THE UNIVERSITY OF MANITOBA

THYROXINE 5'-MONODEIODINATION IN THE HEPATIC MICROSOME FRACTION OF RAINBOW TROUT, SALMO GAIRDNERI; EFFECTS OF MODIFYING AGENTS AND STARVATION ON ENZYME ACTIVITY.

by

Catherine Anne Shields

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ABSTRACT

The properties of the L-thyroxine (T4) 5'-deiodinase enzyme were studied in the liver of the rainbow trout, Salmo gairdneri, by incubating subcellular fractions with radioiodinated thyroxine (*T4) and carrier T4. Any radioiodide ($*I^-$) generated from *T4 by deiodination was separated from the reaction mixture on G-25 Sephadex minicolumns and counted. Deiodination was expressed as pmol T4 converted $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$.

The enzyme has been tentatively localized in the endoplasmic reticulum of the hepatic microsome fraction. Enzyme activity depended on exogenous thiols such as dithiothreitol (DTT) and increased linearly with incubation time and protein concentration. Optimum recorded pH was 7.0.

G-25 Sephadex chromatography of the microsomal fraction incubated with *T4 showed equal production of radioiodinated triiodothyronine (*T3) and $*I^-$ with no reverse T3 or conjugated iodothyronines. The *T3 deiodination rate was 3% of the *T4 deiodination rate.

Km and Vmax values of fed fish were 1.9×10^{-7} M and 3.4 pmol T4 converted $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$ respectively.

Several potential enzyme activators and inhibitors were tested. Deiodination was unaffected by reduced glutathione

(GSH) but was enhanced with diamide, an inhibitor of peripheral deiodination in mammals, and with the chelating agents EDTA and EGTA. Propylthiouracil (PTU) and N-ethyl maleimide, inhibitors that bind to sulfhydryl groups, depressed deiodination suggesting that enzyme sulfhydryls are involved in the reaction mechanism.

Deiodinase activity was significantly lower ($p<0.01$) in the livers of fish starved for 14 days than in fed fish. Kinetic parameters Km and Vmax and overall enzyme efficiency were also depressed in starved fish.

It is concluded that, as in mammals, there is an hepatic microsomal T4 5'-deiodinase that depends on pH, enzyme concentration, and incubation time. Enzyme activity is enhanced by exogenous DTT and inhibited by PTU and N-ethyl maleimide. However, in contrast to mammals, GSH inhibits rather than activates the hepatic trout enzyme and T4 deiodination terminates with the production of T3; thus, a sequential deiodination pathway is not present in rainbow trout.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANS : 8-anilino-1-naphthalene sulfonic acid

BAT : brown adipose tissue

Ci : curie(s)

cpm : counts per minute

5'-D : T4 5'-deiodinase

DTT : dithiothreitol

EDTA : ethylene diamine tetra-acetic acid

EGTA : ethylene glycol-bis-(-amino-ethylether) N,N'-tetra-acetic acid

ER : endoplasmic reticulum

FT4 : free thyroxine

GSH : glutathione (reduced)

GSSG : glutathione (oxidized)

h : hour

*I^- : iodide (^{125}I-labeled)

IRD : inner-ring deiodinase(tion)

Km : K_m; Michaelis-Menten constant

NADPH : nicotinamide adenine dinucleotide phosphate (reduced)

NADP : nicotinamide adenine dinucleotide phosphate (oxidized)

PTU : propylthiouracil

rT3 : reverse T3 = 3,3',5'-triiodothyronine

s.a. : specific activity

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

NPSH : non-protein sulfhydryl

SH : sulfhydryl group

TCA : trichloroacetic acid

T3 : 3.5.3'-triiodo-L-thyronine or triiodothyronine (stable)

*T3 : 3,5,3'-triiodothyronine (^{125}I-labeled)

T3S : sulfate-conjugated T3

T4 : L-thyroxine (stable)

*T4 : L-thyroxine (^{125}I-labeled)

T4S : sulfate-conjugated T4

TRH : thyrotropin-releasing hormone

TSH : thyrotropin = thyroid stimulating hormone

Vmax : V_{max} ; maximum velocity

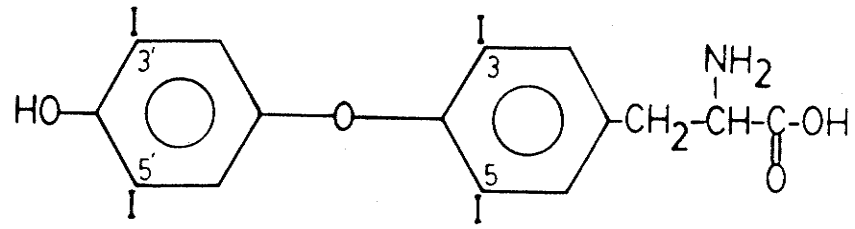
INTRODUCTION

In the mammalian thyroidal system, the two principal iodothyronines, thyroxine (T4) and 3,5,3'-triiodothyronine (T3), are released from the thyroid after endocytosis of the colloid by follicular cells (Fig. 1). Thyroid stimulating hormone (TSH) acts on the follicular cells with the net effect of increasing T4 and T3 secretion from the gland (Fig. 2). In the brook trout, T4 is released from the thyroid upon TSH stimulation although it is possible that T3 secretion also occurs (Chan and Eales, 1975).

