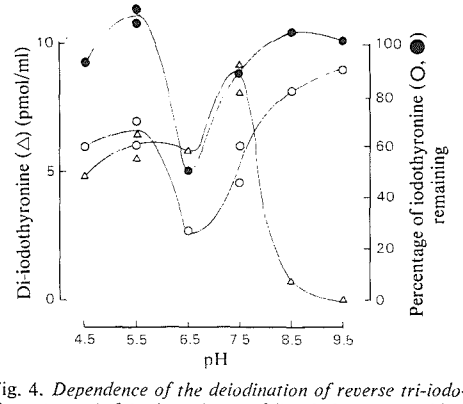


Fig. 4. *Dependence of the deiodination of reverse tri-iodothyronine and of the degradation of di-iodothyronine in dilute rat liver homogenate on pH*

Incubation mixtures contained 0.015 μM-reverse tri-iodothyronine or 0.013 μM-di-iodothyronine and 0.025 mg of homogenate protein/ml. The concentration of dithiothreitol was 3.2 μM. After incubation for 10 min the production of di-iodothyronine (△) and the percentage of added reverse tri-iodothyronine (○) or di-iodothyronine (●) remaining were determined. The buffers used were acetate (pH 4.5–5.5), phosphate (5.5–7.5) and borate (7.5–9.5). For details see the Experimental section.

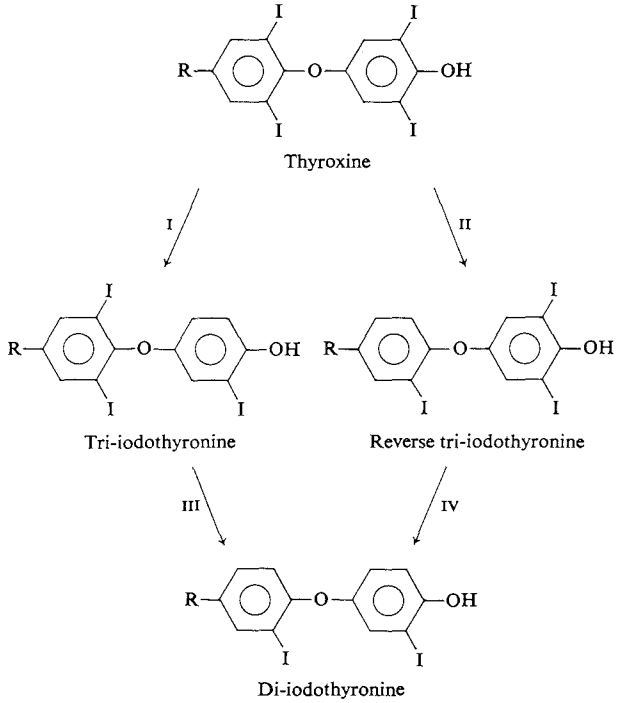
of the degradation of reverse tri-iodothyronine was difficult to interpret, these reactions and the breakdown of di-iodothyronine were investigated by using dilute homogenate (Fig. 4). Both reverse tri-iodothyronine and di-iodothyronine were most rapidly degraded at pH6.5. The pH optimum of the conversion of reverse tri-iodothyronine into di-iodothyronine could not easily be determined, because of the lability of the latter around pH6.5. It was also noted that the extent of degradation of di-iodothyronine was not linearly dependent on the concentration of the homogenate in the reaction mixture (results not shown). This is probably the result of two counteracting processes; dilution of homogenate decreases not only the concentration of enzyme but also that of binding sites in the medium (Visser *et al.*, 1975*a*). Dilution of the homogenate therefore increases the availability of the substrate for interaction with the enzyme, the concentration of which has decreased concomitantly.

Discussion

The reactions in Scheme 1 were studied: reactions I and IV represent deiodination of the phenolic (or outer) ring, whereas in reactions II and III an iodine atom is removed from the tyrosine (or inner) ring.

Under the conditions used reaction II could not be investigated directly, owing to the extreme lability of the product (reverse tri-iodothyronine). The rapid disappearance of the latter was virtually completely accounted for by reaction IV, since addition of reverse tri-iodothyronine to the homogenate resulted in a quantitative yield of di-iodothyronine. However, in the cascade thyroxine $\xrightarrow{II}$ reverse tri-iodothyronine $\xrightarrow{IV}$ di-iodothyronine, at least at pH7.4, reaction II is apparently the rate-limiting step, and conversion of thyroxine into reverse tri-iodothyronine can be estimated by measuring the production of di-iodothyronine. By comparison the production of di-iodothyronine via the pathway thyroxine $\xrightarrow{I}$ tri-iodothyronine $\xrightarrow{III}$ di-iodothyronine is negligible under all conditions tested.

Reaction IV is slowed down by increasing the pH to 9.0, thereby permitting the accumulation of reverse tri-iodothyronine (Fig. 3). Thus, since reaction IV is much faster than reaction II, except above pH9.0, the pH optimum of the production of di-iodothyron-



Scheme 1. *Sequential deiodination of thyroxine as observed in rat liver homogenate*
R = CH_2–$CH(NH_2)$–CO_2H

ine actually reflects optimal conditions for the conversion of thyroxine into reverse tri-iodothyronine. This can also be deduced from the accumulation of the latter as a function of pH and the stability of this compound under these conditions (results not shown).

The pH optimum for reaction IV is probably about 6.5. This is indicated by the rate of accumulation of di-iodothyronine, taking into account the data on the stability of di-iodothyronine in the homogenate, which is least at pH 6.5 (Fig. 4). This is further substantiated by the finding that reverse tri-iodothyronine is most rapidly degraded in the homogenate at pH 6.5 (Fig. 4) and, since this compound is quantitatively converted into di-iodothyronine, this must point to a maximal rate of reaction IV at approx. pH 6.5. Thus it can be concluded that conditions for deiodination of the phenolic ring (reactions I and IV) are optimal at pH 6.0–6.5 and for deiodination of the tyrosine ring (reactions II and III) at pH 8.0. These findings are hard to interpret, since in the homogenate optimal conditions for the reactions will depend not only on the nature of the enzymic reaction but also on the availability of the substrates, since pH may be of importance in the binding of iodothyronines to cytoplasmic proteins (Höffken *et al.*, 1977). Nevertheless, conversion of thyroxine into tri-iodothyronine by rat liver microsomal fraction was also found to be maximal at pH 6.0 (Höffken *et al.*, 1977; T. J. Visser, unpublished work).

In line with our previous observations on the conversion of thyroxine into tri-iodothyronine by the microsomal fraction of rat liver homogenate (Visser *et al.*, 1976), the monodeiodination reactions studied are stimulated by the addition of dithiothreitol. This raises the question whether the enhanced reaction rates observed in the presence of thiol-group-containing compounds is due to their protection of cysteine residues of the enzymes or because these compounds act as cofactors or a combination of both effects. As discussed by Visser *et al.* (1976), if the sequential deiodination is accompanied by the formation of I^-, reactions I–IV can be regarded as reductions. In that case the reducing equivalents may be supplied by thiol-containing-compounds (RSH), such as dithiothreitol. The overall reaction may then be described by eqns. (1) and (2):

$$\text{Thyroxine} + 2\text{RSH} \rightarrow \text{tri-iodothyronine or reverse tri-iodothyronine} + \text{HI} + \text{RSSR} \quad (1)$$

$$\text{Tri-iodothyronine or reverse tri-iodothyronine} + 2\text{RSH} \rightarrow \text{di-iodothyronine} + \text{HI} + \text{RSSR} \quad (2)$$

In this respect it is noteworthy that glutathione is almost as effective as dithiothreitol in supporting the conversion of thyroxine into tri-iodothyronine by rat liver microsomal fraction (T. J. Visser, unpublished work). It may therefore be postulated that glutathione is the endogenous cofactor of the monodeiodination reactions.

All reactions are stimulated in the presence of EDTA, which has also been shown to enhance the production of tri-iodothyronine in the microsomal fraction (Visser *et al.*, 1976).

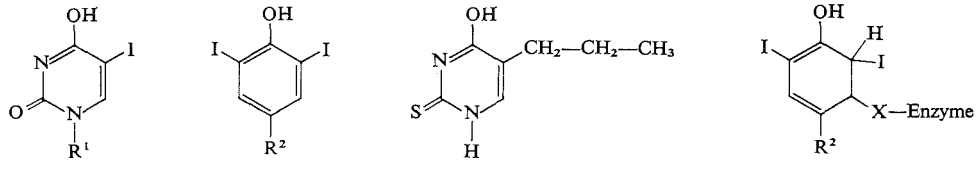
Administration of propylthiouracil to humans has been shown to result in increased reverse tri-iodothyronine and decreased tri-iodothyronine concentrations in plasma (Westgren *et al.*, 1977). This is in accordance with previous findings that administration of propylthiouracil to rats (Oppenheimer *et al.*, 1972), as well as to humans (Saberi *et al.*, 1975; Geffner *et al.*, 1975), blocks the extrathyroidal production of tri-iodothyronine. Our data indicate that propylthiouracil decreases markedly the production from thyroxine of both tri-iodothyronine and reverse tri-iodothyronine (as indicated for the latter by the fall in the generation of di-iodothyronine).

Reaction III was much less affected by propylthiouracil, and its effect on reaction IV is impossible to assess, since initial reaction rates were not observed under the conditions used. Addition of propylthiouracil affected the reactions to a similar extent in both the absence and presence of dithiothreitol. This finding excludes the possibility that propylthiouracil interferes with the interaction of the cofactor with the enzyme or enzyme–substrate complex. It is, however, to be noted that the reaction that is least stimulated by dithiothreitol, i.e. production of di-iodothyronine from tri-iodothyronine, is also least inhibited by propylthiouracil.

Data bearing on the mechanism of deiodination of the several iodothyronines are scarce.

Deiodination of iodotyrosines appears to involve an NADPH-dependent enzymic reaction (Rosenberg & Ahn, 1969). It has also been suggested that thyroxine is deiodinated by tyrosine hydroxylase (Dratman *et al.*, 1976). On the contrary, Hüfner *et al.* (1977) showed that conversion of thyroxine into tri-iodothyronine by rat liver homogenate is not influenced by α-methyltyrosine, a specific inhibitor of tyrosine hydroxylase.

For several reasons comparison of the data reported here with the mechanism of the enzymic and chemical dehalogenation of 5-bromo- and 5-iodo-2′-deoxyuridylate (Wataya & Santi, 1975, 1977) is of interest. First, the structure of 5-iodo-2′-deoxyuridylate (1) resembles not only that of iodothyronines, e.g. thyroxine (2), but also that of 5-propyl-2-thiouracil (3), a strong inhibitor of the conversion of thyroxine into tri-iodothyronine (Visser *et al.*, 1975*a*). Secondly, dehalogenation of 5-bromo- and 5-iodo-2′-deoxyuridylate as catalysed by thymidylate synthetase requires thiol-group-containing compounds, such as dithiothreitol (Wataya & Santi, 1975). In analogy with the



(1) (2) (3) (4)

mechanism of that reaction, we suggest that for example in the 5′-deiodination of thyroxine the primary event is an attack of a nucleophile (X) of the enzyme, e.g. a thiol group, at the 2′- or the equivalent 6′-position of thyroxine (2) yielding the 2′,3′-dihydro derivative (4). The removal of the iodine atom may then take place via different routes, in each of which the thiol group of the cofactor is oxidized (Wataya & Santi, 1975).

Two more observations appear to fit remarkably well with this model.

(1) The pH optima of the conversion of thyroxine into tri-iodothyronine and of the production of di-iodothyronine from reverse tri-iodothyronine were shown to be somewhat below the p*K* of the phenolic hydroxyl group, which is approx. 6.6 (Handwerger *et al.*, 1975). Dissociation of the phenol would render the primary attack of a nucleophile an unlikely event.

(2) Deiodination of the phenolic ring is much more rapid when reverse tri-iodothyronine is the substrate than with thyroxine. This may indicate that substitution of both the 3- and 5-positions hinders the approach of the nucleophile to position 2′. It remains, however, to be investigated whether both reactions I and IV are catalysed by the same enzyme. The resemblance of 5-propyl-2-thiouracil to the substrates of the deiodination reactions suggests that this compound may act as a competitive inhibitor. The resemblance to 6-propyl-2-thiouracil is, however, less apparent. Nevertheless this compound is also an effective inhibitor of the several monodeiodination reactions in the homogenate.

We have found that iodothyronines are not deiodinated by thymidylate synthetase of bacterial origin (amethopterin-resistant strain of *Lactobacillus casei*) (T. J. Visser, unpublished work). We suggest, even so, that removal of iodine atoms from iodothyronines involves a mechanism that is similar to that described for the deiodination of 5-iodo-2′-deoxyuridylate by thymidylate synthetase (Wataya & Santi, 1975) and is mediated by enzymes with similar specificities. This can only be tested when the reactions are investigated with purified enzyme preparations. Such studies will also reveal whether reactions I and IV and reactions II and III are catalysed by the same enzymes.

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CHAPTER 4

Biochimica et Biophysica Acta, 587 (1979) 12—19

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LOCATION OF RAT LIVER IODOTHYRONINE DEIODINATING ENZYMES IN THE ENDOPLASMIC RETICULUM

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Summary

The iodothyronine-deiodinating enzymes (iodothyronine-5- and 5′-deiodinase) of rat liver were found to be located in the parenchymal cells. Differential centrifugation of rat liver homogenate revealed that the deiodinases resided mainly in the microsomal fraction. The subcellular distribution pattern of these enzymes correlated best with glucose-6-phosphatase, a marker enzyme of the endoplasmic reticulum. Plasma membranes, prepared by discontinuous sucrose gradient centrifugation, were found to contain very little deiodinating activity. Analysis of fractions obtained during the course of plasma membrane isolation showed that the deiodinases correlated positively with glucose-6-phosphatase ($r \geqslant 0.98$) and negatively with the plasma membrane marker 5′-nucleotidase (r ranging between —0.88 and —0.97). It is concluded that the iodothyronine-deiodinating enzymes of rat liver are associated with the endoplasmic reticulum.

Introduction

It is now generally accepted that peripheral monodeiodination of thyroxine is the major source of circulating 3,3′,5-triiodothyronine and 3,3′,5′-triiodo-

thyronine in man [1]. In the rat this has been demonstrated only for 3,3′,5-triiodothyronine [2]. However, in vitro conversion of thyroxine into 3,3′5-triiodothyronine and 3,3′,5′-triiodothyronine has been detected in several extrathyroidal tissues of animals, especially in rat liver [3—7]. Several recent studies have demonstrated that monodeiodination of thyroxine into 3,3′,5-triiodothyronine is mainly located in the microsomal fraction of rat liver cells [3,8] and Cavalieri et al. [9] found production of 3,3′,5′-triiodothyronine from thyroxine in the cytoplasm of rat liver homogenate. Leonard and Rosenberg [10] suggested that the enzymatic activity mediating the formation of 3,3′,5-triiodothyronine from thyroxine in rat kidney is associated with the plasma membrane. Up to now no studies have been reported concerning the intracellular location of enzymes converting 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine into 3,3′-diiodothyronine, reactions known to occur in rat liver, too [7,11—14]. In this paper we present data on subcellular localization of iodothyronine-deiodinating enzymes (iodothyroinine-5- and 5′-deiodinase). It was found that these enzymes are located mainly in the endoplasmic reticulum of rat liver parenchymal cells.

Materials and Methods

Materials. L-thyroxine, L-3,3′5-triiodothyronine and dithiothreitol were from Sigma. L-3,3′,5′-triiodothyronine and 3,3′-L-diiodothyronine were obtained by courtesy of Dr. E. Scheiffele, Henning GmbH, Berlin.

Isolation of rat parenchymal and non-parenchymal liver cells. Parenchymal cells were isolated from rat liver essentially according to the method of Berry and Friend [15], using collagenase (0.05%) and Ca^{2+} (2 mM) in Hank's solution. The cells obtained, of which at least 90% excluded trypan blue, were not contaminated with non-parenchymal cells or cell fragments as judged by light microscopy. The non-parenchymal cells (mainly Kupffer cells) were isolated by the second isolation method of Van Berkel et al. [16]. These cells were not contaminated with parenchymal cells as judged by light microscopy. For conversion studies the freshly prepared liver cells were homogenized in Hank's solution using a Potter-Elvehjem homogenizer with a Teflon pestle.

Preparation of rat liver homogenate and subcellular fractions. Male Wistar rats were used after a 24 h period of fasting. The liver was perfused in situ through the portal vein (omission of perfursion resulted in decreased enzyme activities), minced, washed and homogenized (Potter-Elvehjem) in 4 vols. of medium A, consisting of 250 mM sucrose, 3 mM dithiothreitol, 10 mM Tris, pH 7.4. The homogenate was filtered through a fine-mesh nylon sieve and partitioned into subcellular fractions essentially by the method of De Duve et al. [17]. All steps were carried out at 0—5° C. Four particulate fractions and a final supernatant were isolated from the homogenate by subsequent centrifugations for 10 min at $700 \times g_{max}$ (nuclear fraction, N), 10 min at $4460 \times g_{max}$ (mitochondrial fraction, M), 10 min at $25\,200 \times g_{max}$ (lysosomal fraction, L) and 60 min at $110\,000 \times g_{max}$ (microsomal fraction, P and supernatant, S). The N, M and L pellets were washed twice. All pellets were suspended in medium B, consisting

of 10 mM Tris, 3 mM dithiothreitol and 3 mM EDTA, pH 7.4. The purity of the fractions was evaluated by analysis of several marker enzymes. Acid phosphatase (EC 3.1.3.2), glucose-6-phosphatase (EC 3.1.3.9), glutamate dehydrogenase (EC 1.4.1.2), 5′-nucleotidase (EC 3.1.3.5) and lactate dehydrogenase (EC 1.1.1.27) were assayed as described (Refs. 17, 17, 18, 19 and 20, respectively) with minor modifications. DNA [21] and protein [22] were determined using calf thymus DNA and bovine serum albumin as standards, respectively.

Membrane preparations. Liver cell plasma membranes were prepared from a nuclear fraction (the pellet obtained after centrifugation of rat liver homogenate for 10 min at $1500 \times g_{max}$) and from the microsomal fraction. The former membranes were isolated using the Neville procedure [23]. The last steps in this procedure (steps 13—15) were omitted, because these gave no further purification of the membranes. Hardening of the nuclear membrane was achieved by adding 0.5 mM $CaCl_2$ to the bicarbonate medium [24]. The membranes from the microsomal fraction were isolated by the method of Touster et al. [25].

Conversion studies and measurement of thyroxine, 3,3′,5-triiodothyronine, 3,3′,5′-triiodothyronine and 3,3′-diiodothyronine. To a mixture of 0.8 ml of 0.15 M sodium phosphate buffer (pH 6.5, 7.0 or 8.0), containing 4 mM dithiothreitol and 0.1 ml of tissue preparation, 0.1 ml of 10 μM thyroxine, 5 μM 3,3′,5-triiodothyronine or 0.1 μM 3,3′,5′-triiodothyronine was added. Production of 3,3′,5-triiodothyronine from thyroxine and of 3,3′-diiodothyronine from 3,3′,5′-triiodothyronine was analysed at pH 6.5 and production of 3,3′,5′-triiodothyronine and 3,3′-diiodothyronine from thyroxine and of 3,3′-triiodothyronine from 3,3′,5-triiodothyronine at pH 8.0 [7] unless stated otherwise. For the determination of the conversion of thyroxine into 3,3′,5′-triiodothyronine, the amount of 3,3′,5′-triiodothyronine degraded into 3,3′-diiodothyronine was taken into account [7]. After 10 min of incubation at 37°C 0.2 ml portions of the reaction mixture were added to 0.6 ml of 85% (v/v) ice-cold ethanol. Processing of the extracts and measurement of iodothyronines were done as previously described [7]. Recovery estimated with the aid of ^{125}I-labelled iodothyronines varied between 92.5 and 99.8%. Under the conditions tested less than 10% of added thyroxine, 3,3′,5-triiodothyronine and 3,3′-diiodothyronine was degraded. Where tested degradation of 3,3′,5′-triiodothyronine was found to be equal to 3,3′-diiodothyronine formation.

Results

Iodothyronine-deiodinating activity in homogenates of parenchymal and non-parenchymal cells

Table I shows the thyroxine, 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine-deiodinating activities in homogenates of rat parenchymal and non-parenchymal liver cells. The specific activities of all deiodinases in non-parenchymal homogenates were less than 6% of those in the parenchymal cell homogenates.

TABLE I

DEIODINATION OF SEVERAL IODOTHYRONINES IN HOMOGENATES OF RAT PARENCHYMAL AND NON-PARENCHYMAL LIVER CELLS

The reaction mixtures contained 0.14—0.41 mg of liver cell protein/ml. All experiments were done in duplicate.

Reaction	Conversion rate (pmol/mg protein per min) in:	
	Parenchymal cells	Non-parenchymal cells
thyroxine → 3,3′,5-triiodothyronine	2.42	<0.14 *
thyroxine → 3,3′,5′-triiodothyronine	1.72	0.13
3,3′,5-triiodothyronine → 3,3′-diiodothyronine	1.23	0.07
3,3′,5′-triiodothyronine → 3,3′-diiodothyronine	8.48 **	<0.01 *

* Below the limits of detection.