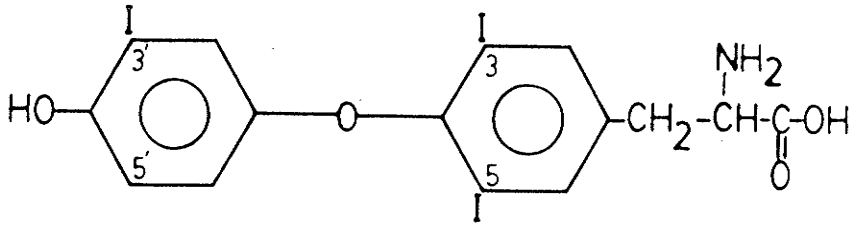
Essentially all of the T4 (99.95%) and T3 (99.7%) in mammalian plasma exists in the bound form associated with thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) and albumin (Refetoff, 1979). The pattern of plasma protein binding in the trout is similar to mammals'. Triiodothyronine binds less strongly than T4 to the TBPA fraction but there is a smaller percentage of free T3 (FT3) and larger percentage of free T4 (FT4) in the blood (0.1% FT3, 0.2% FT4) (Eales, 1979a; Eales and Shostak, 1985).

Free T4 enters the tissues and undergoes enzymatic deiodination and the resulting T3 returns to the circulation in which it is transported to the target tissues. Surks *et al*. (1973) estimated that in humans 43% of the daily T4 production is peripherally deiodinated to T3. The extensive peripheral conversion of T4 to T3 and the 4 to 10 times

Figure 1. Tetraiodo-L-thyronine (T4) and triiodo-L-thyronine (T3).

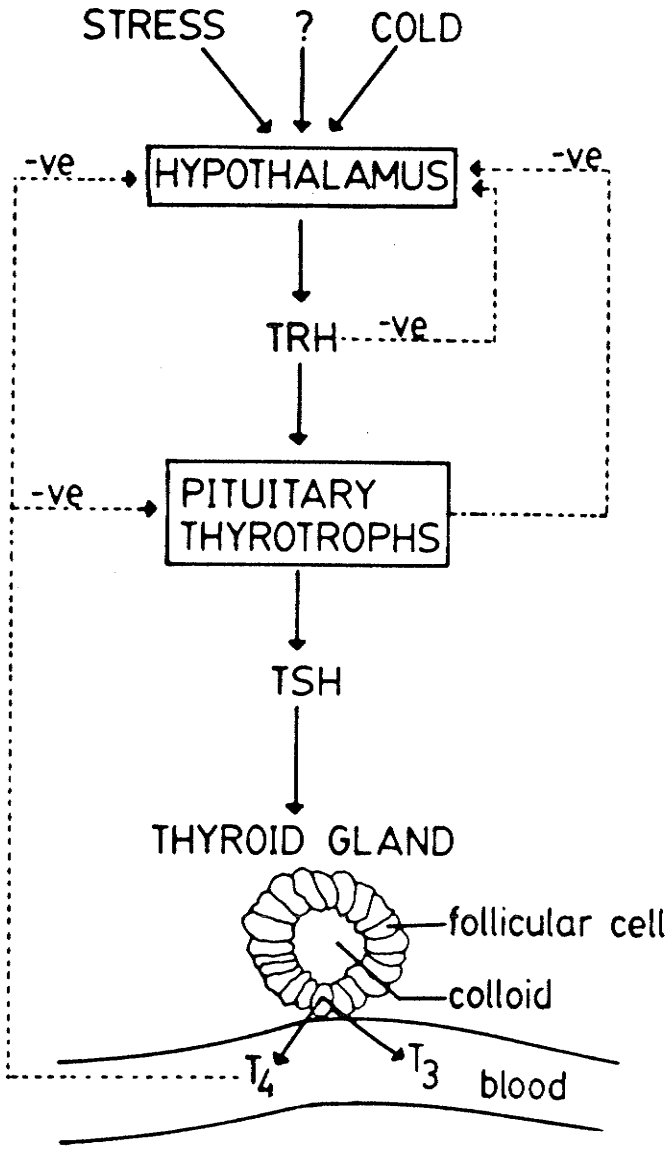


T_4 (3,5,3',5'-TETRAIODO-L-THYRONINE)



T_3 (3,5,3'-TRIIODO-L-THYRONINE)

Figure 2. Hypothalamic-pituitary-thyroid axis in mammals.