** Representing a minimal value due to exhaustion of the substrate.

Subcellular distribution of iodothyronine-deiodinating enzymes in rat liver cells

The subcellular distribution of the several deiodinases is shown in Fig. 1 and is compared to that of glucose-6-phosphatase and 5′-nucleotidase. All other marker enzymes and DNA also showed a characteristic distribution pattern (not shown here). Most of the deiodinating activity was recovered in the microsomal fraction. Less than 10% was found in the nuclear fraction. There is a striking resemblance between the distribution of the deiodinases and that of glucose-6-phosphatase. 5′-Nucleotidase activity was distributed somewhat differently. This is more obvious if the ratios of the specific activities in the microsomal and nuclear fraction are considered (Table II). Table II illustrates that the microsomal fraction is more enriched with the deiodinases (6—7-fold) and glucose-6-phosphatase (four-fold) than with 5′-nucleotidase (1.7-fold), whereas compared to whole homogenate, the nuclear fraction contains less deiodinases and glucose-6-phosphatase than 5′-nucleotidase.

TABLE II

RATIOS OF THE SPECIFIC ENZYME ACTIVITIES IN WHOLE HOMOGENATE (H), NUCLEAR (N) AND MICROSOMAL (P) FRACTIONS

Results shown are the mean ± S.E. of four separate experiments.

Enzyme of enzymatic reaction	Ratio		
	N/H	P/H	P/N
glucose-6-phosphatase	0.42 ± 0.03	3.88 ± 0.10	9.62 ± 0.54
5′-nucleotidase	0.80 ± 0.04	1.71 ± 0.13	2.22 ± 0.36
thyroxine → 3,3′,5-triiodothyronine	0.50 ± 0.02	6.37 ± 0.14	12.76 ± 0.62
thyroxine → 3,3′,5′-triiodothyronine	0.47 ± 0.04	7.02 ± 0.49	15.33 ± 1.64
3,3′,5-triiodothyronine → 3,3′-diiodothyronine	0.65 ± 0.04	7.08 ± 0.54	10.75 ± 1.38
3,3′,5′-triiodothyronine → 3,3′-diiodothyronine	0.48 ± 0.03	5.68 ± 0.22	12.00 ± 0.77

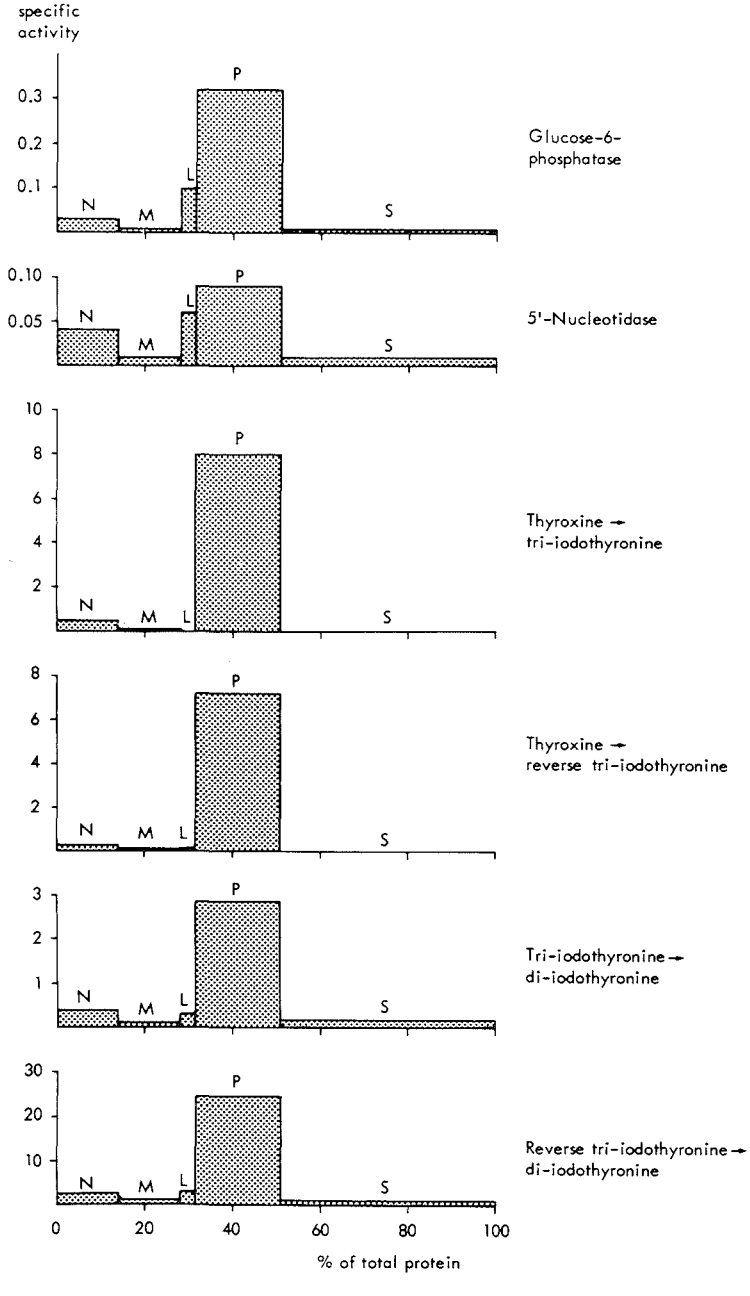

Fig. 1. Subcellular distribution pattern of iodothyronine-deiodinating enzymes and marker enzymes. The specific activities of the deiodinases are defined as pmol of deiodinated product formed/mg of protein per min and of glucose-6-phosphatase and 5'-nucleotidase as μmol of inorganic phosphate formed/mg of protein per min. For details see Materials and Methods.

Activity of deiodinases in the plasma membrane preparations

Several fractions, more or less enriched in the plasma membrane marker 5′-nucleotidase were isolated from the nuclear fraction. Among these were the floating layer and a dense layer, obtained according to Neville [23]. In Table III the specific activities of glucose-6-phosphatase, 5′-nucleotidase and some deiodinases of these three fractions are shown. From the microsomal fraction (P) we obtained a fraction (P2), which was enriched in plasma membranes and two fractions (P5 and P6) enriched in endoplasmic reticulum, according to Touster et al. [25]. The specific activities of glucose-6-phosphatase, 5′-nucleotidase and the deiodinases of these fractions and of P are also shown in Table III. From these data it is obvious that the deiodinases correlate positively with glucose-6-phosphatase ($r = 0.98$—0.99). On the other, a negative correlation between the deiodinases and 5′-nucleotidase is shown, with r ranging between -0.88 and -0.97.

TABLE III

COMPARISON OF SPECIFIC ACTIVITIES OF GLUCOSE-6-PHOSPHATASE AND 5′-NUCLEOTIDASE WITH DEIODINASE ACTIVITIES

For definition of specific activities see Fig. 1. Conversion studies were done at pH 7.0. The concentrations of thyroxine and 3,3′,5′-triiodothyronine were 5.0 and 0.5 μM, respectively. Further conditions are described in Materials and Methods.

Fraction	glucose-6-phosphatase	5′-nucleotidase	Converting activity		
			thyroxine → 3,3′,5-triiodothyronine	thyroxine → 3,3′,5′-triiodothyronine	3,3′5′-triiodothyronine → 3.3′-diiodothyronine
Nuclear fraction	0.167	0.133	2.84	1.70	7.6
Floating layer	0.090	0.680	2.28	1.14	5.0
Dense layer	0.246	0.034	3.62	2.52	9.6
P	0.387	0.085	8.38	4.66	53.8
P2	0.180	0.344	2.12	2.52	23.2
P5	0.507	0.070	13.88	5.78	76.2
P6	0.611	0.007	16.96	6.02	82.0

Discussion

Our results demonstrate in which rat liver cells the thyroxine-deiodinating enzymes are located and their subcellular location. It was found that the specific activities of the deiodinases in non-parenchymal cells are less than 6% of those in parenchymal cells. However, if we consider the whole rat liver, consisting of about 90% of parenchymal cell protein and 10% of non-parenchymal cell protein [26], it can be concluded that the parenchymal cells are responsi-

ble for at least 99% of the deiodination of thyroxine into 3,3′-diiodothyronine via 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine.

The subcellular fractionation studies revealed that both thyroxine-monodeiodinating enzymes and the enzymes converting 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine into 3,3′-diiodothyronine are mainly located in the microsomal fraction of rat liver homogenate. These results are in conformity with those of Hesch et al. [3] and Visser et al. [8], who have shown thyroxine-5′-deiodinase to be located in the same fraction. However, Maciel et al. [27] reported about integration of the enzyme in the plasma membrane and Leonard and Rosenberg [10] also showed a plasma membrane location in rat kidney. On the other hand Cavalieri et al. [9] found the enzyme converting thyroxine into 3,3′,5′-triiodothyronine in the soluble fraction of rat liver homogenate. These results are based on very smal conversion rates, being of the same order of magnitude we found in cytosol. Conversion rates in microsomes are at least 50 times higher than in cytosol (Fig. 1).

Because of cofractionation of 5′-nucleotidase with the deiodinases in the microsomal fraction, we tried to separate endoplasmic reticulum (glucose-6-phosphatase activity) from plasma membranes (5′-nucleotidase activity) by means of discontinuous sucrose gradient centrifugation. We obtained relatively pure plasma membranes and fractions enriched in glucose-6-phosphatase activity. From the data in Tables II and III it is obvious that the above-mentioned deiodinases are all associated with the endoplasmic reticulum of rat liver cells. These observations are in contrast with those of Maciel et al. [27], who found a plasma membrane location of thyroxine- and 3,3′,5′-triiodothyronine-5′-deiodinase activities.

The specific activities of the deiodinating enzymes mentioned in this paper have to be interpreted with caution, because these values are, among others, dependent on the concentration of substrate. Ideally, for estimation of specific activities saturating concentrations of substrate should have been used. Because of the very high concentrations of thyroxine and 3,3′,5-triiodothyronine needed and the insolubility of these compounds under these conditions [28], this was not possible. Also under these circumstances, due to cross-reaction of substrate in the assay of the products, estimation of the conversion rate is less accurate. However, except for the 5′-deiodination of 3,3′,5′-triiodothyronine in Table I, it was assessed that conversion rates were proportional to the protein concentration in the incubation mixture. Therefore, although not representing maximal velocities, the reported figures on enzyme activities in the several preparations may be interpreted as specific activities.

Subcellular fractionation does not result in separation of the deiodinating enzymes. However, it has also been found that thyroxine → 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine → 3,3′-diiodothyronine converting activities are more sensitive to omission of dithiothreitol during preparation of rat liver microsomes than the thyroxine → 3,3′,5′-triiodothyronine and 3,3′,5-triiodothyronine → 3,3′-diiodothyronine converting activities (not detailed here). These findings are compatible with the concept that the sequential deiodina-

tion of thyroxine is mediated by two enzymes, viz. iodothyronine-5-deiodinase catalyzing conversion of thyroxine into 3,3′,5′-triiodothyronine and 3,3′,5-triiodothyronine into 3,3′-diiodothyronine and iodothyronine-5′-deiodinase catalyzing conversion of thyroxine into 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine into 3,3′-diiodothyronine [29]. However, confirmation of this hypothesis must await the final purification of these enzymes.

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CHAPTER 5

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SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF RAT LIVER IODOTHYRONINE DEIODINASES

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Summary

Rat liver cells contain iodothyronine deiodinating enzymes (iodothyronine-5 and 5′-deiodinases), which are associated with the endoplasmic reticulum. In the present study, the iodothyronine deiodinases have been solubilized from the microsomal fraction of rat liver with 1.0% cholate and 0.25% of the polyoxyethylene ether W-1. Cholate can be effectively removed from the cholate extract with a mixture of the polystyrene beads XAD-2 and XAD-7. However, after some time, aggregation of proteins occurred.

Cholate solubilized iodothyronine-5′-deiodinase has an apparent molecular weight of 65 000 and a Stokes radius of 36—37 Å. The sedimentation coefficient is 4.3 S in 0.4—0.6% cholate, 7.6 S in 0.05% W-1 ether and 12.8 S in the absence of detergent. The enzyme solubilized with W-1 ether has an apparent molecular weight of approx. 200 000 and a Stokes radius of 52—56 Å in 0.025% W-1 ether. In the latter extract, the sedimentation coefficient of the deiodinase is 4.3—5.2 S under different conditions.

On DEAE-Sepharose chromatography, 70% of the bound deiodinases eluted with 0.1 M NaCl. The purification of this fraction was only 2-fold. Covalent chromatography, using activated thiol-Sepharose, resulted in approximately 3-fold purification of the deiodinases solubilized with W-1 ether, whereas in case of the cholate extract, no purification at all was obtained. Glutathione-Sepharose affinity chromatography resulted in no enrichment of the deiodinases.

Introduction

The parenchymal cell of rat liver can deiodinate thyroxine to 3,3′-diiodothyronine via 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine [1,2]. This deiodination pathway is composed of four reactions, which are catalyzed by enzymes. Several reports favour the concept that these reactions are catalyzed by two enzymes, viz. an iodothyronine-5′-deiodinase, responsible for the 5′-deiodination of thyroxine and 3,3′,5′-triiodothyronine, and an iodothyronine-5-deiodinase, catalyzing the 5-deiodination of thyroxine and 3,3′,5-triiodothyronine [1,3—5]. Upon subcellular fractionation, all deiodinase activities were found to be associated with the endoplasmic reticulum of the rat liver cell [2,6] and, at the present, no separation of deiodinase activities has been obtained.

In the present study, we have solubilized the iodothyronine deiodinases from the microsomal fraction, determined some of their physico-chemical properties and made preliminary attempts at purification.

Materials and Methods

Materials. L-Thyroxine, L-3,3′,5-triiodothyronine, dithiothreitol, Triton X-405, cholate, deoxycholate, Brij 35, polyoxyethylene ether W-1 (W-1 ether), trypsin (bovine pancreas, crystallized twice) and trypsin inhibitor (soya bean) were obtained from Sigma. L-3,3′,5′-Triiodothyronine was obtained by courtesy of Dr. E. Scheiffele (Henning GmbH, Berlin). Triton X-100 and digitonin were purchased from B.D.H. Lubrol PX was from I.C.I. and taurocholate from Calbiochem. Sephadex G-25 (Fine), DEAE-Sepharose CL-6B, activated thiol-Sepharose 4B, Sepharose 6B, Sephacryl S-200 SF, Sephadex G-150, and the marker proteins ribonuclease A, chymotrypsinogen A, ovalbumin, albumin aldolase, catalase, ferritin and thyroglobulin were obtained from Pharmacia, Uppsala, Sweden. Glutathione-Sepharose was obtained by elution of an activated thiol-Sepharose column with dithiothreitol. The resins XAD-2 and XAD-7 were purchased from Serva, Heidelberg, F.R.G.

Assay of iodothyronine deiodinase activity. Iodothyronine deiodinase activity was determined by incubating 5 μM thyroxine, 2.5 μM 3,3′,5-triiodothyronine or 0.5 μM 3,3′,5′-triiodothyronine with appropriate dilutions of the fraction to be tested in the presence of 0.08 M sodium phosphate (pH 7.0) and 2.5 mM dithiothreitol in a total volume of 0.2 ml. After incubation at 37°C for 10 min with shaking, the reaction was stopped by addition of 0.8 ml 0.06 M barbitone buffer, (pH 8.6), 0.1% bovine serum albumin, 0.1% SDS. The amounts of iodothyronine produced were measured directly in 50 μl of the extract with specific radioimmunoassays [1]. For the determination of the conversion of thyroxine into 3,3′,5′-triiodothyronine, the amount of 3,3′,5′-triidothyronine degraded into 3,3′-diiodothyronine was taken into account.

Solubilization of enzymes. Microsomes were prepared as described previously [2]. Solubilization was carried out using various procedures, viz. solvent extraction, treatment with detergents, high salt concentrations, trypsin or

EDTA. For solvent extraction, acetone [7] and *n*-butanol [8] were used. The detergents used were Triton X-100 and X-405, Lubrol, cholate, deoxycholate, taurocholate, Brij 35, digitonin and W-1 ether. W-1 ether is a 1—1.78 mixture of cetyl 10 ether (Brij 56) and cetyl 20 ether (Brij 58). Detergent (in Tris-HCl/dithiothreitol buffer, consisting of 10 mM Tris-HCl (pH 7.4), 3 mM dithiothreitol) and an equal volume of microsomal fraction (containing approx. 15 mg protein/ml) were mixed and incubated for 45 min at 0° C. Final detergent concentrations ranged between 0.05 and 1.3% (w/v). Deiodinase activity was determined in the suspension and, following centrifugation (1 h at 110 000 × *g*), in the supernatant and in the pellet which was suspended in Tris-HCl/dithiothreitol buffer. Salt extraction with 0.2—4.0 M KCl (final concentration) [9] and treatment with 3—50 mM EDTA were carried out similarly. Solubilization with trypsin was attempted by incubating approx. 6 mg microsomal protein with 25—100 μg trypsin in a total volume of 0.9 ml (in Tris-HCl/dithiothreitol buffer). After incubation at 20° C for 2.5 h, or at 37° C for 1 h, the reaction was stopped by the addition of a 3-fold excess of trypsin inhibitor. Determination of deiodinases was carried out as described above.

Protein determinations. Protein was estimated by a modification of the Bradford method [10] using bovine serum albumin as standard [11]. This method is not influenced by several laboratory reagents, especially by dithiothreitol, which is used throughout all procedures. In preparations containing detergent, a modification [12] of the method of Lowry et al. [13] was used.

Removal of cholate. Cholate was removed from solubilized microsomal proteins by using a mixture of the polystyrene beads XAD-2 and XAD-7 (4 : 1, w/w) [14]. The column (1.0 × 19.5 cm) was equilibrated and eluted with 15 mM Tris-HCl (pH 9.0), 3 mM dithiothreitol (flow rate, 18 ml/h). Cholate was determined by liquid scintillation counting of [^{14}C]cholic acid (New England Nuclear, Boston, MA) of which a tracer quantity had been added to 1% sodium cholate.

Gel filtration. Gel filtration was carried out at 8° C on columns of Sepharose 6B (1.5 × 26 cm), Sephacryl S-200 (1.6 × 30 cm) and Sephadex G-150 (1.5 × 25 cm). The elution buffer contained 0.05 M Tris-HCl (pH 7.2), 0.1 M NaCl, 2 mM dithiothreitol with or without detergent (flow rates, 14, 28 and 9.5 ml/h, respectively). The molecular weight and the Stokes radius, R_s, were calculated from the plot of K_{av} vs. log molecular weight and of $\sqrt{-\log(K_{av})}$ vs. R_s, respectively, using marker proteins from Pharmacia calibration kits.

Sucrose gradient centrifugation. Linear sucrose gradients (4—24%, w/w) contained 20 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol and in some experiments 0.6% cholate or 0.05% W-1 ether. A Beckman SW41 rotor was used for 16 h at 5° C and 153 000 × *g*. The sedimentation coefficients were determined by comparison with several marker proteins.

Chromatography on DEAE-Sepharose. The microsomal proteins solubilized with W-1 ether (approx. 15 mg protein) were loaded on a colum (1.6 × 8 cm) of DEAE-Sepharose CL-6B, which had been equilibrated with 20 mM Tris-HCl (pH 8.0)/2 mM dithiothreitol. The column was then eluted (flow rate, 20 ml/h) with 35 ml buffer and, subsequently, with similar volumes of buffer con-

taining 0.1, 0.2 and 0.4 M NaCl.

Chromatography on thiol-Sepharose 4B. Approximately 20 mg of solubilized microsomal protein was added to the top of a Sephadex G-25 column (1 × 12 cm), which had been equilibrated with 40 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA, 0.15 M NaCl. The fractions containing deiodinase activity were pooled and this solution was then added to the top of a column (0.7 × 9 cm) activated thiol-Sepharose 4B, which had been equilibrated with the Tris-HCl buffer. The column was washed with the same buffer (flow rate, 6 ml/h) until the absorbance at 277 nm of the effluent was approx. zero. Elution was achieved with the equilibration buffer containing 8 mM dithiothreitol. Conditions for chromatography on glutathione-Sepharose were the same as for activated thiol-Sepharose.

Results

Solubilization

In the control experiment (treatment with Tris-HCl/dithiothreitol only) the supernatant contained less than 10% of deiodinase activity (Table I). Treatment with Lubrol, Triton X-100, Triton X-405, deoxycholate, KCl, EDTA, acetone, *n*-butanol or trypsin yielded a soluble preparation displaying less than 15% of the activity of untreated microsomes. Taurocholate, digitonin and Brij 35 were able to solubilize 20—40% of the deiodinases. Of these detergents Brij 35 inactivated the deiodinases most strongly. In Table I are summarized results obtained with the detergents cholate and W-1 ether, which were most successful in solubilizing microsomes.

As demonstrated in Table I, cholate and W-1 ether are most effective at a final concentration of 1.0 and 0.25% (w/v), respectively. However, the apparent yield of solubilized enzyme was low. It was found that cholate or W-1 ether, added directly to the assay, caused a concentration-dependent decrease in microsomal deiodinase activity (Fig. 1). Therefore, the low yield of enzymatic activity after solubilization is at least partly due to a direct inhibitory effect of the detergent. Indeed, when the solubilized microsomes were assayed in a

TABLE I

DEIODINASE ACTIVITY AFTER SOLUBILIZATION OF MICROSOMES

Conversion studies were done at pH 7.0 using thyroxine as the substrate. Values for the supernatant are indicated as a percent of those for the mixture of microsomes and detergent before centrifugation and represent the mean of three experiments.

Detergent	Protein (% of initial)	5′-Deiodinase activity (% of initial)	5-Deiodinase activity (% of initial)
—	24	8	8
0.25% Cholate	44	20	19
0.50% Cholate	66	58	52
1.00% Cholate	80	80	78
1.30% Cholate	86	82	81
0.10% W-1 ether	49	22	26
0.25% W-1 ether	68	78	81
0.50% W-1 ether	85	82	84
1.00% W-1 ether	90	84	86

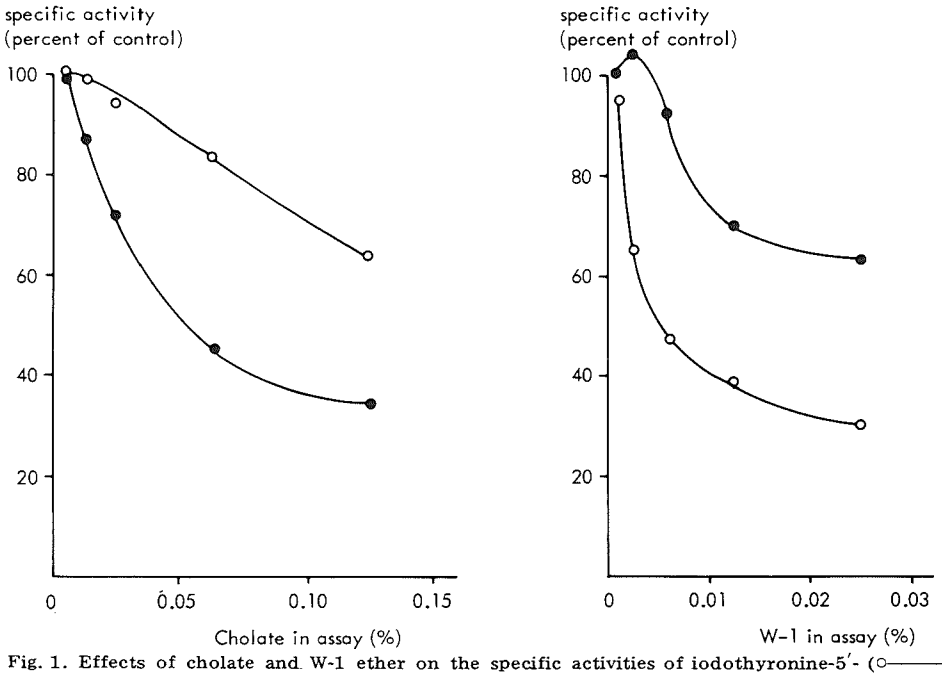

Fig. 1. Effects of cholate and W-1 ether on the specific activities of iodothyronine-5′- (○———○) and 5-deiodinase (●———●). Thyroxine was used as a substrate. Values are indicated as a percent of untreated microsomes.

suitable dilution (yielding 0.005—0.025% cholate and 0.0025% W-1 ether, final concentrations), most of the deleterious action of cholate and W-1 ether was prevented (see specific activities in Table II). The data in Fig. 1 suggest that the 5-deiodinase activity is more affected by cholate than the activity of 5′-deiodinase, whereas with W-1 ether the reverse appears to be the case. Table II summarizes the isolation and solubilization of the 5′- and 5-deiodinase. It can be seen that cholate decreases the specific activity of the deiodinases by approx. 30%, whereas the decrease in case of W-1 ether is less than 20%.

TABLE II

ISOLATION AND SOLUBILIZATION OF RAT LIVER MICROSOMAL IODOTHYRONINE-5′- AND 5-DEIODINASE

The conversion studies were performed at pH 7.0, with thyroxine as the substrate.

Fraction	Total protein (mg)	Specific activity (pmol/mg protein per min)		Recovery (%)	
		5′-Deiodinase	5-Deiodinase	5′-Deiodinase	5-Deiodinase
Homogenate	1544	1.32	0.76	—	—
Microsomes	284	7.65	4.24	106	102
Supernatant fluid after solubilization with cholate	222	5.62	2.73	61	53
Supernatant fluid after solubilization with W-1 ether	173	6.23	3.63	53	55

Removal of cholate

Elution of microsomal proteins solubilized with 1.0% cholate on a mixture of the polystyrene beads XAD-2 and XAD-7 resulted in a more than 99% removal of cholate. Recovery of protein was approx. 95% and that of 5′-deiodinase activity (measured with 3,3′,5′-triiodothyronine as the substrate) was 190%. This is probably due to stripping of cholate from the enzyme. In this manner a crude soluble enzyme preparation was obtained essentially free of detergent. However, after some time aggregation occurred.

Gel filtration

Microsomal proteins solubilized with cholate or W-1 ether were applied to columns of Sepharose 6B, Sephacryl S-200 and Sephadex G-150. Apparent molecular weights and Stokes radii of the 5′-deiodinase after gel filtration on Sepharose 6B and Sephacryl S-200 are shown in Table III. In the absence of cholate in the solvent, the actual cholate concentration in the fractions containing deiodinase activity after chromatography on Sephacryl S-200 was approx. 0.05 mM (measured by using ^{14}C-labeled cholate). Apparently, this amount is sufficient to keep the protein in a soluble state. No cholate was measured in deiodinase containing fractions after Sepharose 6B chromatography. The results obtained with Sephadex G-150 are not included in this table, because the molecular weight of the deiodinase was, under all conditions, higher than 200 000 on this column. It is evident that the molecular weight and Stokes radius of the 5′-deiodinase are not only dependent on the solubilizing agent, but also on the type of gel filtration and the presence of detergent in the elution buffer. The apparent molecular weight and the Stokes radius of the 5′-deiodinase in the cholate extract are approx. 65 000 and 36—37 Å, respectively. For the W-1 ether extract, these values are 160 000—230 000 and 52—56 Å, respectively.

TABLE III

SEPHAROSE 6B AND SEPHACRYL S-200 GEL FILTRATION DATA OF MICROSOMAL 5′-DEIODINASE

The molecular weight and Stokes radius (R_S) of the 5′-deiodinase solubilized with cholate and W-1 ether are given. 3,3′,5′-Triiodothyronine was used as a substrate. n.d., not determined.

Sample	Sepharose 6B chromatography		Sephacryl S-200 chromatography	
	Molecular weight	R_S (Å)	Molecular weight	R_S (Å)
Cholate extract	>700 000	>100	60 000—70 000	34—37
Cholate extract (0.4% cholate in eluent)	60 000—70 000	36—39	65 000—75 000	35—38
W-1 Ether extract	160 000	53	>300 000	>60
W-1 Ether extract (0.025% W-1 ether in eluent)	n.d.	n.d.	200 000—230 000	52—56

TABLE IV

SEDIMENTATION COEFFICIENTS ($s_{20,w}$) OF SOLUBILIZED 5'-DEIODINASE

3,3',5'-Triidothyronine was used as substrate.

Sample	Detergent in gradient	$s_{20,w}$ ($\times 10^{-13}$)
Cholate extract	none	12.8
Cholate extract	0.6% cholate	4.3
Cholate extract	0.05% W-1 ether	7.6
Cholate extract after removal of cholate	none	12.8
W-1 Ether extract	none	4.3
W-1 Ether extract	0.6% cholate	5.2
W-1 Ether extract	0.05% W-1 ether	5.2

Sucrose gradient centrifugation

In Table IV, the sedimentation coefficients of the 5'-deiodinase under several conditions are shown. The deiodinase solubilized with W-1 ether had a sedimentation coefficient (4.3—5.2 S), which was almost independent of the presence of detergent in the gradient. However, in the cholate extract the enzyme had a high sedimentation coefficient (12.8 S) in the absence of any detergent in the gradient, pointing to aggregation. This also appeared to be the case after removal of cholate with the XAD-resin. In the presence of 0.05% W-1 ether, the sedimentation coefficient was 7.6 S and in the presence of 0.6% cholate it was 4.3 S.

DEAE-Sepharose chromatography

The DEAE-Sepharose column bound approx. 80% of added 5'-deiodinase at pH 8.0 (Fig. 2). Nearly 70% of this bound enzyme could be eluted with 0.1 M NaCl. At concentrations higher than 0.2 M NaCl, no deiodinase activity was

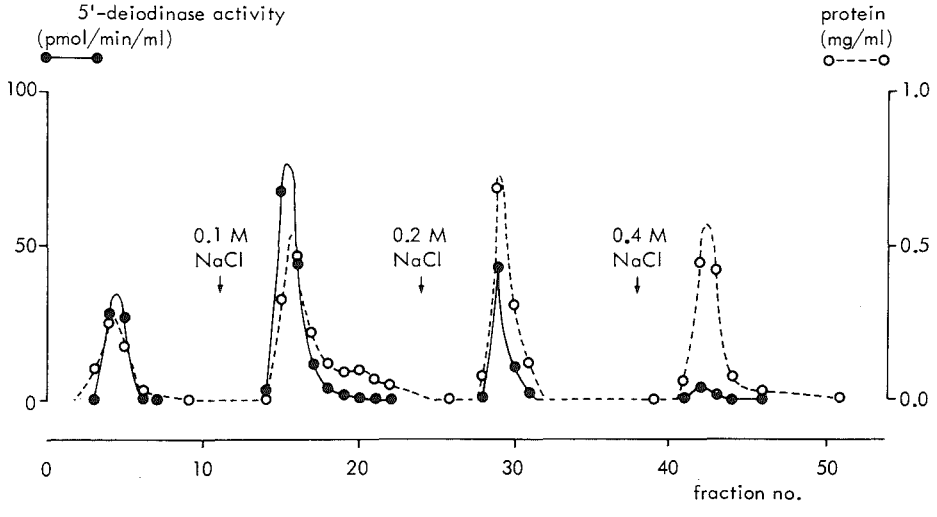

Fig. 2. DEAE-Sepharose chromatography, pH 8.0, of microsomes solubilized with W-1 ether. The column was developed with a discontinuous gradient of KCl in column buffer. 3,3',5'-Triiodothyronine was used as the substrate.

bound to the column, whereas protein was still present. The 5-deiodinase showed a similar profile. Recovery of total deiodinase activity was approx. 50% (protein recovery 100%) and enrichment of the enzyme in the fraction eluted with 0.1 M NaCl was only 2-fold.

Chromatography on thiol-Sepharose 4B

After removal of dithiothreitol from W-1 ether-solubilized microsomal proteins by Sephadex G-25, the sample was applied to activated thiol-Sepharose. The elution pattern is seen in Fig. 3. About 25% of the deiodinase activity appeared in the column volume, whereas the remainder could be eluted with 8 mM dithiothreitol. However, many other proteins were bound to thiol-Sepharose too, resulting in little purification of the 5′-deiodinase (approx. 3-fold). 5-Deiodinase showed a similar profile. Protein and deiodinase recoveries were higher than 75%. After application of microsomes solubilized with cholate and prechromatographed on Sephadex G-25, about 90% of the deiodinase activity (together with approx. 75% protein) appeared in the column volume. The deiodinases were not adsorbed onto glutathione-Sepharose using microsomes solubilized with W-1 ether as well as with cholate.

Discussion

Effective solubilization of the microsomes was obtained with the detergents cholate and W-1 ether. The results with cholate are in agreement with those of Köhrle et al. [9] and Leonard and Rosenberg [15], although the latter studied

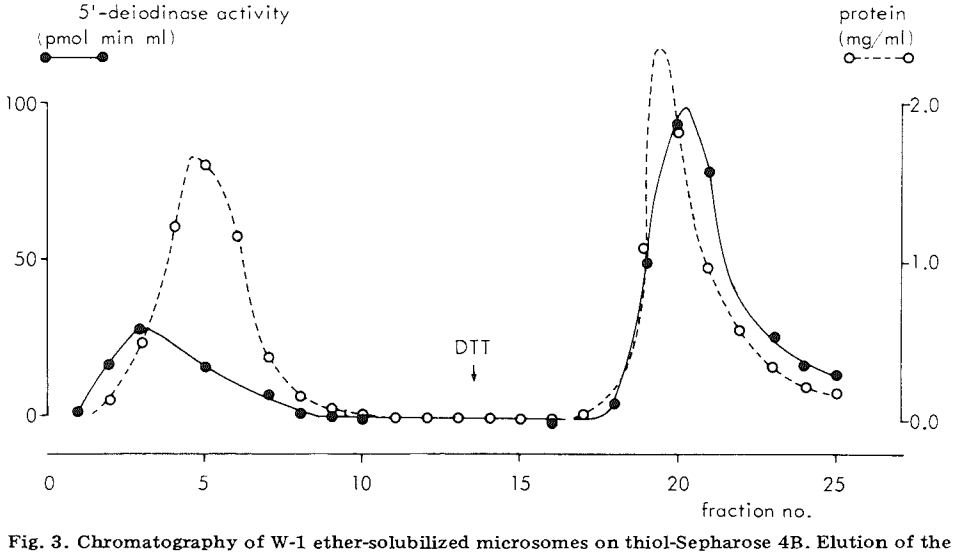

Fig. 3. Chromatography of W-1 ether-solubilized microsomes on thiol-Sepharose 4B. Elution of the bound enzyme was performed with buffer containing 8 mM dithiothreitol. 3,3′,5′-Triiodothyronine was used as a substrate. DTT, dithiothreitol.

rat kidney microsomes. In the presence of 1.0% cholate, 58 and 52% of the active form of iodothyronine-5′- and 5-deiodinase, respectively, could be solubilized. For 0.25% W-1 ether, these values are 50 and 54%, respectively. From our observations, it can be concluded that the thyroxine-deiodinating enzymes are integral (intrinsic) and not peripheral (extrinsic) membrane proteins because the enzymes cannot be dislodged from the membrane by the chelating agent EDTA nor by increasing the ionic strength (salt extraction) with KCl. However, Köhrle et al. [9] reported the solubilization of the deiodinase from microsomal fractions with 4 M KCl. We repeated the experiments and indeed we found deiodinating activity in the supernatant after centrifugation. But this turbid supernatant apparently had a similar density as the microsomes. Upon dilution, all deiodinating activity could be pelleted. Therefore, no actual solubilization had occurred. Although solubilization does not result in separation of the deiodinating enzymes, the detergents cholate and W-1 ether affect the specific activity of iodothyronine-5′- and 5-deiodinase in a different manner (Fig. 1).

In many cases it is desirable to have a soluble enzyme preparation which is essentially free of detergent, especially in case of an ionic detergent. Therefore, we attempted to remove cholate from the soluble microsomal proteins. A mixture of the polystyrene beads XAD-2 and XAD-7 (4 : 1, w/w) appeared to be very successful for this purpose. However, after storage for a few days at 4° C, the proteins obtained in this manner aggregated visibly. Probably, aggregation occurred immediately after stripping of cholate from the proteins. In almost all chromatography procedures described in this paper, the applied cholate extract aggregated during elution, which was accompanied by considerable loss of activity of the deiodinases. The latter fact remained even in the presence of cholate during chromatography. For this reason, most column chromatography was carried out with microsomes solubilized with the non-ionic detergent, W-1 ether. Even in the absence of W-1 ether during chromatography, no observable aggregation occurred.

The apparent molecular weight of the 5′-deiodinase as determined by gel filtration turned out to be dependent on the presence and type of detergent and surprisingly, also on the gel filtration support. The results suggest that aggregation took place during chromatography of the cholate extract on the Sepharose 6B column in the absence of detergent in the eluent. Measurement of cholate in the deiodinase-containing fractions revealed that these were devoid of the detergent. On the other hand, some cholate was present in the deiodinase-containing fractions after chromatography on Sephacryl S-200, which, apparently, was sufficient to keep the deiodinase in a soluble form. Also, in case of the W-1 ether extract, differences in apparent molecular weights were observed to be dependent on the gel filtration support. Since we were not able to measure the amount of W-1 ether in the fractions containing deiodinase activity, we cannot conclude whether this was due to loss of detergent during chromatography.

The molecular weight of the deiodinase as determined by Sephadex G-150 chromatography was in all cases higher than 200 000. An explanation for this

finding cannot be given at the moment. Nevertheless, from the gel filtration experiments on Sepharose 6B and Sephacryl S-200, it can be concluded that the apparent molecular weight of the 5′-deiodinase in the cholate extract is lower than in the W-1 ether extract. This may be explained by the fact that the deiodinase has more binding sites for W-1 ether than for cholate. Another possibility is that the enzyme binds to a whole micelle. In the latter case, the protein-detergent complex formed with W-1 ether is larger than that formed with cholate, because cholate has a micellar weight of only 900—1800, whereas for W-1 ether, having a similar structure as Lubrol and Brij, this value is higher than 50 000 [16].

The sedimentation coefficient of the 5′-deiodinase in the cholate extract in the presence of 0.6% cholate was 4.3 S. Similar values were obtained by centrifugation of the W-1 ether extract under various conditions. This value is near the sedimentation coefficient of bovine serum albumin, which is 4.4. S. The approximate molecular weight of the enzyme is therefore similar to the one determined by gel filtration. Omission of cholate in the sucrose gradient resulted in aggregation, whereas exchange of cholate for a low concentration of W-1 ether, yielded partial aggregation of the deiodinase. The sedimentation coefficients of the 5′-deiodinase in the cholate and W-1 ether extract show only a slight difference, whereas their apparent molecular weights as determined by gel filtration show a much greater difference. This may be explained by their different hydrodynamic properties. Protein-W-1 ether complexes have a low sedimentation coefficient and a high Stokes radius, whereas protein-cholate complexes lack a similar combination [16].

DEAE-Sepharose chromatography of the W-1 ether-solubilized deiodinases at pH 8.0 yielded essentially three activity peaks. A minor peak eluted with buffer, a major peak with 0.1 M NaCl and a minor peak with 0.2 M NaCl. However, purification was very modest (approx. 2-fold). This is also true for chromatography on activated thiol-Sepharose. Since it is known that iodothyronine deiodinases contain essential sulfhydryl groups [17], it seemed worthwile trying to purify these enzymes by chromatography on thiol-Sepharose. It appeared that approx. 75% of the enzymes solubilized with W-1 ether, were absorbed onto this column. The bound deiodinases could be eluted from the column with the activating sulfhydryl reagent dithiothreitol [1,17]. However, many other proteins were bound to thiol-Sepharose too, resulting in very little purification of the deiodinases. The deiodinases were neither resolved by DEAE- nor by thiol-Sepharose. Unexpectedly, no binding occurred on a glutathione-Sepharose column, whereas glutathione is thought to be the endogenous cofactor of iodothyronine deiodinases [1,4].

In conclusion, iodothyronine deiodinases of the endoplasmic reticulum of rat liver cells can be solubilized with cholate and W-1 ether, and these enzymes are integral and not peripheral membrane proteins. The 5′-deiodinase has a molecular weight of about 65 000 in cholate and of approx. 200 000 in W-1 ether, a Stokes radius of 36—37 Å in cholate and of 52—56 Å in W-1 ether, a sedimentation coefficient of 4.3 S in cholate and 5.2 S in W-1 ether; it contains accessible sulfhydryl groups and has negligible affinity for glutathione under

the conditions used. Up to now, no molecular evidence has been obtained for the existence of two deiodinating enzymes. This is not surprising, because the purification of 5′-deiodinase activity after column chromatography was only very modest.

Acknowledgements

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CHAPTER 6

PROPERTIES OF DETERGENT-DISPERSED IODOTHYRONINE 5- and 5'-DEIODINASE ACTIVITIES FROM RAT LIVER

EVIDENCE FOR A COMMON ENZYME

Durk Fekkes, Georg Hennemann and Theo J. Visser

Biochimica et Biophysica Acta, submitted for publication

ABSTRACT

In order to obtain more knowledge about the regulation and mechanism of thyroid hormone deiodination, some properties of detergent-solubilised iodothyronine deiodinase have been studied. Moreover, a starting point for its purification has been made. Several chromatography media were tested for their ability to purify the deiodinases. In some instances, a 4-fold purification was obtained. Treatment of cholate-solubilised microsomes with 35% ammonium sulphate resulted in quantitative precipitation of the deiodinase activities and concomitant removal of phospholipid. The pellet could be solubilised with 0.3% W-1 ether and the deiodinase in this ammonium sulphate extract exhibited approximately 10-fold higher apparent K_m and V_{max} values for its substrate compared with the cholate extract. Readdition of some phospholipids was shown to decrease enzyme activity. Isoelectric focusing of W-1 ether-solubilised microsomes resulted in a major activity peak around pH 6.4 and a minor peak at pH 5.2, while in the ammonium sulphate extract the deiodinase had an isoelectric point at pH 9.3. Refocusing of this activity peak yielded a preparation with a specific activity being only 3 times higher than in the ammonium sulphate extract. However, after sodium dodecyl sulphate gel electrophoresis only 5 bands could be detected.

The elevated kinetic parameters as well as the higher isoelectric point of the deiodinase after ammonium sulphate treatment were caused by delipidation of the enzyme. Both the change in isoelectric point and the behaviour on several column materials were found to be similar for the 5- and 5'-deiodinase activities. These results suggest that a single enzyme is operative in the deiodination of iodothyronines in rat liver and that its activity may be regulated by phospholipids.

INTRODUCTION

Deiodination is an important route in the metabolism of thyroid hormone in humans as well as in rats (1-3). At least one half of circulating thyroxine (T_4) undergoes deiodination, where 3,3',5-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) are the immediate products. In normal humans, 80% of T_3 and an even greater fraction of rT_3 are derived from deiodination of T_4 in peripheral tissues, the remainder being secreted by the thyroid (2,4). The biological effects of thyroid hormone are only exerted after conversion of T_4 to T_3, while rT_3 is devoid of thyromimetic activity (5-7).

T_3 is produced from T_4 by deiodination of the phenolic ring, a process termed 5'-deiodination. Via the same type of reaction rT_3 is converted to 3,3'-diiodothyronine (3,3'-T_2). Deiodination of the tyrosyl ring (5-deiodination) of T_4 yields rT_3, and 5-deiodination of T_3 is another way to generate 3,3'-T_2. In several clinical situations, commonly referred to as the low T_3 syndrome, characteristic changes in the metabolism of thyroid hormone are observed, leading to low serum T_3 and elevated rT_3, while, usually, T_4 concentrations are normal (2). Studies of the plasma disappearance curves of injected tracers are compatible with a decreased production but normal clearance of T_3, and a normal production but decreased clearance of rT_3 (2). These findings have led several investigators to imply the involvement of distinct enzymes, i.e. iodothyronine 5- and 5'-deiodinase, in the sequential breakdown of T_4 (2,3,8). A reduction in the 5'-deiodination of T_4 and rT_3 by a selective decrease in the activity of the latter enzyme could then explain the abnormal metabolism of thyroid hormone in the low T_3 syndrome.

Iodothyronine deiodinase activities are most abundant in the liver and the kidneys of the rat (2,3,8). The enzyme activities of rat liver are associated with the endoplasmic reticulum (9-11) and are thought to be intrinsic membrane proteins (12,13). Soluble deiodinase preparations have been obtained with retention of activity by treatment of microsomes with detergents (12-14) and some of the physicochemical properties have been determined (13).

Here we report a starting point for the purification of a hepatic iodothyronine deiodinase. All techniques employed sofar have not resulted in a dissociation of 5- and 5'-deiodinase activities. This is compatible with previous, circumstantial evidence that, at least in rat liver, both types of deiodination are catalyse by a single enzyme (15).

MATERIALS AND METHODS

Materials

T_3, rT_3 and 3,3'-T_2 were purchased from Henning GmbH. (Berlin, FRG). [3',5'-^{125}I] rT_3 was from the Radiochemical Centre (Amersham, UK). Minicon B15 units and Matrex gel red A were products from Amicon Co. (Lexington, USA). All other chromatography media and Pharmalytes were from Pharmacia Fine Chemicals (Uppsala, Sweden). Ultrodex and Ampholines were obtained from LKB Produkter (Bromma, Sweden). Dithiothreitol (DTT), sodium cholate, polyoxyethylene ether W-1 (W-1 ether), N-ethyl - N'-(3-dimethylaminopropyl) carbodiimide hydrochloride, Coomassie brilliant blue R 250 and G 250, phosphatidylcholine, -ethanolamine, -inositol and - serine were products from Sigma Chemical Co. (St. Louis, MO, USA). Sphingomyelin was purchased from Supelco Inc. (Bellafonte, USA) and 2- thiouracil-6-propionic acid was a gift from Dr. D.S. Cooper, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA. Ammonium sulphate (for enzyme work) and sodium dodecyl sulphate (specially pure) were obtained from BDH Chemical Co. (Poole, UK). All other chemicals were of analytical grade.

Assays of Iodothyronine Deiodinase Activities

Iodothyronine deiodinase activities were determined at pH 7.2 and 37° C using T_3 (5 μM) or rT_3 (0.5 μM) as substrate in the presence of 3 mM DTT by the method described previously (16). One unit of activity is defined as the amount which produces 1 pmol 3,3'-T_2/min from T_3 (5-deiodination) or rT_3 (5'-deiodinatior under the standard assay conditions. The product 3,3'-T_2 was measured by a specific radioimmunoassay (16). Specific activity is expressed as enzyme units/mg protein.

In later experiments, rT_3 5'-deiodinase activity was assayed by a rapid procedure based on the measurement of radioactive iodide released from [3',5'-^{125}I]rT_3.

Reaction mixtures consisted of 0.06 M sodium phosphate, pH 7.2, 3 mM EDTA, 3 mM DTT, 0.4 μM [^{125}I]rT_3 and the enzyme preparation to be tested in a final volume of 0.12 ml. The reaction was allowed to proceed for 20 min at 37^{o}C and was stopped by the addition of 0.5 ml human serum. After 30 min at 0^{o}C the iodothyronine substrate (rT_3) and product (3,3'-T_2), bound to serum proteins, were precipitated with 0.5 ml 10% trichloroacetic acid. After centrifugation for 10 min at 1500 x g, the radioiodide liberated was quantitated by counting 0.4 ml of the supernatant using a Nuclear Enterprise NE 1600 gamma spectrometer. In control experiments the enzyme preparation was added only after the serum. Net enzymatic deiodination was calculated by subtraction of the $^{125}I^-$ in the control experiment from that produced in the complete reaction mixture, and multiplication of the difference by 2 as the radioactivity is equally distributed among the products 3,3'-T_2 and I^-. This procedure yields the same value for the rT_3 5'-deiodinase activity as that based on the determination of 3,3'-T_2 by radioimmunoassay.

Preparation of solubilised enzymes

Microsomes were prepared as described previously (9). For solubilisation, the microsomal fraction was treated with detergent in reaction mixtures containing (at final concentrations): 7-8 mg microsomal protein/ml, 40 mM Tris/HCl (pH 7.5), 3 mM EDTA, 3 mM DTT and 1% (w/v) sodium cholate or 0.25% (w/v) W-1 ether. The suspension was stirred at 0^{o} C for 1 h and centrifuged at 110 000 x g for 1 h. The clear yellow supernatants are designated as the cholate or W-1 ether extracts, respectively.

Ammonium sulphate fractionation

Solid ammonium sulphate was added with stirring to the cholate extract at 0^{o} C to give a 35% saturated solution. After gentle stirring for 1 h, the suspension was centrifuged at 20 000 x g for 20 min. The supernatant was discarded and the pellet was solubilised in a minimum volume of 50 mM Tris/HCl buffer (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 2.5 mM DTT and 0.3% W-1 ether. After 1 h incubation at 0^{o} C and centrifugation (110 000 x g, 1 h), the supernatant (designated as ammonium sulphate extract) retained most of the deiodinase activity.

Analytical Methods

Protein was measured by the Bradford method (17) as modified (18), using bovine serum albumin as the standard. When determinations were done in the

presence of detergent, the protein standards were prepared in a solvent containing an equal concentration of the detergent.

The phospholipid contents of protein fractions and phospholipid dispersions were quantitated by measurement of lipid phosphorus (19).

Gel electrophoresis in presence of sodium dodecyl sulphate (0.2%) was performed using 5% polyacrylamide gels (20). The protein bands were stained with 0.25% Coomassie brilliant blue R 250 in 45% methanol and 9% acetic acid.

Chromatographies

Ion exchange chromatographies (DEAE-Sephacel and CM-Sepharose CL-6B) were performed on 1.6 x 5 cm (5 ml) columns and all other chromatographies on 0.7 x 8 cm ($\sim$3 ml) columns at 4-8^{o} C. In most instances the crude W-1 ether extract was applied to the several columns, but on some occasions the cholate or the ammonium sulphate extracts were chromatographed as well. The chromatography media and buffer systems used, are reported in the Results section.

Preparation of affinity columns

T_4- and T_3-Sepharose were prepared by coupling T_4 or T_3 to activated CH-Sepharose 4B at pH 8.3 and room temperature according to the procedure described by the manufacturers (21). The use of tracer amounts of [^{125}I]T_4 or [^{125}I]T_3 allowed for the estimation of the degree of coupling. This was found to be 7-8 μmol T4 or T_3/ml gel.

Thiouracil-Sepharose was prepared by coupling of 2-thiouracil-6-propionic acid to AH-Sepharose 4B using N-ethyl-N'-(3-dimethyl-aminopropyl) carbodiimide hydrochloride. The coupling procedure was performed around pH 5 at room temperature as described by the manufacturers (21). This affinity adsorbent contained approximately 10 μmol ligand/ml gel, as determined spectrophotometrically (measurement of absorbance of reaction mixture and wash fluids at 285 nm).

Isoelectric focussing

Preparative flat-bed electrofocusing was performed at 1^{o} C with the LKB 2117 Multiphor system (LKB, Bromma, Sweden), according to manufacturers' instructions. As solid support Ultrodex was used, which was equilibrated with 10% ethylene glycol containing 2% of carrier ampholytes and W-1 ether at a final concentration of 0.25%. Ampholine or Pharmalyte gradients were used as supplied, or by appropriate mixing of commercially available products. Samples were concen-

trated with a Minicon B-15 unit before loading on the gel. Focusing was carried out for 16 h at 12 W and 1200 V constant. The initial amperage was 18 mA and this decreased steadily to about 3 mA.

After the run the gel was fractionated using the grid provided and the pH gradient was measured with the aid of a surface glass electrode (type 10403 3005, Ingold AG, Urdorf, Switzerland). Thereafter the gel of each fraction was transferred to small plastic columns and the proteins were eluted with 2.5 ml 0.05 M sodium phosphate (pH 7.3) containing 2.5 mM EDTA, 2mM DTT and 15% ethylene glycol.

Effects of phospholipids on deiodinase activity

Phospholipid dispersions were prepared by the addition of buffer (50 mM Tris/HCl, 0.1 M NaCl,1 mM EDTA, 3 mM DTT, pH 7.3) to a dry lipid film (rotary evaporation) and shaking of the suspension for 3 h at 37° C. Various concentrations of phospholipid were added to the ammonium sulphate extract (1.2 µg protein) and after preincubation for 1 h at 37° C rT_3 or T_3 was added to a final concentration of 0.1 or 5 µM, respectively. Incubation (at 37° C) was performed for 20 min at pH 7.3 (using rT_3 as substrate) or for 60 min at pH 8.0 (using T_3 as substrate) in a total volume of 0.25 ml. The reaction was stopped and the 3,3'-T_2 produced was measured as described previously (16).

Treatment of kinetic data

Kinetic constants were determined by drawing the straight lines of double-reciprocal (Lineweaver-Burk) plots by the least-squares method applied to unweighted means.

RESULTS

Ammonium sulphate fractionation

The supernatant, obtained after treatment of the cholate extract with 35% ammonium sulphate, contained negligible deiodinase activity (Table 1). The pellet could be solubilised best with 0.3% W-1 ether, yielding a soluble preparation with approximately ten times higher specific activity than the cholate extract. The overall recovery of 5'-deiodinase activity was 310%. The W-1 ether extract could not succesfully be fractionated with ammonium sulphate.

Table 1. AMMONIUM SULPHATE FRACTIONATION AND SUBSEQUENT SOLUBILISATION OF rT_3 5'-DEIODINASE ACTIVITY FROM CHOLATE SOLUBILISED RAT LIVER MICROSOMES

Fraction	Protein	rT_3 5'-deiodinase activity	Specific activity
	mg	units	units/mg protein
cholate extract (5.0 ml)	25.0	1412	56.5
supernatant after 35% $(NH_4)_2$ SO_4 treatment (5.0 ml)	7.0	2	0.3
supernatant after solubilisation of $(NH_4)_2$ SO_4-pellet (3.3 ml)	6.6	3804	576.4
pellet after solubilisation of $(NH_4)_2$ SO_4-pellet	10.6	578	54.5

Chromatographies

Results of several chromatographies are summarized in Table 2. Ionexchange chromatography on DEAE-Sephacel at pH 8.0 resulted in elution of 3% of activity in the void volume with the W-1 ether extract, whereas with the ammonium sulphate extract this was 11%, representing virtually all activity recovered. With the former sample 30% of activity was eluted with 0.1 M NaCl and with the latter this was only 1%. The behaviour of these two extracts on CM-Sepharose was the reverse of that on DEAE-Sepharose; i.e. with the W-1 ether extract most of the activity recovered from this column appeared in the void volume (Table 2). Total enzyme recoveries were in case of the ammonium sulphate extract very low.

Deiodinase activity was strongly retained on octyl-Sepharose and could not be eluted with ethylene glycol. Results with phenyl-Sepharose were similar. However, Sephadex LH-60, a gel possessing both hydrophilic and lipophilic properties, did neither retain activity nor protein (results not shown).

Con A-Sepharose and heparin-Sepharose retained very little activity (Table 2), while 2',5'-ADP-Sepharose retained no activity at all and less than 5% of

TABLE 2. CHROMATOGRAPHIES OF IODOTHYRONINE DEIODINASE ACTIVITY SOLUBILISED FROM RAT LIVER MICROSOMES.
The samples applied on the columns were the cholate extract (a), the ammonium sulphate (b) and in all other cases the W-1 ether extract. Pi, phosphate buffer n.d., not determined

Column material	Equilibration buffer	Fraction eluted with	Activity/protein (%)	Recovery of activity/protein (%)
DEAE-Sephacel	30 mM Pi, 2 mM DTT, pH 8.0	0.1 M NaCl, pH 7.0	30/18	58/68
"	"	"	1/3	13/53 b
CM-Sepharose CL-6B	30 mM Pi, 2 mM DTT, pH 5.6	0.1 M NaCl, pH 6.5	12/4	50/99
"	"	"	6/11	12/48 b
Octyl-Sepharose CL-4B	15 mM Pi, 2 mM DTT, pH 7.0	0-50% ethylene glycol, pH 7.8	1/8	10/65
"	" + 0.8 M $(NH_4)_2 SO_4$		<1/n.d.	<1/n.d.
"	15 mM Pi, 2 mM DTT, pH 7.0	0-50% ethylene glycol, pH 7.8	<1/4	7/50 a
"	" + 0.6 M $(NH_4)_2 SO_4$		<1/2	2/10 a
Con A-Sepharose 4B	40 mM Pi, 150 mM NaCl, 2mM DTT, pH 7.2	0.2 M α-D-methyl-glucopyranoside	3/5	64/76
Heparin-Sepharose CL-6B	30 mM Tris, 150mM NaCl, 2mM EDTA, 2 mM DTT, pH 7.2	0-1.5 M NaCl	5/18	94/83
Thiopropyl-Sepharose 6B	50 mM Tris, 100 mM NaCl, 1 M EDTA, pH 7.3	8 mM DTT	30/40	49/75
Thiol-Sepharose 4B	40 mM Tris, 150 mM NaCl, 1mM EDTA, pH 7.4	8 mM DTT	33/43	84/83
Blue-Sepharose CL-6B	40 mM Pi, 100 mM NaCl, 2mM EDTA, 2mM DTT, pH 6.5	1 M NaCl, pH 7.7	58/32	68/74
"		"	56/41	57/59 b
Matrex gel red A	40 mM Pi, 100 mM NaCl, 1mM EDTA, 2 mM DTT, 0.1% W-1 ether, pH 6.7	1 M NaCl, pH 7.9	97/62	107/88
T_4-Sepharose 4B	40mM Tris, 500mM NaCl, pH 8.0	50% ethylene glycol	2/30	3/35
"	"	" + 5 mM T_4	2/n.d.	3/n.d.
T_3-Sepharose 4B	40 mM Tris, 100 mM NaCl, 2 mM EDTA, pH 7.0	50% ethylene glycol+ 4 mM DTT	71/35	72/50
Thiouracil-Sepharose 4B	40 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.015 mM 3',5'-T_2, pH 7.4	50% ethylene glycol+ 12 mM DTT	7/10	47/59

protein (not shown). Covalent chromatography using thiopropyl-Sepharose or activated thiol-Sepharose did not result in enrichment of enzyme activity (Table 2).

Blue-Sepharose and Matrex gel red A both retained most of the enzyme activity applied to the column. Elution could be achieved with 1 M NaCl. Some of these eluted fractions exhibited a three-fold increase in specific activity compared with the original sample. The ammonium sulphate extract was adsorbed almost completely onto the blue-Sepharose column and eluted with 1 M NaCl with 60% recovery of both protein and enzyme activity.

Both T_4- and T_3-Sepharose strongly bound deiodinase activity (see Table 2). However, enzyme could not be eluted from the T_4-Sepharose column, whereas more than 70% of activity, together with 35% of protein, could be eluted from T_3-Sepharose using 50% ethylene glycol. Thiouracil-Sepharose retained very little enzyme and only some of the eluted fractions showed an increase in specific activity.

There was no difference in behaviour between the 5- and 5'-deiodinase activit on the column materials tested.

Isoelectric focusing

Electrofocusing of the W-1 ether extract revealed that the enzyme activity wa resolved into two peaks, a major one around pH 6.4 and a minor one at pH 5.2 (Fig. 1). Overall recovery of deiodinase activity was more than 90%.

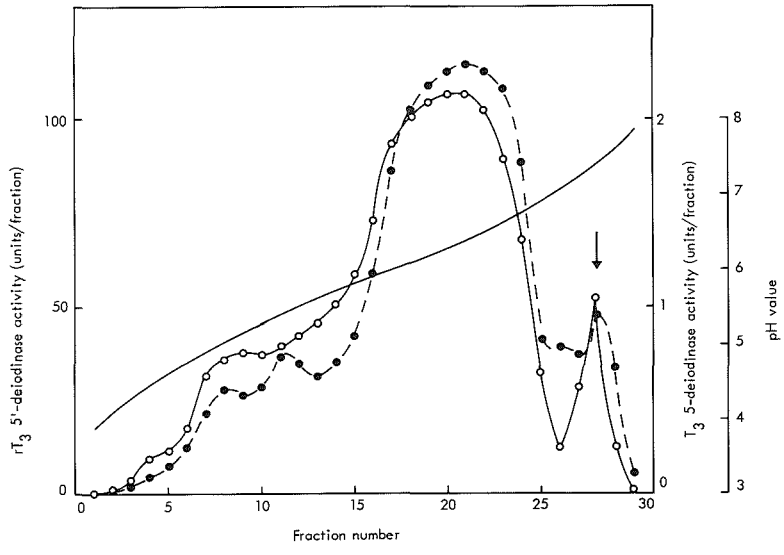

Fig. 1. Preparative isoelectric focusing of iodothyronine deiodinase activity. The W-1 ether extract (50 mg protein in 0.6 ml) was applied to the gel at the position indicated by the arrow. Focusing was carried out for 16 h at 1° C. An aliquot of the 2.5 ml fraction was used to determine deiodinase activity as described in Materials and Methods. (o—o) rT_3 5'-deiodinase activity ; (●—●) T_3 5-deiodinase activity ; (——) pH ampholyte gradient.

In the absence of detergents, the isoelectric focusing profile was diffuse and exhibited maximum activity between pH 5.7 and 6.3. Focusing of the ammonium sulphate extract resulted in one major peak of deiodinase activity around pH 9.3 (Fig. 2A). Overall recoveries after focusing of this extract were between 50 and 60%. Refocusing of the latter activity peak again showed a single dominant species with an isoelectric point of 9.1 (Fig. 2B). There was no dissociation between the 5- and 5' - deiodinase activities by isoelectric focusing of both the W-1 ether extract and the ammonium sulphate extract. Peak fractions after electrofocusing showed only a three-fold increase in specific activity. Refocusing of the material around pH 9.3 did not result in a higher specific deiodinase activity. Despite its low specific activity, the latter preparation consisted of only 5 protein bands as demonstrated by sodium dodecyl sulphate gel electrophoresis. In the crude W-1 ether extract of the microsomal fraction, containing the same amount of protein, about 30 protein bands could be detected (not shown).

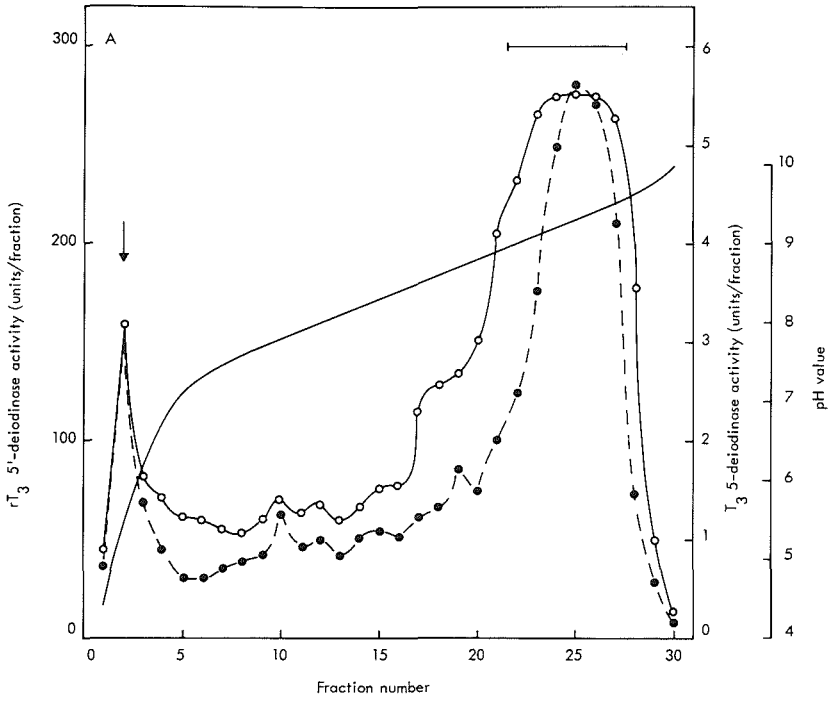

Fig. 2A. Preparative isoelectric focusing of iodothyronine deiodinase activity in the ammonium sulphate extract (17 mg protein in 0.3 ml was applied). The arrow indicates the position of sample application. Focusing experiments were performed for 16 h at 1° C. An aliquot of the fraction (2.5 ml) was assayed for deiodinase activity as described in Materials and Methods. (o——o) rT_3 5'-deiodinase activity (•---•) T_3 5-deiodinase activity; (——) pH ampholyte gradient. The bar above the activity peak indicates the fraction which was combined and used in the refocusing experiment (see Fig. 2B).

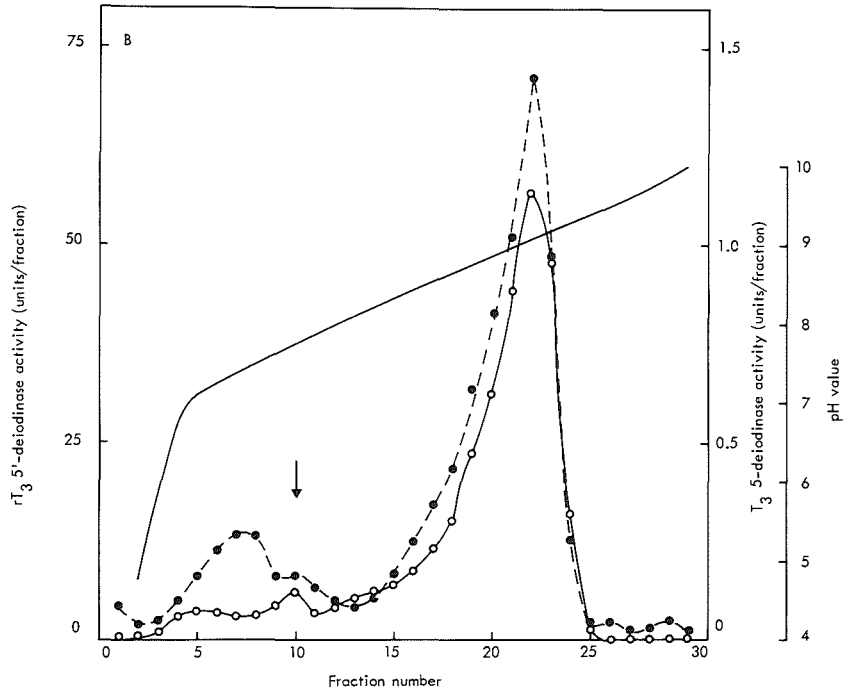

Fig. 2B. Preparative isoelectric refocusing of iodothyronine deiodinase activity i the peak fraction (0.6 mg protein in 0.3 ml) obtained after isoelectric focusing c the ammonium sulphate extract. See legend to Fig. 2A.

Isoelectric focusing of the desalted and concentrated eluate, obtained after chromatography of the W-1 ether extract on Matrex gel red A, resulted in a major activity peak around pH 9 (not shown).

Characteristics of solubilised iodothyronine deiodinase activity

Table 3 presents the K_m and V_{max} values for rT_3 of membrane-bound and solubi ised iodothyronine deiodinase activity. The kinetic parameters were not greatly altered by solubilisation with cholate or W-1 ether. However, solubilisation of the ammonium sulphate precipitate with W-1 ether greatly increased both kinetic constants. In addition, the phospholipid content of the several preparations is shown in Table 3. It is obvious that treatment with ammonium sulphate results in nearly complete depletion of phospholipid from the deiodinase. After ammonium sulphate fractionation approximately 97% of phospholipid remained in solution. The supernatant contained 49 μg lipid phosphorus per mg protein.

Table 3. K_M AND V_{MAX} VALUES FOR RT_3 AND PHOSPHOLIPID CONTENT OF MEMBRANE-BOUND AND SOLUBILISED RAT LIVER IODOTHYRONINE DEIODINASE ACTIVITY. Values are average of three separate experiments.

Origin of enzyme	Apparent K_m	V_{max}	Phospholipid content
	µM	units/mg protein	µg lipid phosphorus/mg protein
microsomal fraction	0.10	0.36	21.8
W-1 ether extract	0.09	0.26	12.3
cholate extract	0.11	0.29	11.8
ammonium sulphate extract	0.69	4.09	1.0

Storage of the W-1 ether extract for 23 weeks at 4^{o} C resulted in a 97% loss of deiodinase activity (Table 4). Ethylene glycol in concentrations of 20-50% (v/v) partially prevented this loss. After storage under the same conditions in 50% ethylene glycol, remaining activity was 25%. Storage in the frozen state at -25^{o} C proved to be a better means to keep enzyme activity, with only 15 and 20% loss after 15 and 25 weeks, respectively. However, after 15 weeks at -25^{o} C deiodinase activities of the cholate and ammonium extracts had fallen to 20 and 30%, respectively. At -25^{o} C no protection was provided by ethylene glycol.

Table 4. EFFECT OF ETHYLENE GLYCOL ON THE STABILITY OF W-1 ETHER SOLUBILISED IODOTHYRONINE DEIODINASE ACTIVITY.

Ethylene glycol	Activity after 23 weeks at 4^{o}C	Activity after 25 weeks at -25^{o}C
%	% of original	
-	3	80
20	8	81
30	14	77
40	20	78
50	25	78

Effects of phospholipids

Treatment of the delipidated deiodinase in the ammonium sulphate extract with several classes of phospholipid showed that phosphatidylserine inhibited the 5'-deiodinase activity strongest (Fig. 3). Phosphatidylcholine and -ethanolamine inhibited the enzyme to a lesser extent, while phosphatidylinositol was without effect. On the other hand, sphingomyelin stimulated 5'-deiodinase activity. Similar effects of these phospholipids were observed on T_3 5-deiodinase activity (not shown).

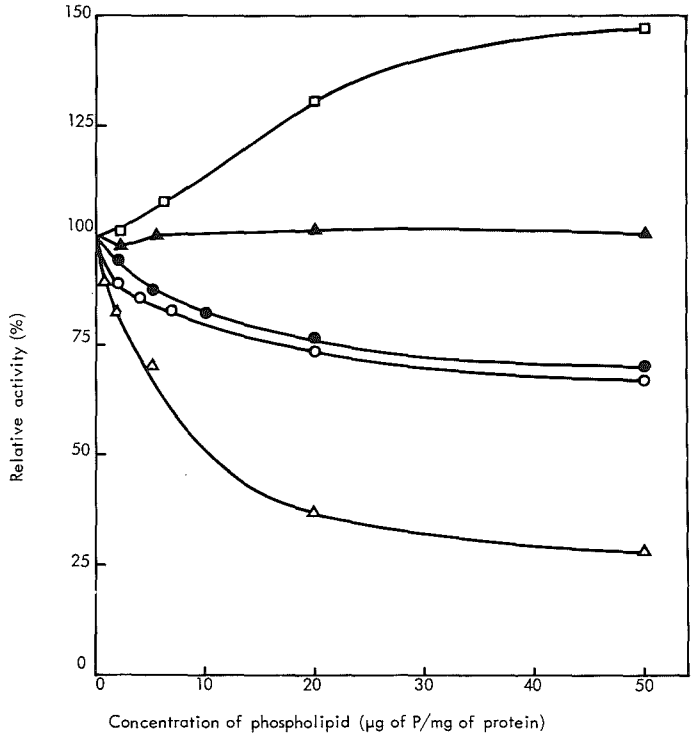

Fig. 3. Effect of phospholipids on rT_3 5'-deiodinase activity.
The ammonium sulphate extract was treated with various concentrations of phosphatidylcholine(o), phosphatidylethanolamine (●), phosphatidylserine (Δ), phosphatidylinositol (▲) and sphingomyeline (□) as described in the Materials and Methods. Enzyme activity was assayed by the standard method and the values were expressed as a percentage of the activity of untreated enzyme.

DISCUSSION

None of the chromatographic methods tested resulted in a significant purification of iodothyronine deiodinase activity. Even affinity chromatography on T_4- and T_3-Sepharose failed to separate this activity from other proteins. In case of T_4-Sepharose, enzyme activity as well as the bulk of protein were bound very strongly. After elution with 50% ethylene glycol only 3% of the enzyme and 35% of protein could be recovered. In this regard T_4-Sepharose behaved similarly as octyl-Sepharose. On the other hand, 71% of activity and 35% of protein could be eluted from T_3-Sepharose with 50% ethylene glycol. This may reflect the higher affinity of the enzyme for T_4 than for T_3, which is in accordance with the K_m values of these substrates for the deiodinase (16).

The results of the chromatography on DEAE-Sephacel are similar to those obtained previously with DEAE-Sepharose (13). Chromatography on activated thiol-Sepharose did not result in an appreciable enrichment of deiodinase activity, somewhat in contrast to the meagre 3-fold purification previously reported (13). The reason for this is unclear, but slight differences in sample preparation cannot be excluded. The strong adsorption of the deiodinase to phenyl- and octyl-Sepharose suggests that the enzyme is a hydrophobic membrane protein.

Since 2-thiouracil and 6-propyl-2-thiouracil are strong inhibitors of enzymatic deiodination (22), we decided to prepare an affinity column by coupling 2-thiouracil-6-propionic acid to Sepharose. As it is known that substrate is required for the inhibition of iodothyronine deiodinase by thiouracil (23,24), 3',5'-T_2 was incorporated in the equilibration buffer. However, approximately 50% of the enzyme was adsorbed to this column and only 7% could be eluted with high DTT concentrations. Even after batchwise incubation of the W-1 ether extract for 1 h at 37° C, less than 50% of the activity was bound to thiouracil-Sepharose of which only a few percent could be recovered after incubation for 1 h at 20° C with 20 mM DTT to reduce the enzyme-thiouracil mixed disulphide.

Only the chromatographies on DEAE-Sephacel, CM-Sepharose, Blue-Sepharose, Matrex gel red A and T_3-Sepharose yielded 2- or 3-fold purification of deiodinase activity. However, these findings should be interpreted with caution, because removal of any inhibiting substance during chromatography leading to an apparent purification cannot be ruled out. The 10-fold purification of the enzyme in the ammonium sulphate extract over the cholate extract is not only due to loss of non-enzyme protein, but also to loss of some inhibitor, because the overall recovery of deiodinase activity was over 300%.

An indication for the kind of inhibitor removed by ammonium sulphate fractionation came from the isoelectric focusing experiments. Focusing of the W-1

ether extract yielded two activity peaks with relatively low isoelectric points (Fig. 1). However, most of the activity in the ammonium sulphate extract was focused around pH 9.3 (Fig. 2). This rise in isoelectric point indicates that ammonium sulphate treatment results in removal of some acidic inhibitor. As most phospholipids are acidic substances which may influence the activity of membrane-bound enzymes, we decided to determine total phospholipid in the several preparations. Indeed, most of the phospholipid was found in the supernatant after ammonium sulphate fractionation and the phospholipid: protein ratio was 12 times higher in the W-1 ether extract than in the ammonium sulphate extract (Table 3). The K_m and V_{max} of rT_3 5'-deiodination in the delipidated ammonium extract were also greatly altered. Thus, delipidation results in increases of the isoelectric point and the maximal deiodination rate, but, unfortunately, in a less stable enzyme preparation.

Addition of some phospholipids to the ammonium sulphate extract leads to an inhibition of deiodinase activity (Fig. 3). Of these lipids phosphatidylserine appeared to be the strongest inhibitor. However, no significant difference in inhibitory activity between phosphatidylcholine, -ethanolamine and -serine was observed when these compounds were tested at a phospholipid: protein ratio equivalent to that in the microsomal membrane of rat liver (25). Phosphatidylinositol and sphingomyelin showed under these circumstances no effect. On the other hand, the phospholipid composition of the solubilised preparations needs not be similar to that of the microsomal fraction as exposure to mild detergents may release proteins in association with specific phospholipids not representative of the total membrane lipid. Consequently, the activity of the deiodinase in the membrane of the endoplasmic reticulum may be regulated in part by its association with different phospholipids.

Our stability studies suggest that phospholipids also have a stabilising effect on deiodinase activity, whereas Leonard and Rosenberg (26) found that iodothyronine 5'-deiodinase showed an absolute requirement for phospholipid. Apparently, the W-1 ether we used for solubilisation of the enzyme in the ammonium sulphate pellet can substitute in part for phospholipid to provide a suitable matrix for deiodinase activity. Clearly, this does not hold for both deoxycholate, the detergent used by Leonard and Rosenberg (26) and cholate, which we also employed.

Studying the effects of soybean phospholipid, Leonard and Rosenberg (26) obtained evidence that besides an absolute dependence of deiodinase activity on phospholipids, these substances may also apparently inhibit deiodination by the sequestration of substrate. This latter effect may have contributed to the decreased deiodination rates we observed after addition of some phospholipids.

The enrichment in deiodinase activity after chromatography of the W-1 ether

extract on Matrex gel red A may also be due to removal of phospholipids, as the isoelectric point of this preparation was approximately 9. The differences in behaviour on DEAE-Sephacel and CM-Sepharose between the deiodinase activity in the W-1 ether extract and in the ammonium sulphate extract square completely with the higher isoelectric point of the enzyme in the latter preparation.

The ultimate enzyme preparation obtained after repeated isoelectric focusing of the ammonium sulphate extract appeared to be relatively pure as judged by electrophoresis in the presence of sodium dodecyl sulphate. However, the specific activity was increased only 3-fold, which may be owing to the lability of the enzyme.

Under all conditions used in the present study iodothyronine 5- and 5'-deiodinase activities appeared to be affected in identical fashions. Neither chromatography on various columns nor isoelectric focusing of the different enzyme preparations resulted in a dissociation of the two activities. This is strong support of previous, circumstantial evidence that both deiodinations are mediated by a single enzyme entity (15). Thus, among other things, both 5- and 5'-deiodinase activities have been localized in the same subcellular fraction of rat liver (endoplasmic reticulum) (9,10,27), have very similar cofactor requirements (thiols) (3), are inhibited in the same way by thiouracil (uncompetitive with substrate, competitive with cofactor) (2,3) and are inhibited to the same extents by a number of competitive inhibitors (radiographic contrast agents and iodothyronine analogues) (15). The latter point is further substantiated by the finding that the K_m values for substrates undergoing 5'-deiodination are equal to the K_i values in their inhibition of the 5-deiodination of other substrates and vice versa.

It should be emphasized that the above conclusion only applies to rat liver. Recently, Chopra et al (28) arrived at the same conclusion on the basis of similar, indirect evidence. These findings make it implausible to explain the low T_3 syndrome (see Introduction) solely on the basis of changes in the deiodinase activity of the liver and, perhaps, the kidneys.

It has, however, been demonstrated that in other tissues such as rat anterior pituitary (29,30), rat brain (31) and rat and human placenta (32,33), enzymes specific for the inner or the outer ring of iodothyronines do exist. It is not excluded that the 5-deiodinase activity of the liver enzyme is not expressed in vivo and that most 5-deiodinations occur in extrahepatic tissues.

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CHAPTER 7

ONE ENZYME FOR THE 5′-DEIODINATION OF 3,3′,5′-TRIIODOTHYRONINE AND 3′,5′-DIIODOTHYRONINE IN RAT LIVER

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Abstract—Many studies suggest that one enzyme is involved in the phenolic ring deiodination of iodothyronines in rat liver and kidney and another one in the tyrosyl ring deiodination. This study describes some characteristics of the phenolic ring (5′-) deiodination of rT_3 and 3′,5′-T_2 by rat liver microsomes. At pH 7.2 the K_m values of the 5′-deiodination of rT_3 and 3′,5′-T_2 were 0.103 and 0.77 μM, respectively. 3′,5′-T_2 and rT_3 inhibited the respective 5′-deiodination reactions competitively, the K_i values being 1.05 and 0.134 μM, respectively. Several radiographic contrast agents markedly inhibit the 5′-monodeiodination of rT_3 and 3′,5′-T_2, the type of inhibition being competitive. Of these compounds iopanoic acid, ipodic acid and iophenoxic acid are the most potent inhibitors with K_i values of approximately 2 μM for both reactions. The non-iodine containing compound 8-anilino-1-naphthalene sulphonic acid (ANS) appeared to be a very strong competitive inhibitor of both 5′-deiodinations (K_i 4.3–4.7 μM), whereas salicylic acid, which as ANS inhibits the binding of iodothyronines to T_4-binding globulin, inhibited these reactions to a much lesser extent (K_i 300–500 μM). On the other hand, diiodosalicylic acid was a very strong inhibitor. The β-adrenergic blocker D,L-propranolol was a weak noncompetitive inhibitor of both 5′-deiodinations (K_i 0.4–0.7 mM). These reactions were also inhibited by various 2,6-diiodophenol derivatives, triiodophenol being the strongest and diiodotyrosine the weakest inhibitor tested. Comparing the K_i values of various inhibitors for the 5′-deiodination of rT_3 and 3′,5′-T_2, a positive correlation between these values was found ($r = 0.97$). It was concluded that rT_3 (to 3,3′-T_2) and 3′,5′-T_2 (to 3′-T_1) monodeiodinating activities are very similar to each other and that there may just be one monodeiodinase catalyzing the 5′-deiodination of iodothyronines in rat liver.

Deiodination of T_4† is an important pathway for the production of T_3, rT_3 and lower substituted iodothyronines [1–3]. The deiodination reactions in rat liver are catalyzed by iodothyronine deiodinase activity, which is associated with the endoplasmic reticulum [4–6]. Since the deiodination is a reductive process and is enhanced by thiol compounds, reduced glutathione is thought to be the endogenous cofactor for this reaction [7–9].

Two types of deiodination reactions may be distinguished, viz. deiodination of the phenolic ring (5′- or 3′-deiodination) and deiodination of the tyrosyl ring (5- or 3-deiodination). Many investigators suggest that 5- and 5′-deiodinations in rat liver and kidney are mediated by separate enzymes, viz. an iodothyronine 5- and 5′- deiodinase, respectively [8, 10, 11]. However, subcellular fractionation [4], as well as solubilization and partial purification [12]‡ did not result in separation of the 5- and 5′-deiodinating activities. In this study, the 5-deiodination of rT_3 and 3′,5′-T_2 by rat liver microsomes has been studied to establish whether the effect of various inhibitors is similar by comparing the inhibitor constants on these reactions. The results in the present paper suggest that in rat liver one iodothyronine deiodinase catalyzes the 5′-monodeiodination of rT_3 and 3′,5′-T_2.

MATERIALS AND METHODS

Materials. All iodothyronines and thyronine except for 3-T_1 were purchased from Henning GmbH (Berlin, F.R.G.). 3-T_1 was obtained by courtesy of Dr. P. Block, Jr. (University of Toledo, OH, U.S.A.). Iopanoic acid (Telepaque) and sodium tyropanoate (Bilopaque) were kindly provided by Sterling Winthrop Laboratories (New York, NY). Sodium ipodate (Oragrafin), amidotrizoic acid (Urografin), iotroxic acid (Biliscopin), ioglycamic acid (Bilivistan) and iodipamide (Biligrafin) were a gift from Schering (Berlin, F.R.G.). Diatrizoic acid (Angiografin), iocetamic acid (Cholebrin), iodamide (Urombrin), metrizoic acid (Isopaque), acetrizoic acid (Plexombrine) and iophenoxic acid (Trilombrine) were kindly supplied by the Research Lab-

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† Abbreviations: T_4, thyroxine; T_3, 3,5,3′-triiodothyronine; rT_3 (reverse T_3), 3,3′,5′triiodothyronine; 3′,5′-T_2, 3′,5′-diiodothyronine; 3,3′-T_2, 3,3′-diiodothyronine; 3,5-T_2, 3,5-diiodothyronine; 3′-T_1, 3′-monoiodothyronine; 3-T_1, 3-monoiodothyronine; DTT, dithiothreitol; ANS, 8-anilino-1-naphthalene sulphonic acid.

‡ Fekkes, Hennemann and Visser, in preparation.

..ratories of Dagra (Diemen, Holland). Salicylic acid was purchased from B.D.H. (Poole, U.K.), sulfosalicylic acid from Pierce Chemical Company (Rockford, IL) and D,L-propranolol from I.C.I. (Macclesfield, U.K.). The following substances were obtained from Sigma (St. Louis, MO): 3,5-diiodotyrosine, 3,5-diiodosalicylic acid, DTT and ANS. 2,4,6-Triiodophenol, 2,6-diisopropylphenol and 3,5-diiodo-4-hydroxybenzoic acid were purchased from I.C.N. Pharmaceuticals (Plainview, NY) and 2,6-diiohydroquinone and 2,6-diiodo-4-nitrophenol were from Pfaltz & Bauer (Stamford, CT).

Preparation of rat liver microsomes. Male Wistar rats weighing approx. 200 g were used. Preparation of rat liver microsomal fraction in 25 mM Tris/HCl–3 mM EDTA–3 mM DTT (pH 7.4) was done essentially as described before [7]. The protein content of this fraction was measured after solubilization in 0.5 M NaOH, by using the method of Spector [13] with bovine serum albumin as a standard.

Deiodination studies. Conversion of rT_3 and T_3 into 3,3′-T_2 and of 3′,5′-T_2 into 3′-T_1 by rat liver microsomal fraction were studied essentially as by Visser *et al.* [11]. In short, the iodothyronine was reacted with microsomes (5–15 μg protein/ml) and various substances to be tested for 10–20 min at 37° in 0.1 M sodium phosphate–3 mM EDTA–3 mM DTT (pH 6.5 or 7.2), unless stated otherwise. The reaction was stopped by the addition of 4 vol. 0.06 M barbitone buffer (pH 8.6 at 20°) containing 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.1% (w/v) bovine serum albumin. The amounts of 3,3′-T_2 and 3′-T_1 produced were measured in duplicate with a specific radioimmunoassay in 50 μl of the extract [14, 15]. The amounts of 3,3′-T_2 and 3′-T_1 measured varied between 2 and 200 pg/tube (detection limit for both iodothyronines is 1 pg/tube). Cross-reactivities of the precursors and of the various agents tested with the products in the radioimmunoassay were in most instances negligible [14, 15]. Only in the case of the effect of rT_3 on the 5′-deiodination of 3′,5′-T_2, the amount of 3,3′-T_2 produced from rT_3 cross-reacted considerably in the radioimmunoassay of 3′-T_1. In this special case, different amounts of 3,3′-T_2 were included in the 3′-T_1 standards (this resulted in a modified 3′-T_1 standard curve), both the amounts of 3,3′-T_2 generated from rT_3 and of 3′-T_1 from 3′,5′-T_2 were measured and the actual amount of 3′-T_1 formed was gathered from the modified 3′-T_1 standard curve. In control experiments substrate was added only after the addition of the barbitone–SDS buffer. The amounts of 3,3′-T_2 and 3′-T_1 measured in the control experiments were less than 5 and 20%, respectively of those produced in the complete reaction mixture and were subtracted from these latter values. Under all conditions tested, both 3,3′-T_2 and 3′-T_1 were degraded for less than 5% during the incubation period.

Treatment of data. For the determination of K_m and V_{max} values the straight lines of double reciprocal plots were drawn by the method of least squares applied to unweighted means. In case of competitive inhibition K_i values were estimated by using the equation: apparent $K_m = K_m\,(1 + [I]/K_i)$, where the apparent K_m is -1/intercept on the abscissa in the Lineweaver–Burk plot and $[I]$ the concentration of the inhibitor. In case of noncompetitive inhibition K_i values were determined by using the equation: 1/apparent $V_{max} = 1/V_{max}\,(1 + [I]/K_i)$, where the apparent V_{max} is 1/intercept on the ordinate in the Lineweaver–Burk plot.

RESULTS

Deiodination studies of rT_3 and 3′,5′-T_2. Table 1 shows the K_m, V_{max} and K_i values of the 5′-deiodination of rT_3 and 3′,5′-T_2 at pH 6.5 and 7.2. The results demonstrate that at both pH values 3′,5′-T_2 inhibits the 5′-deiodination of rT_3 competitively with a K_i value being close to its K_m value in the 5′-deiodination to 3′-T_1. rT_3 also inhibits the 5′-deiodination of 3′,5′-T_2 competitively with a K_i value similar to the K_m value of the rT_3 5′-deiodination. The data shown in Table 1 were obtained in the presence of 3 mM DTT. Repetition of these experiments with varying concentrations of DTT yielded 2–3-fold higher K_m and V_{max} values after extrapolating the data to infinite DTT concentrations. The K_m values of DTT varied between 2 and 4 mM for both 5′-deiodinations (results not shown).

Effects of iodothyronines. In Table 2 the K_i values of various iodothyronines for the 5′-deiodination of rT_3 and 3′,5′-T_2 are shown. All inhibitions are competitive and K_i values of these analogs are similar

Table 1. K_m, V_{max} and K_i values for rT_3 and 3′,5′-T_2 5′-deiodinations catalyzed by rat liver microsomal fraction

Substrate	Inhibitor	pH	K_m (μM)	K_i (μM)	V_{max} (nmoles/min/mg protein)
rT_3		6.5	0.063 ± 0.011		0.271 ± 0.113
	3′,5′-T_2			0.42 ± 0.14	
3′,5′-T_2		6.5	0.36 ± 0.07		0.155 ± 0.040
	rT_3			0.033 ± 0.007	
rT_3		7.2	0.103 ± 0.046		0.380 ± 0.107
	3′,5′-T_2			1.05 ± 0.21	
3′,5′-T_2		7.2	0.77 ± 0.41		0.270 ± 0.055
	rT_3			0.134 ± 0.040	

Values are means ± S.E. (n = 6).

Table 2. K_i values of various competitive inhibitors for the 5′-deiodination of rT_3 and 3′,5′-T_3 by rat liver microsomal fraction

Inhibitor	K_i of inhibitor (μM)	
	rT_3-5′-deiodination	3′,5′-T_2-5′-deiodination
rT_3	—	0.13 ± 0.04
3′,5′-T_2	1.0 ± 0.3	—
T_4 (▼)	2.7 ± 1.0	2.4 ± 0.6
T_3	17.3 ± 9.5	19.6 ± 6.1
3,5-T_2	9.6 ± 1.0	10.1 ± 2.3
3-T_1	—*	12.3 ± 2.3
Thyronine	103 ± 46	90 ± 16
Diiodotyrosine	191 ± 107	177 ± 29
ANS	4.7 ± 1.1	4.3 ± 1.2
Salicylic acid	476 ± 191	302 ± 54
Sulfosalicylic acid	114 ± 16	142 ± 30
Diiodosalicylic acid	0.3 ± 0.1	0.4 ± 0.2
Iopanoic acid (○)	1.8 ± 0.5	1.5 ± 0.4
Ipodic acid (●)	2.2 ± 0.8	2.0 ± 0.3
Iophenoxic acid (△)	2.1 ± 0.5	1.8 ± 0.7
Tyropanoic acid (▲)	27.9 ± 6.4	21.5 ± 14.2
Ioglycamic acid (□)	107 ± 26	24.9 ± 9.2
Iodipamide (■)	25.2 ± 5.6	16.2 ± 3.1

Conversion studies were done at pH 7.2.
Values are means ± S.E. ($n = 4$).
Symbols refer to Fig. 1.
* Could not be determined due to experimental problems.

for both deiodinations. In addition diiodotyrosine was tested. It can be seen (Table 2) that this compound is about 100 times less active than T_4 and even two times less active than thyronine.

Effects of radiographic agents. Figure 1 shows the effect of increasing concentrations of various radiographic agents and T_4 on the conversion of rT_3 into 3,3′-T_2. The K_i values of these compounds, except for diatrizoic acid, are given in Table 2. At the same time the K_i values for the 5′-deiodination of 3′,5′-T_2 are shown. All inhibitions with these agents are competitive with respect to substrate. An example of the type of inhibition of iopanoic acid and ipodic acid is shown in Fig. 2. As can be extracted from Fig. 1 and Table 2, 5′-deiodination of rT_3 and 3′,5′-T_2 were inhibited best by iopanoic acid, ipodic acid and iophenoxic acid and to a somewhat lesser

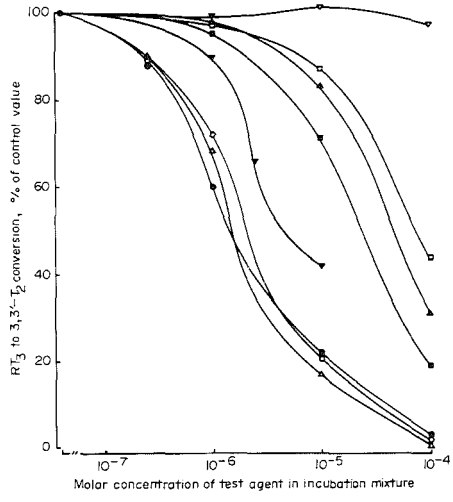

Fig. 1. Inhibition of the conversion of rT_3 into 3,3′-T_2 by increasing concentrations of radiographic agents and T_4 (▼). For explanation of symbols see Table 2; in addition, the effect of diatrizoic acid is shown (△). Conversion studies were done with 0.1 μM rT_3 at pH 7.2. Data are mean of two closely agreeing experiments performed in duplicate.

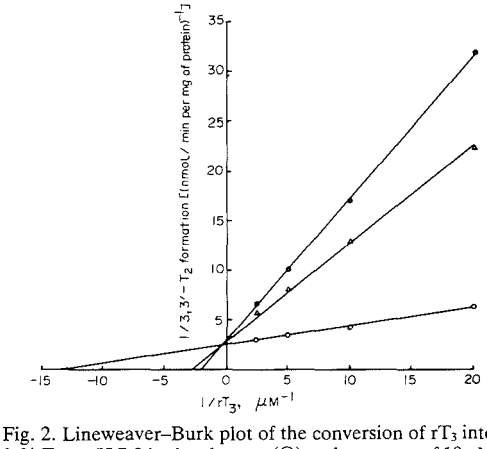

Fig. 2. Lineweaver–Burk plot of the conversion of rT_3 into 3,3′-T_2 at pH 7.2 in the absence (○) and presence of 10 μM iopanoic acid (●) and 10 μM ipodic acid (△). Results are mean of two experiments performed in duplicate.

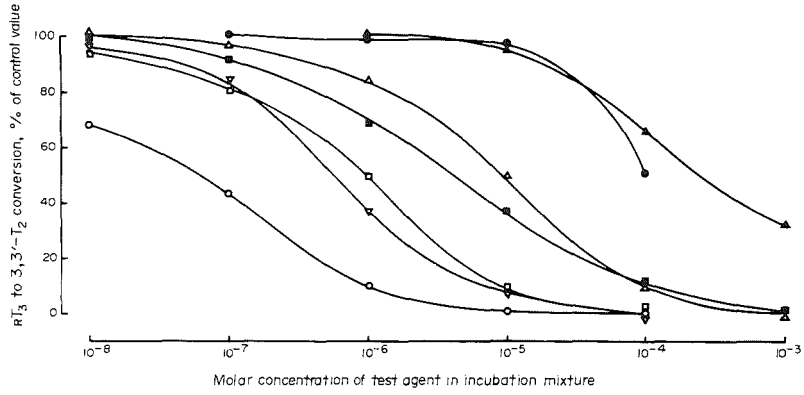

Fig. 3. Inhibition of the conversion of rT_3 into 3,3'-T_2 by increasing concentrations of 2,6-diiodophenol derivatives. Deiodination studies were performed at pH 6.5 using 0.1 μM rT_3. ○, 2,4,6-Triiodophenol: ●, 2,6-diisopropylphenol; ▼, 3,5-diiodosalicylic acid; △, 3,5-diiodo-4-hydroxybenzoic acid; ▲, 3,5-diiodotyrosine; □, 2,6-diiodohydroquinone; ■, 2,6-diiodo-4-nitrophenol. All points represent the mean of three separate determinations.

extent by T_4. There was no significant difference between the inhibitory potency of these three radiographic agents. The radiographic agents amidotrizoic acid, iotroxic acid, iodamide, metrizoic acid and acetrizoic acid inhibited the 5'-deiodination of rT_3 and 3',5'-T_2 for less than 20% at a concentration of 100 μM, while iocetamic acid at this concentration inhibited these reactions for approximately 50% (results not shown).

Effects of other inhibitors. Salicylic acid and ANS, agents known to displace T_4 from serum binding proteins, were competitive inhibitors, the latter being about 100 times more active than salicylic acid (see Table 2). Two derivatives of salicylic acid, viz. sulfosalicylic acid and especially diiodosalicylic acid, have a greater inhibitory activity than salicylic acid (Table 2). Addition of D,L-propranolol, a β-adrenergic blocker commonly used in treating hyperthyroid patients, was found to inhibit both 5'-deiodinations noncompetitively with K_i values of 0.4–0.7 mM.

From the data in Table 2 it can be deduced that the K_i values of the various inhibitors for the 5'-deiodination of rT_3 correlate positively with those for the 5'-deiodination of 3',5'-T_2 ($r = 0.97$).

Figure 3 shows the effect of increasing concentrations of 2,6-diiodophenol derivatives on the conversion of rT_3 into 3,3'-T_2. The inhibitory activity is strongly dependent on the substituent at C_4, iodine being the best of the substituents tested. The order of activity was I ≫ OH > NO_2 > COOH ≫ alanine. Unfortunately, 2,6-diiodophenol was not available. Instead, 2,6-diisopropylphenol was tested, the activity of which was found to be similar to that of diiodotyrosine (see Fig. 3).

DISCUSSION

In vitro studies of the enzymatic reductive deiodination of iodothyronines in rat liver and kidney have led to the hypothesis that T_4 is sequentially deiodinated by two enzymes, viz. an iodothyronine 5- and

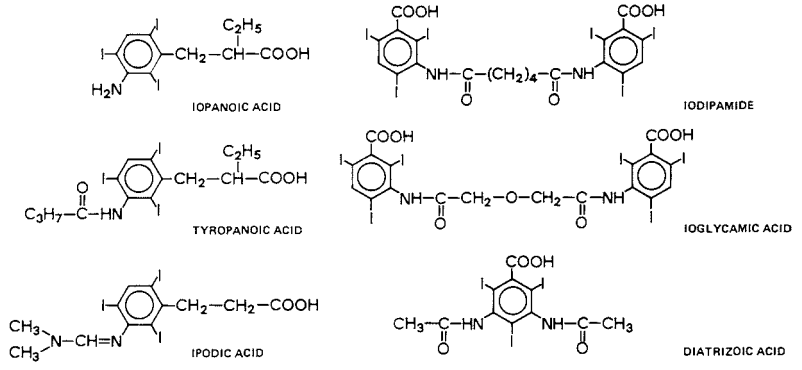

Fig. 4. Structures of some radiographic contrast agents.

5′-deiodinase [8, 10, 11]. The fact that T_4 and rT_3 are 5′-monodeiodinated by the same enzyme has been confirmed by several investigators [10, 11, 16, 17]. Recently, Chopra [18] showed that 3′,5′-T_2 and 3,3′-T_2 are similarly substrates for a common 5′-deiodinase, and the involvement of a single enzyme in the 5′-deiodination of rT_3 and 3′,5′-T_2 has also been suggested by Visser [15]. In this study a similarity of the K_m and K_i for rT_3, and the K_m and K_i for 3′,5′-T_2 at two pH values was found. This is a strong indication that a single hepatic enzyme catalyzes 5′-monodeiodination of both rT_3 and 3′,5′-T_2. When taking these data together one could conclude that the 5′- (or 3′-) monodeiodinations of T_4, rT_3, 3′,5′-T_2 and of 3,3′-T_2 in rat liver are all mediated by one enzyme. This is strengthened by the fact that the 5′-monodeiodination of rT_3 and 3′,5′-T_2 are inhibited in a similar manner by a number of iodothyronines, several radiographic agents and various other substances as tested in this study. We have chosen radiographic agents, because these iodine-containing compounds have been reported to influence thyroid hormone deiodination *in vivo* [19] and, in addition, some of these agents are potent inhibitors of the liver system converting T_4 to T_3 [2, 10].

We found that iopanoic acid, iopodic acid and iophenoxic acid are very strong competitive inhibitors of the 5′-deiodination of rT_3 and 3′,5′-T_2 with K_i values of approx. 2 μM irrespective of the agent or reaction tested. The inhibitory activity of these compounds was similar to that of T_4 (Table 2). The structure of iophenoxic acid differs from that of iopanoic acid (Fig. 4) in that it contains an amino instead of a hydroxyl group in the *meta* position to the side chain. Clearly this does not affect inhibitory potency. The structural differences between iopanoic acid and iopdic acid are much more pronounced, though this is not reflected by their activity (Fig. 4 and Table 2). Comparing the structures of iopanoic acid, tyropanoic acid and ipodic acid (Fig. 4), one should expect tyropanoic acid to be about as active as the other compounds. However, tyropanoic acid appeared to have less than a tenth of the activity of iopanoic acid and ipodic acid (Table 2). As can be gathered from Fig. 4 this difference is situated in the acyl group of tyropanoic acid, which strongly diminishes the electron-donating properties of the amino group. The activities of iodipamide and ioglycamic acid are comparable to that of tyropanoic acid (Table 2). These compounds are also structurally related to tyropanoic acid as they contain two *N*-acyl triiodobenzoic acid groups (Fig. 4). Addition of a second *N*-acetyl amino group to such a ring as in diatrizoate (Fig. 4) results in a virtually inactive structure (Fig. 1). As can be concluded from Table 2 all inhibitors, except for ioglycamic acid, have similar activity towards the 5′-deiodination of rT_3 and 3′,5′-T_2. An explanation for the difference in inhibitory activity of ioglycamic acid cannot be given. Deletion of the side-chain of iophenoxic acid yields 2,4,6-triiodophenol, a compound which is even more inhibitory to the 5′-deiodinase than the most potent radiographic agent (see Figs. 1 and 3). Replacement of the 4-iodine atom by a nitro group, a carboxyl group or by an alanine side-chain (yielding diiodotyrosine) strongly reduces inhibitor activity. Replacement by a hydroxyl group (yielding diiodohydroquinone) affects the inhibitory potency least (Fig. 3). Very interesting is the difference in inhibitory activity between 3,5-diiodo-4-hydroxybenzoic acid and 3,5-diiodosalicylic acid (3,5-diiodo-2-hydroxybenzoic acid), as shown in Fig. 3. The only difference in structure is the position of the hydroxyl group. The reason for the high activity of diiodosalicylic acid is unclear. It does, however, closely resemble the highly potent triiodophenol from which it is derived by substituting COOH for I in one of the *ortho* positions to the OH group.

We found that ANS is a very strong competitive inhibitor (K_i ~4 μM) of iodothyronine 5′-deiodination. This is in discordance with previous reports [20, 21] on 100–1000-fold higher values for the K_i of ANS. However, these studies were performed with homogenate containing an abundance of ANS binding proteins [3], whereas we used low amount of microsomal fraction. On the other hand, our data concerning salicylic acid are in agreement with these other studies [20, 21]. The effects of D,L-propranolol on the 5′-deiodination are contradictory [20, 22, 23]. We found that this compound is a weak noncompetitive inhibitor (K_i 0.4–0.7 mM) of the 5′-deiodination of rT_3 and 3′,5′-T_2 and is therefore of no importance for the *in vivo* inhibition of the deiodination, because the plasma levels of D,L-propranolol during treatment of hyperthyroid patients never exceed a concentration of 0.6 μM [25].

In conclusion, our results strongly suggest that a single hepatic enzyme catalyzes the 5′-deiodination of rT_3 and 3′,5′-T_2. Consequently, all iodothyronines may be 5′-monodeiodinated by one enzyme in rat liver.

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CHAPTER 8

INHIBITION OF IODOTHYRONINE DEIODINASE BY PHENOLPHTHALEIN DYES

Structure–activity relationship

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1. Introduction

It has been found that several iodine-containing radiographic contrast agents are potent inhibitors of the 5′-deiodination of thyroxine (T_4) to 3,3′,5-triiodothyronine (T_3) in peripheral tissues in vivo [1] as well as in vitro [2,3]. In rat liver microsomes the 5′-deiodination of 3,3′,5′-triiodothyronine (rT_3) to 3,3′-diiodothyronine (3,3′-T_2) and of 3′,5′-diiodothyronine to 3′-monoiodothyronine as well as the 5-deiodination of T_3 to 3,3′-T_2 are also inhibited by these radiographic agents [4,5]. It is still uncertain whether a single enzyme mediates both types of deiodination or that two separate enzymes are involved, i.e., iodothyronine 5- and 5′-deiodinase [5,6].

During attempts to purify the enzyme(s) from rat liver by electrophoresis, it was noted that the tracking dye bromophenol blue strongly inhibited deiodination. This compound is structurally related to iodothyronines and several X-ray contrast agents in that it also contains two halogen substituents in the ortho positions to an electron-donating group (OH or NH_2). It was therefore thought of interest to study the structure-activity relationship of phenolphthalein derivatives as inhibitors of iodothyronine 5′-deiodination. Another point of consideration for this study was the wide use of these compounds as acid-base indicators. It was found that bromophenol blue is a very strong competitive inhibitor of iodothyronine 5′-deiodinase activity. At the same time sulfobromophthalein (BSP) and bilirubin, both compounds known to displace T_4 and T_3 from cytosol binding proteins [7], were found to inhibit the 5′-deiodination of rT_3.

2. Materials and methods

2.1. *Materials*

Phenolphthalein, chlorophenol red, thymol blue, cresol red, bromocresol purple, 2-bromophenol, 2-iodophenol and BSP were purchased from Merck, Darmstadt; bromophenol blue, bromothymol blue and bromocresol green from BDH Chemicals, Poole; bilirubin from Fluka AG, Buchs; 2,4,6-tribromophenol and 2,4,6-triiodophenol from ICN Pharmaceuticals Inc., Plainview, NY; phenol red from Serva, Heidelberg; 2,6-dibromophenol from Eastman Kodak Co., Rochester, NY; rT_3 and 3,3′-T_2 from Henning GmbH, Berlin.

2.2. *Methods*

Deiodination of rT_3 to 3,3′-T_2 by rat liver microsomal fraction was studied essentially as in [8]. In short, 0.1 μM rT_3 was reacted with 2-5 μg microsomal protein and various substances to be tested in 0.25 ml 0.06 M phosphate, 3 mM EDTA and 1 mM dithiothreitol (pH 6.5). After incubation for 10-20 min at 37°C, the reaction was stopped by the addition of 1 ml 0.06 M barbitone buffer containing 0.1% bovine serum albumin and 0.1% SDS (pH 8.6). The amount of 3,3′-T_2 produced was measured with a specific radioimmunoassay in 50 μl of the extract [9]. All values were corrected for non-enzymatically formed 3,3′-T_2 by incorporating appropriate controls, as previously described [8]. Under all conditions tested, less than 5% of added 3,3′-T_2 was degraded during the incubation period. Moreover, it was found that in all experiments the amount of 3,3′-T_2 formed is equivalent to the amount of rT_3 disappearing from the system. Consequently, nonenzymatic deiodination was not promoted by the various phenolphthalein dyes tested.

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For the determination of K_i values, conversion studies were done at pH 7.2 in the presence of 3 mM dithiothreitol and the straight lines of double-reciprocal (Lineweaver-Burk) plots were drawn by the method of least squares applied to unweighted means. Estimation of the K_i value was done by using the equation: apparent $K_m = K_m (1 + [I]/K_i)$, where the apparent K_m is -1/intercept on the abscissa in the Lineweaver-Burk plot and [I] the concentration of the inhibitor.

3. Results

Figure 1 shows the effect of increasing concentrations of various phenolphthalein derivatives on the conversion of rT_3 into 3,3'-T_2. The inhibitory activities and the structures of these compounds are given in table 1. Replacement of the carboxyl group in phenolphthalein by sulfonic acid, yielding phenol red, does not alter inhibitory activity. Addition of methyl (Me) groups to the 5′ and 5″ positions of phenol red, yielding cresol red, does not affect inhibitory activity, whereas addition of Cl, yielding chlorophenol red, enhances activity sixfold. Addition of Br to the 3′ and 3″ positions of cresol red, yielding bromocresol purple, strongly increases activity, and so does further substitution of Br for Me in bromocresol purple as well as for isopropyl (iPr) in bromothymol blue, yielding bromophenol blue and bromocresol green, respectively. Comparing the latter compounds, it is obvious that Me in the 2′ and 2″ positions has a deleterious effect. Despite the presence of this Me group in thymol blue its activity is similar to chlorophenol red and tenfold higher than phenol red, demonstrating the favourable effect of iPr in the 5′ and 5″ positions. (It should be noted that in the absence of 2′ and 2″ substituents, positions 3′ and 3″ are equivalent with 5′ and 5″). The negative logarithm of the ID_{50} (pID_{50}) of the phenolphthalein derivatives was inversely correlated with their pK values ($r = -0.84$; $p < 0.005$). In fig.2 the effect of three bromine-containing phenolphthalein derivatives on the 5′-deiodination of rT_3 is shown. The results demonstrate that all three compounds inhibit this reaction competitively with rT_3. From the change in apparent K_m for rT_3 the K_i values were calculated (table 2). In addition, BSP and bilirubin were found to be inhibitory to the 5′-deiodination of rT_3, the type of inhibition being competitive. The K_i values for these agents are shown in table 2.

To find out whether bromine attached to a rela-

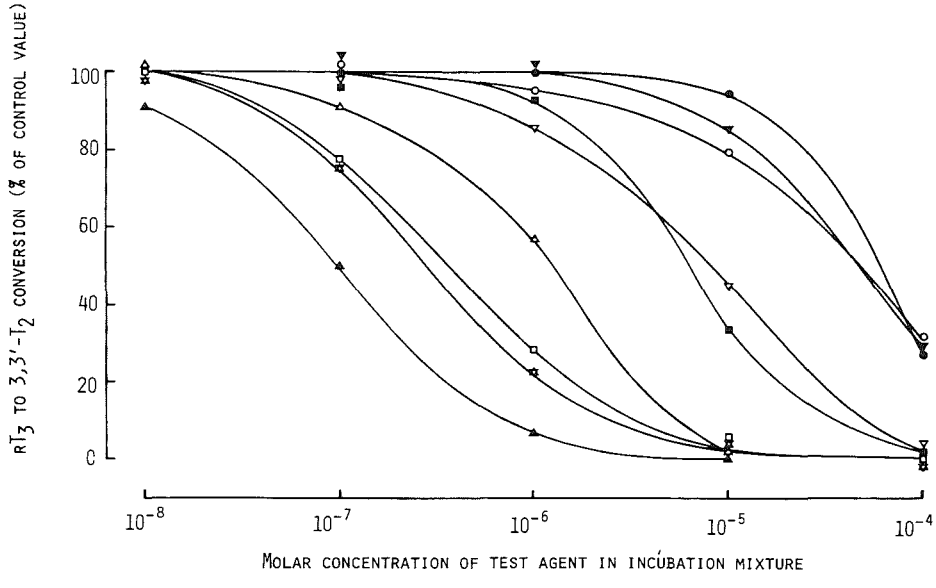
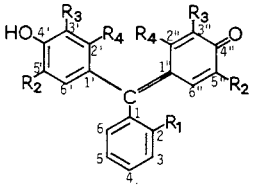


Fig.1. Inhibition of the conversion of rT_3 into 3,3'-T_2 by increasing concentrations of phenolphthalein derivatives. For details see section 2 and for explanation of symbols see table 1. Results are mean of 3 closely agreeing experiments performed in duplicate.

Table 1
Inhibitory activity of phenolphthalein derivatives



		R_1	R_2	R_3	R_4	pID_{50}	pK
○	phenolphthalein	COO^-	H	H	H	4.3	9.6
●	phenol red	SO_3^-	H	H	H	4.2	7.9
▽	chlorophenol red	SO_3^-	Cl	H	H	5.1	6.0
▼	cresol red	SO_3^-	CH_3	H	H	4.3	8.3
▫	bromocresol purple	SO_3^-	CH_3	Br	H	6.4	6.3
▪	thymol blue	SO_3^-	$CH(CH_3)_2$	H	CH_3	5.2	8.9
△	bromothymol blue	SO_3^-	$CH(CH_3)_2$	Br	CH_3	5.9	7.0
▲	bromophenol blue	SO_3^-	Br	Br	H	7.0	4.0
⧈	bromocresol green	SO_3^-	Br	Br	CH_3	6.5	4.7

pID_{50}: the negative logarithm of the concentration giving 50% inhibition of deiodinase activity. Symbols refer to fig.1.

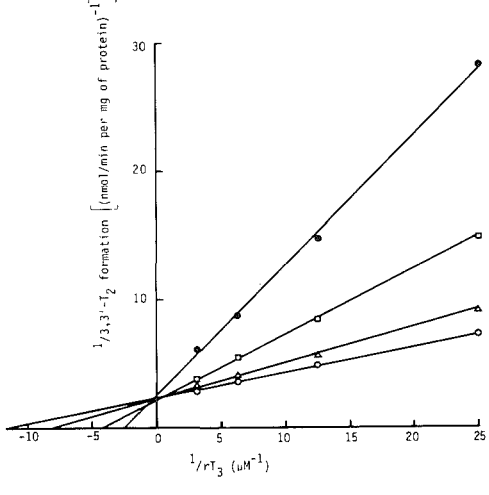


Fig.2. Lineweaver-Burk plot of the conversion of rT_3 into 3,3'-T_2 at pH 7.2 in the absence (○) and presence of 0.2 μM bromophenol blue (●), 0.4 μM bromothymol blue (△) or 0.2 μM bromocresol green (▫). For details see section 2. Results are means of 3 experiments performed in duplicate.

tively simple aromatic ring is also inhibitory or that a more complicated structure is required, 2-bromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol were tested. The results are shown in fig.3 and are compared with the effect of 2-iodophenol, 2,6-diisopropylphenol (unfortunately, 2,6-diiodophenol was not available) and 2,4,6-triiodophenol. This last mentioned agent appeared to be a very strong inhibitor [4].

Table 2
K_i values of bromine-containing phenolphthalein derivatives and bilirubin in the 5'-deiodination of rT_3

Inhibitor	K_i of inhibitor (μM)
bromophenol blue	0.04 ± 0.01
bromothymol blue	1.36 ± 0.49
bromocresol green	0.11 ± 0.01
BSP	1.33 ± 0.48
bilirubin	2.12 ± 0.94

The K_m of the 5'-deiodination of rT_3 was 0.10 ± 0.04 μM. The type of inhibition is competitive. Values are means ± SE (n = 4)

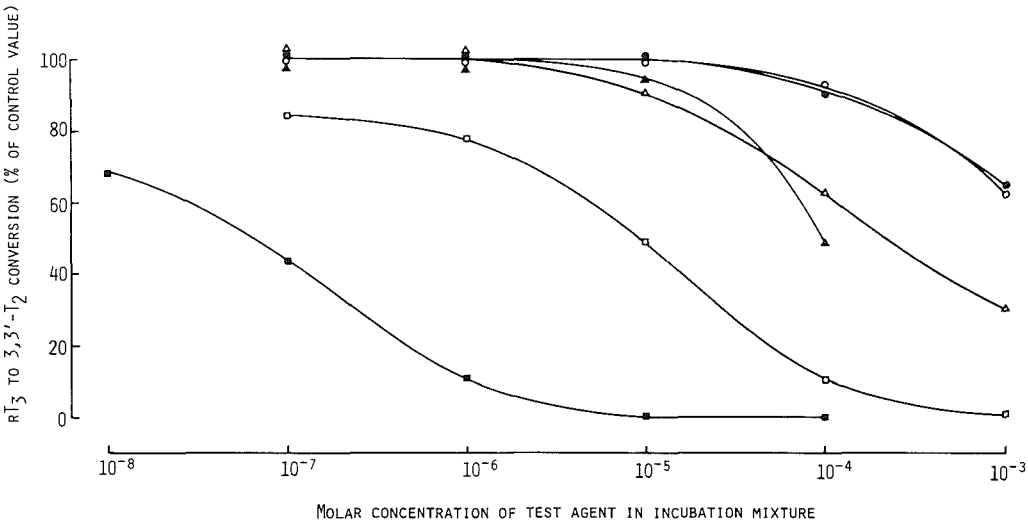


Fig.3. Inhibition of the conversion of rT_3 into 3,3'-T_2 by increasing concentrations of 2-bromophenol (○), 2-iodophenol (●), 2,6-dibromophenol (△), 2,6-diisopropylphenol (▲), 2,4,6-tribromophenol (□) and 2,4,6-triiodophenol (■). For details see section 2. Results are mean of 2 closely agreeing experiments performed in duplicate.

4. Discussion

The present study indicates that 5'-monodeiodination of rT_3 is strongly inhibited by phenolphthalein derivatives. Inhibitory potency of the various dyes is enhanced, in this order, by Me, Cl, iPr and Br substituents in the ortho positions of either phenolic ring. The most active compound tested is the tetra-bromo derivative bromophenol blue. The presence of a methyl group in the meta position has a deleterious effect. All bromine-containing phenolphthalein derivatives are competitive inhibitors of the rT_3 5'-deiodination (fig.2, table 2).

The reason why bromophenol blue is such a strong competitive inhibitor of iodothyronine 5'-deiodination is unclear. Part of the structure of this dye is similar to that of T_4 and rT_3, viz. the 3,5-dihalogen-4-hydroxyphenol group. However, comparing the activity of 2,4,6-tribromophenol with that of 2,4,6-triiodophenol, it is clear that for a proper fit on the enzyme I is a better substituent than Br. This is in agreement with the finding that iodothyronines are better substrates for the deiodinase than bromoiodothyronines [10]. Yet, the K_i for bromophenol blue (0.04 μM) is less than the K_m for rT_3 (0.1 μM). The structure shown in table 1 for phenolphthalein derivatives is that present in solution below the pK of these compounds. Above the pK the rings equilibrate between the quinoid and phenolate resonance structures. Our finding of a negative relationship between the dissociation and inhibition constants for these compounds suggests that the phenolate anion is the preferred form for interaction with the enzyme. This may also explain the increased activity of 2,4,6-tribromophenol compared with 2,6-dibromophenol considering the higher acidity of the OH in the former. An important implication of these findings is that one should be careful with the use of these dyes in deiodination studies. A well known example is the application of bromophenol blue as a marker in protein separation studies. The concentration used in these experiments often amounts to 0.1 mM, which lies far beyond the K_i value (0.04 μM) of this dye. One should also keep in mind the use of phenol red as an indicator in various growth media. This latter compound is mostly present in a concentration of about 0.3 mM, which amount results in more than 80% inhibition of deiodinase activity as can be gathered from fig.1.

BSP, which is used for liver function test and bilirubin, an intermediate in the degradation of hemoglo-

bin, were also tested. It has been shown that BSP and bilirubin can displace both T_4 and T_3 from the cytosol binding proteins Y (ligandin) and Z [7]. However, the affinity of these binding proteins for T_4 and T_3 was much ($\sim 10^4$-times) higher than for BSP. Furthermore, BSP and bilirubin are able to inhibit uptake of T_4 and T_3 by rat liver slices [11] and BSP can displace T_4 from the liver in vivo [12]. BSP is a bromine-containing phenolphthalein derivative with only one bromine-containing phenyl ring, which is substituted with four bromine atoms, while the two remaining positions in this ring are also occupied. Nevertheless, we found BSP to be a relatively strong competitive inhibitor (K_i 1.33 μM) of the 5′-deiodination of rT_3. Bilirubin also inhibited this reaction, though less effectively than BSP (table 2). A reason for the inhibitory activity of the seemingly structurally unrelated bilirubin cannot be given at the moment. The finding that bilirubin inhibits the 5′-deiodination of rT_3 could be of clinical importance. If we take the line that one enzyme is responsible for the 5′-deiodination of all iodothyronines, bilirubin would also be inhibitory to the 5′-monodeiodination of T_4 [4,6]. In hepatic cirrhosis serum T_3 is unusually low relative to T_4, which is probably due to a reduction in peripheral conversion of T_4 to T_3 [3]. This inhibition of the 5′-monodeiodination of T_4 may be caused in part by the increased bilirubin levels.

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CHAPTER 9

Evidence for a single enzyme in rat liver catalysing the deiodination of the tyrosyl and the phenolic ring of iodothyronines

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The enzymic 5'-deiodination of 3',5'-di-iodothyronine and 5-deiodination of 3,3',5-tri-iodothyronine by rat liver microsomal fractions were found to be characterized by apparent K_m values of 0.77 and 17.4 μM respectively. 3',5'-Di-iodothyronine was a competitive inhibitor of 3,3',5-tri-iodothyronine 5-deiodination (K_i 0.65 μM) and 3,3',5-tri-iodothyronine was a competitive inhibitor of 3',5'-di-iodothyronine 5'-deiodination (K_i 19.6 μM). In addition, several radiographic contrast agents and iodothyronine analogues inhibited both reactions competitively and with equal potencies ($r = 0.999$). These results strongly suggest the existence of a single hepatic deiodinase acting on both the tyrosyl and phenolic ring of iodothyronines.

The biologically active form of thyroid hormone, 3,3',5-tri-iodothyronine (tri-iodothyronine), is produced predominantly by enzymic phenolic-ring- or 5'-deiodination of thyroxine in peripheral tissues like the liver and the kidney, whereas the inactive isomer, 3,3',5'-tri-iodothyronine (reverse tri-iodothyronine), is formed by tyrosyl-ring- or 5-deiodination of thyroxine. Both tri-iodothyronines are further degraded by a cascade of deiodinations, with 5'-deiodination to 3,3'-di-iodothyronine as the main initial step in the metabolism of reverse tri-iodothyronine (Visser, 1980).

In a variety of clinical settings a concomitant decrease in tri-iodothyronine production and reverse tri-iodothyronine clearance has been observed, presumably owing to a specific decrease in the 5'-deiodinase activity of the tissues (Braverman & Vagenakis, 1979). This has led to the hypothesis that the sequential deiodination of thyroxine is catalysed by two distinct enzymes, i.e. an iodothyronine 5-deiodinase and an iodothyronine 5'-deiodinase (Schimmel & Utiger, 1977; Visser, 1978). However, subcellular fractionation of rat liver homogenate (Fekkes *et al.*, 1979) and partial purification of detergent extracts of microsomal fractions thereof (Fekkes *et al.*, 1980), have not resulted in a separation of these two activities.

In the present paper we describe the effect of various inhibitors on the 5-deiodination of tri-iodothyronine and the 5'-deiodination of 3',5'-di-iodothyronine by rat liver microsomal fractions. Evidence is obtained that a single enzyme is responsible for both deiodination reactions in rat liver.

Materials and methods

Materials

All iodothyronines and thyronine were from Henning G.m.b.H., Berlin, Germany. Iopanoic acid (Telepaque) and sodium tyropanoate (Bilopaque) were kindly supplied by Sterling–Winthrop Laboratories (Amsterdam, The Netherlands) and iophenoxic acid (Trilombrine) was a gift from the Research Laboratories of Dagra, Diemen, The Netherlands.

Deiodination studies

Rat liver microsomal fraction was prepared as previously described (Fekkes *et al.*, 1979). The protein content of this fraction was mesured after solubilization in 0.5 M-NaOH, by a modification of the method of Bradford (1976), with bovine serum albumin as the standard (Spector, 1978). Reaction mixtures contained 0.1 M-sodium phosphate/3 mM-EDTA/3 mM-dithiothreitol, pH 7.2, 1–10 μM-tri-iodothyronine or 0.4–3.2 μM-3',5'-di-iodothyronine, 2–4 μg of microsomal protein and various inhibitors to be tested, all in a volume of 0.25 ml. The reaction was initiated by addition of microsomal fraction and incubation was carried out for 15 min at 37°C. The reaction was stopped by the addition of 1 ml of 0.06 M-barbitone/0.15 M-NaCl/0.1% (w/v) sodium dodecyl sulphate/0.1% (w/v) bovine serum albumin, pH 8.6. In control experiments, microsomal fraction

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was added after the detergent solution. Each experimental point was determined in duplicate. The amounts of 3,3′-di-iodothyronine (5-deiodination product of tri-iodothyronine) and 3′-iodothyronine (5′-deiodination product of 3′,5′-di-iodothyronine) were measured in duplicate by specific radioimmunoassays in 50 μl of the extract (Visser *et al.*, 1978; Visser & van Overmeeren-Kaptein, 1980).

Data analysis

The amount of product formed was corrected for non-enzymic deiodination by subtraction of the respective control value, which was always less than 10% of enzymic deiodination. Regression lines of the double-reciprocal plots were calculated by unweighted least-squares analysis. K_i values for competitive inhibitors were estimated by using the equation:

$$K'_m = K_m(1 + [I]/K_i)$$

where K_m and K'_m are -1/intercept on the abscissa in the absence or presence of inhibitor respectively, and [I] is the concentration of inhibitor.

Results

The enzymic 5-deiodination of tri-iodothyronine was characterized by a mean apparent K_m value of 17.4 μM, and the 5′-deiodination of 3′,5′-di-iodothyronine by a mean apparent K_m value of 0.77 μM (Table 1). Tri-iodothyronine was a competitive inhibitor of the 5′-deiodination of 3′,5′-di-iodothyronine (Fig. 1), with a mean apparent K_i of 19.6 μM (Table 1). Similarly, 3′,5′-di-iodothyronine was a competitive inhibitor of tri-iodothyronine 5-deiodination (Fig. 2) with a mean apparent K_i of 0.65 μM (Table 1).

As potential inhibitors of enzymic 5- and 5′-deiodination were tested 3,5-di-iodothyronine, thyronine and the radiographic contrast agents iophenoxic acid, iopanoic acid and tyropanoic acid. All these compounds inhibited both tri-iodothyronine 5-deiodination and 3′,5′-di-iodothyronine 5′-deiodination competitively with respect to the substrates (see tyropanoic acid as an example in Figs. 1 and 2). Spanning over two orders of magnitude, inhibitor constants of the different compounds were very similar for the two types of deiodination (Table 1). Among the inhibitors, the 2,4,6-tri-iodophenol derivatives, iophenoxic acid, and the

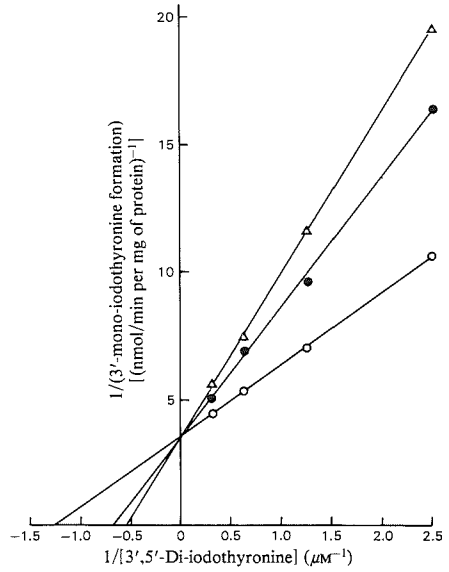

Fig. 1. *Lineweaver–Burk plot of the 5′-deiodination of 3′,5′-di-iodothyronine to 3′-mono-iodothyronine in the absence* (○) *or presence of 20 μM-tri-iodothyronine* (●) *or 30 μM-tyropanoic acid* (△)
For experimental details, see the text. Results shown are means of two closely agreeing values obtained from experiments performed in duplicate.

Table 1. *K_m values for substrates and K_i values for competitive inhibitors of tri-iodothyronine 5-deiodination and 3′,5′-di-iodothyronine 5′-deiodination* (*mean* ± S.D., $n = 4$)

	K_m* or K_i value (μM)	
Compound	Tri-iodothyronine 5-deiodination	3′,5′-Di-iodothyronine 5′-deiodination
Tri-iodothyronine	17.4 ± 4.2*	19.6 ± 6.1
3′,5′-Di-iodothyronine	0.65 ± 0.16	0.77 ± 0.41*
3,5-Di-iodothyronine	11.5 ± 3.1	10.1 ± 2.3
Thyronine	125 ± 29	90 ± 16
Iopanoic acid	1.9 ± 0.6	1.5 ± 0.4
Iophenoxic acid	1.9 ± 0.8	1.8 ± 0.7
Tyropanoic acid	27.2 ± 11.6	21.5 ± 14.2

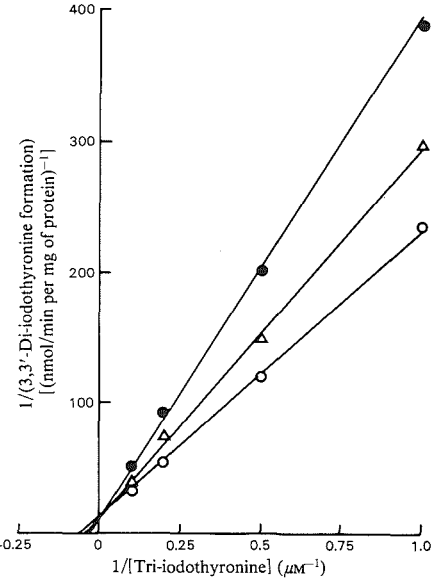

Fig. 2. *Lineweaver–Burk plot of the 5-deiodination of tri-iodothyronine to 3,3'-di-iodothyronine in the absence* (○) *or presence of 0.5 μM-3',5'-di-iodothyronine* (●) *or 20 μM-tyropanoic acid* (△)
For experimental details, see the text. Results are means for three experiments performed in duplicate.

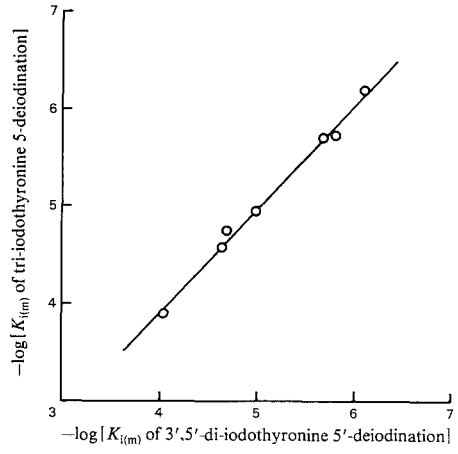

Fig. 3. *Correlation between the negative logarithms of mean apparent K_m and K_i values for substrates and competitive inhibitors of tri-iodothyronine 5-deiodination and 3',5'-di-iodothyronine 5'-deiodination*
For details, see Table 1.

analogous 2,4,6-tri-iodoaniline derivative, iopanoic acid, were the most potent, with apparent K_i value of 1.5–1.9 μM. Thyronine was at least effective, with an apparent K_i of approx. 100 μM.

As a corollary of these findings, Fig. 3 shows the highly significant ($r = 0.999$) correlation between the mean K_m and K_i values for the substrate and competitive inhibitors of tri-iodothyronine 5-deiodination and the corresponding values for 3',5'-di-iodothyronine 5'-deiodination. A similar relationship ($r = 0.996$) was observed when we studied the 5'-deiodination of reverse tri-iodothyronine instead of 3',5'-di-iodothyronine (results not shown).

Discussion

Investigations *in vitro* involving rat liver preparations have revealed striking similarities between enzymic 5- and 5'-deiodination of iodothyronines.

(1) Both 5- and 5'-deiodinase activities have been localized in the endoplasmic reticulum (Fekkes *et al.*, 1979; Auf dem Brinke *et al.*, 1979, 1980).

(2) Both types of deiodination are reductive processes, and are driven by thiols like dithiothreitol (Visser *et al.*, 1978).

(3) Enzymic 5- as well as 5'-deiodinations are inhibited by derivatives of 2-thiouracil (Visser *et al.*, 1978), and in both cases this inhibition is uncompetitive with the iodothyronine substrate (Chopra *et al.*, 1978). Thiouracil is a mechanism-based inhibitor, since it reacts, under formation of a mixed disulphide, with an enzyme–sulphenyl iodide ('E–SI') complex that is an intermediate in the deiodination process (Leonard & Rosenberg, 1978; Visser, 1979). These findings therefore indicate that 5- and 5'-deiodination follow the same reaction mechanism.

(4) Reaction of rat liver microsomal fractions with 3,5-di-iodothyronine, a substrate of which only the tyrosyl ring can be deiodinated, yields the sulphenyl iodide complex of the enzyme catalysing the phenolic-ring deiodination of other iodothyronines as evidenced by the persistent inactivation of 5'-deiodinase activity in the presence of thiouracil (Visser & Van Overmeeren-Kaptein, 1981).

(5) Iodothyronine 5- and 5'-deiodinase activities in detergent-treated rat liver microsomal fractions have very similar chromatographic and sedimentation properties, although, admittedly, none of the methods used has resulted in a considerable increase in specific enzyme activity (Fekkes *et al.*, 1980).

(6) However, a major improvement in enzyme purification has recently been achieved applying the technique of isoelectric focusing to W-1 ether [a 1:1.78 (v/v) mixture of cetyl 10 ether (Brij 56) and cetyl 20 ether (Brij 58)] extracts of rat liver

microsomal fractions. Both before and after removal of phospholipids, 5- and 5′-deiodinase activities were found to co-migrate, indicating identical isoelectric points (Fekkes & Visser, 1981).

(7) We demonstrate here that rat liver 5- and 5′-deiodinase activities are equally affected by several competitive substances covering a wide range of inhibitory potencies. Furthermore, the K_i value for tri-iodothyronine as a competitive inhibitor of 3′,5′-di-iodothyronine 5′-deiodination is equal to its K_m value in the 5-deiodination to 3,3′-di-iodothyronine. Conversely, the K_i value for 3′,5′-di-iodothyronine as a competitive inhibitor of the 5-deiodination of tri-iodothyronine is equal to the apparent K_m for 3′,5′-di-iodothyronine in its 5′-deiodination to 3′-iodothyronine.

In conclusion, the present study in conjunction with previously presented findings seriously challenges the generalization of two specific enzymes, i.e. iodothyronine 5- and 5′-deiodinase, being involved in the sequential deiodination of thyroxine in peripheral tissues (Schimmel & Utiger, 1977; Visser, 1978). Evidence is now compelling that, at least in rat liver, a single enzyme is capable of carrying out both phenolic- and tyrosyl-ring deiodination. If the same holds true for human liver and if this organ plays an important role in the extra-thyroidal conversion of thyroid hormone, then our results imply causes other than changes in deiodinase activity for the regulation of this process.

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SUMMARY

Chapter 1 is an introduction on sources and metabolism of thyroid hormone in humans and defines the scope of the study. Secretion by the thyroid gland is probably the only source of circulating thyroxine (T_4), while the biologically active 3,3',5-triiodothyronine (T_3) and the metabolically inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3) are released from this gland in small and negligible amounts, respectively. These triiodothyronines are produced mainly by enzymatic deiodination of T_4 in peripheral tissues, which process is a major pathway in the degradation of T_4. Two types of deiodination reactions can be distinguished, viz. deiodination of the tyrosyl ring (3- or 5-deiodination) and deiodination of the phenolic ring (3'- or 5'-deiodination). The reactions are thought to be catalyzed by two separate enzyme activities, viz. iodothyronine 5- and 5'-deiodinase. These enzyme activities also mediate the further deiodination of both T_3 and rT_3 to thyronine via the di- and monoiodothyronines. The reciprocal changes in serum T_3 and rT_3 levels observed in several situations indicate that the enzymatic deiodination is subject to a certain regulatory mechanism. The aim of my study was to obtain more knowledge about the regulation and mechanism of thyroid hormone deiodination. This has been attempted by the determination of some of the characteristics of the deiodination reactions with partially purified enzyme activities.

In Chapter 2, *in vitro* observations from others and ourselves on the monodeiodination of iodothyronines are described. Nearly all possible deiodination reactions have been demonstrated in a variety of tissues. Liver and kidney are the most important sites for the metabolism of thyroid hormone by deiodination. The deiodinating activity is enzymatic in nature and requires thiols as cofactor. The enzyme activities of rat liver are located in the endoplasmic reticulum, while those of rat kidney appear to be associated with the plasma membrane. All deiodinations, except for the 5'-monodeiodination of T_4, proceed maximally at slightly alkaline pH, when high substrate concentrations are used. From the kinetics of stimulation by thiols and inhibition by thiouracil derivatives, it is concluded that enzymatic deiodination follows a ping-pong mechanism. Iodothyronine deiodinating enzymes are intrinsic membrane proteins, which can be solubilized in an active form with several detergents.

Chapter 3 deals with the conversion of T_4 into 3,3'-diiodothyronine (3,3'-T_2) via T_3 and rT_3, using rat liver homogenate. It was found that the formation of rT_3 from T_4 is difficult to quantitate, because of the very rapid conversion of rT_3 into 3,3'-T_2. The 5-deiodination of T_3 was found to be the slowest reaction

At low substrate concentrations, the 5'-deiodinations of T_4 and rT_3 were highest between pH 6 and 6.5, while the 5-deiodinations of T_4 and T_3 were most effective at pH 8. All deiodination reactions studied were stimulated by dithiothreitol and inhibited by 6-propyl-2-thiouracil.

Chapter 4 concerns the localization of the iodothyronine deiodinating enzyme(s). Activity was found solely in the parenchymal cells of rat liver. Furthermore, these enzymes appeared to be associated with the endoplasmic reticulum of these cells.

In Chapter 5, the solubilization of the membrane-bound iodothyronine deiodinase(s) in an active form is described. In addition, several characteristics such as the sedimentation coefficient, apparent molecular weight and Stokes' radius are determined. At the same time, preliminary attempts at purification are presented.

In Chapter 6, further investigations of the purification and characterization of solubilized deiodinase activities are reported. It was found that treatment of a solubilized extract with ammonium sulphate removes phospholipids from the enzyme, which results in a marked change in its kinetic parameters. Moreover, the isoelectric point of the deiodinase was shifted towards pH 9.3. Both this change in isoelectric point and the behaviour on several column materials were found to be similar for the 5'- and the 5-deiodinating activities.

Chapter 7 deals with the 5'-deiodination of rT_3 and 3',5'-diiodothyronine (3',5'-T_2). Both reactions are inhibited competitively and to the same extent by several iodothyronine analogues, radiographic contrast agents and some non-iodine containing compounds.

Phenolphthalein dyes, such as bromophenol blue, appeared to be competitive inhibitors of the 5'-deiodination of rT_3. These dyes are structurally related to iodothyronines. Therefore, the structure-activity relationship of phenolphthalein derivatives was investigated. The results of this study are presented in Chapter 8.

In the last chapter, a study concerning the 5-monodeiodination of T_3 and the 5'-monodeiodination of 3',5'-T_2 is described. Comparison of the type and magnitude of inhibition of both reactions by various iodothyronine analogues and radiographic contrast agents led to the conclusion that both 5- and 5'-deiodinations of iodothyronines in rat liver are catalyzed by a single enzyme.

SAMENVATTING IN OMGANGSTAAL

De schildklier is een orgaan dat een bepaalde stof, schildklierhormoon of thyroxine (T_4) genaamd, maakt en vervolgens in de bloedbaan afscheidt. Dit thyroxine wordt wel eens afgekort tot T_4,omdat het een molekuul is wat vier jodiumatomen bevat. Eén van de voornaamste werkingen (biologische aktiviteit) van schildklierhormoon is de stimulering van het energieverbruik van de lichaamscellen. De schildklier kan ook nog een hormoon met drie jodiumatomen maken, het zgn. 3,3',5-trijodothyronine, kortweg T_3 genoemd (zie figuur 1.2.). Dit hormoon wordt in veel kleinere hoeveelheden aan de bloedbaan afgegeven dan T_4. De biologische aktiviteit van T_3 is echter vele malen groter dan die van T_4 en sommige onderzoekers denken zelfs dat T_4 helemaal geen aktiviteit bezit. Het lijkt dus in eerste instantie zinloos dat de schildklier T_4 maakt. Als we echter eens kijken naar wat er met T_4 gebeurt in het lichaam, dan wordt al gauw duidelijk dat dit laatste toch niet het geval is. Het is namelijk gebleken dat veel organen in staat zijn om T_4 om te zetten in het aktieve T_3. Dit omzettingsproces (reaktie) is niets anders dan het verwijderen van een jodiumatoom uit het T_4 molekuul en wordt daarom dejodering genoemd. Deze dejodering wordt bewerkstelligd en versneld (gekatalyseerd) door een enzym (dit is een eiwit met een bepaalde werking), dat 5'-dejodase is genoemd. Het voorvoegsel 5' slaat op de plaats van het jodiumatoom, dat door dit enzym van T_4 wordt afgeplukt.

Zo kan er ook een jodiumatoom van een andere plaats in het T_4-molekuul worden afgehaald. Dit leidt tot een stof met verwaarloosbare biologische aktiviteit, het zgn. 3,3',5'-trijodothyronine, kortweg "reverse" (omgekeerd) T_3 of rT_3 genoemd, omdat het jodiumatoom van de 5-plaats wordt verwijderd (zie figuur 1.2.). De enzymaktiviteit die deze reaktie katalyseert heet 5-dejodase. Beide T_3-molekulen kunnen aldus in verscheidene organen uit T_4 worden gevormd en komen ondermeer in de bloedbaan terecht. Nu is in een aantal omstandigheden, zoals na operaties en tijdens vasten, gevonden dat de hoeveelheid T_3 in het bloed verlaagd is, terwijl de hoeveelheid rT_3 juist verhoogd is. Kennelijk heeft het lichaam tijdens deze gebeurtenissen minder behoefte aan het biologisch aktieve hormoon T_3. Met het vermogen minder van dit hormoon te maken zorgt het lichaam er dus zelf voor dat het energieverbruik van de weefsels wordt verminderd. Dit vermogen wordt regulatie genoemd. Aldus heeft men deze veranderingen vertaald in een verschuiving van de vorming van T_3 uit T_4 ten gunste van de vorming van rT_3 uit T_4. De zaak ligt echter iets minder simpel dan nu is voorgesteld. Het is namelijk gebleken dat de vorming van rT_3 uit T_4 niet is veranderd, maar dat de afbraak van rT_3 verlaagd is. Deze afbraak wordt voornamelijk gekatalyseerd

door de eerdergenoemde 5'-dejodase aktiviteit. De bovenstaande bevindingen hebben daarom geleid tot de veronderstelling dat het enzym wat T_4 omzet in T_3 (het 5'-dejodase) niet hetzelfde is als het enzym wat de omzetting van T_4 in rT_3 katalyseert (het 5-dejodase). De eerdergenoemde omstandigheden leiden kennelijk tot een lagere aktiviteit van het 5'-dejodase, terwijl het 5-dejodase hiervan geen hinder ondervindt.

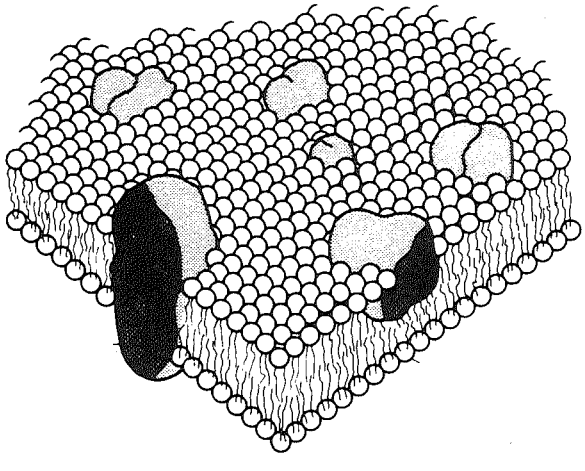
Om meer te weten te komen omtrent de regulering van de dejoderingsreakties in het lichaam, is het noodzakelijk een dieper inzicht te krijgen in de werking en eigenschappen van de daarbij betrokken enzymen. Hiervoor is het nodig om deze enzymen te isoleren (scheiden van andere stoffen) uit een orgaan dat het vermogen bezit om T_4 te dejoderen. Bovendien kunnen we dan te weten komen of er inderdaad twee enzymen (een 5- en 5'-dejodase) zijn, zoals door klinische waarnemingen wordt gesuggereerd. Aangezien het verkrijgen van menselijk weefsel niet eenvoudig is, heb ik mijn toevlucht genomen tot de lever van een rat. Dit dier wordt erg veel gebruikt in laboratoria.

Allereerst heb ik onderzocht welke dejoderingen er in rattelever plaatsvinden en of deze omzettingen gevoelig zijn voor bijvoorbeeld veranderingen in de zuurgraad. Hiertoe werd gebruik gemaakt van zgn. homogenaten, die verkregen waren door fijnwrijven van stukjes lever. Deze bewerking maakt de celstruktuur kapot, maar laat de subcellulaire deeltjes (onderdeeltjes van de cel), zoals de kern, grotendeels intakt. Er werd gevonden dat, behalve de dejoderingen van T_4 in T_3 en rT_3, ook de 5'-dejodering van rT_3 en de 5-dejodering van T_3 (beide omzettingen leiden tot de vorming van het 3,3'-dijodothyronine) plaatsvinden in dit homogenaat (zie figuur 1.4.). De 5'-dejodering van rT_3 bleek de snelste reaktie te zijn. Voor de 5'-dejodering van T_4 en rT_3 was een iets hoger dan normale zuurgraad het meest gunstig, terwijl de 5-dejoderingen van T_4 en T_3 juist het beste bij een wat lagere zuurgraad verliepen. Alle dejoderingen konden worden gestimuleerd door stoffen met een SH-groep, die mogelijk een ondersteunende funktie (als cofaktor) hebben in de enzymatische reaktie. Deze experimenten zijn beschreven in Hoofdstuk 3.

Vervolgens werd onderzocht waar in de rattelever de dejoderende enzymen zitten. Hiertoe werden de subcellulaire deeltjes in het homogenaat van elkaar gescheiden met behulp van een centrifuge. Het bleek dat alle eerdergenoemde dejodases in de zgn. microsomen zitten. Deze microsomen komen niet als zodanig in de cel voor maar worden gevormd tijdens het maken van een homogenaat. Het zijn voornamelijk fragmenten van het endoplasmatische reticulum (dit is een soort samenhangend buizenstelsel van zgn. membranen met vertakkingen in de gehele cel) en van de celmembraan. Nader onderzoek wees uit dat de dejoderende

enzymen verankerd zijn in het endoplasmatische reticulum en niet in de plasma- of celmembraan (zie Hoofdstuk 4).

Voor de zuivering van enzymen is het nodig deze in een oplosbare vorm te hebben. Daarom werden de dejodases als het ware uit de membraan geweekt met zgn. detergentia (een soort zeep). Vervolgens werd met een aantal chemische technieken getracht de dejoderende enzymen te scheiden van andere eiwitten. Ik kon echter hooguit 70% van die andere eiwitten kwijtraken. Met een andere techniek, behandeling met het zout ammoniumsulfaat, raakte ik niet alleen ongeveer 70% van die andere eiwitten kwijt, maar tevens een aantal zure stoffen die de dejodase-aktiviteit remmen. Deze remmende stoffen bleken zgn. fosfolipiden te zijn. Dit zijn vetachtige stoffen met een hydrofiele (water aantrekkende) kop en twee hydrofobe (waterafstotende) staarten, die samen met eiwitten membranen kunnen vormen, zoals bijvoorbeeld het endoplasmatische reticulum waarin het dejoderende enzym zit. Een schematische voorstelling van zo'n membraan is weergegeven in de figuur.



Nu lijkt het een beetje tegenstrijdig dat een enzym, als we dit uit zijn natuurlijke omgeving (membraan) halen en fosfolipiden verwijderen een reaktie sneller kan laten verlopen. Echter de manier waarop het schildklierhormoon door het dejodase wordt omgezet gebeurt met een lagere affiniteit voor dit hormoon, d.w.z. schildklierhormoon past slechter op het enzym na verwijdering van de fosfolipiden. Het bleek dat alle hierboven beschreven bewerkingen dezelfde invloed hebben op de 5- en de 5'-dejodaseaktiviteit; met andere woorden, de toegepaste technieken resulteerden niet in een scheiding tussen de 5- en de 5'-dejoderende

enzymaktiviteiten (zie de Hoofdstukken 5 en 6). Dit gegeven zou erop kunnen wijzen dat er niet twee maar één enzym is dat beide dejoderingsreakties kan katalyseren.

In Hoofdstuk 7 is de invloed van een aantal röntgenkontrastmiddelen (röntgenkontrastmiddelen zijn stoffen die worden toegepast bij het maken van een röntgenfoto van bepaalde organen en wat struktuur betreft een beetje lijken op schildklierhormoon) op de aktiviteit van het dejoderende enzym bekeken. Het bleek dat het merendeel van deze stoffen de dejodering remmen. Ook een aantal kleurstoffen, de zgn. fenolftaleïne kleurstoffen, bleken de dejodases te remmen (zie Hoofdstuk 8). Ik heb naar de remming van bovengenoemde stoffen gekeken om meer te weten te komen over de manier waarop schildklierhormoon wordt omgezet.

In Hoofdstuk 9 zijn nog een aantal experimenten beschreven die ook leiden tot de konklusie, dat er in rattelever kennelijk één enzym is dat zowel de 5- als de 5'-dejodering van schildklierhormoon katalyseert. Als dit laatste ook het geval is voor de lever van de mens en als dit orgaan tevens belangrijk is voor de vorming van T_3 en rT_3 uit T_4, dan is het onwaarschijnlijk dat de regulering van de dejoderingsreakties in het lichaam plaatsvindt door middel van veranderingen in de aktiviteit van het dejodase.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 8 maart 1952 te Workum geboren. Na aldaar de lagere school te hebben doorlopen werd in 1964 een aanvang gemaakt met de Middelbare Schoolopleiding aan het Christelijk Bogerman Lyceum (later Bogerman College) te Sneek. In mei 1969 werd het diploma HBS-B behaald. Een paar maanden later werd begonnen met de scheikundestudie aan de Rijksuniversiteit te Groningen. Het kandidaatsexamen werd in april 1973 afgelegd, terwijl het doctoraalexamen met als hoofdvak Biochemie en als bijvak Biologische Psychiatrie in februari 1977 werd afgelegd. In mei 1977 werd begonnen met een promotieonderzoek bij de afdeling Inwendige Geneeskunde III van het Academisch Ziekenhuis Dijkzigt te Rotterdam. Op laatstgenoemde afdeling werd tevens dit proefschrift bewerkt. Sinds december 1981 is hij werkzaam bij de afdeling Farmacologie van de Erasmus Universiteit te Rotterdam.