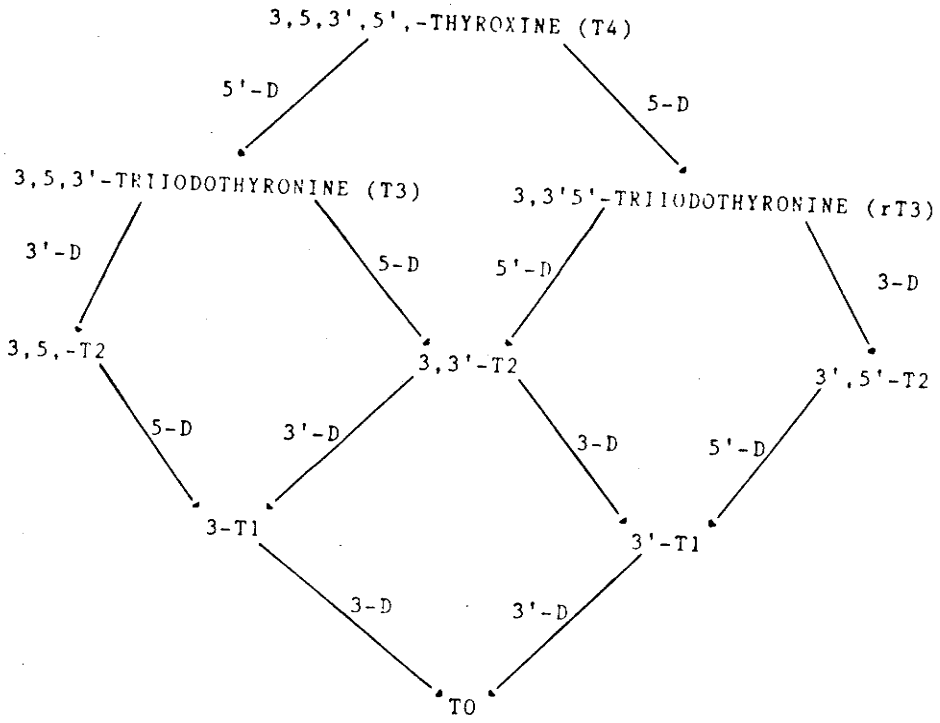

STRESS
?
COLD
-ve
HYPOTHALAMUS
-ve
TRH
-ve
-ve
PITUITARY
THYROTROPHS
TSH
THYROID GLAND
follicular cell
colloid
T_4
T_3
blood

greater thyroid hormone receptor affinity for T3 than for T4 suggest that T4 acts as a prohormone while T3 is the active form of the hormone (DeGroot, 1979).

Several enzymes are involved in catalyzing the removal of iodine from the inner and outer ring of iodothyronines. This sequential deiodination ultimately results in the production of thyronine (T0) (Fig. 3). Thyroxine 5'-deiodinase, for example, catalyzes the removal of iodine from the 5'-position on T4 to yield T3. The enzyme is membrane-bound and located in the endoplasmic reticulum (ER) and plasma membrane of liver and kidney respectively (Chiraseveenuprapund *et al.*, 1978; Leonard and Rosenberg, 1978a; Fekkes *et al.*, 1979; Maciel *et al.*, 1979). Thiol compounds for example, dithiothreitol (DTT), act as a cosubstrate for this reaction which proceeds *via* a bisubstrate mechanism (Visser, 1979; Leonard and Rosenberg, 1980). Propylthiouracil (PTU) is a potent inhibitor of 5'-deiodination both *in vivo* (Silva *et al.*, 1984) and *in vitro* (Visser, 1979).

A second type of mammalian 5'-deiodinase is PTU-insensitive, requires high concentrations of exogenous thiols for activity and exhibits sequential reaction kinetics (Visser *et al.*, 1981,1982,1983). Brown adipose tissue (BAT), cerebral cortex, pituitary, and recently placenta are documented locations for this enzyme (Kaplan, 1980; Leonard *et al.*, 1983; Kaplan and Shaw, 1984).

Figure 3. Sequential deiodination of thyroxine to thyronine.



T2 = DIIODOTHYRONINE

T1 = MONOIODOTHYRONINE

TO = THYRONINE

D = DEIODINASE

Inner-ring deiodination (IRD) of T4 via 5-deiodinase results in the production of the non-thyromimetic reverse T3 (rT3).

Trout liver and kidney possess significant 5'-deiodinating activity (Leatherland, 1981; Pimlott and Eales, 1983). Thiouracils such as PTU inhibit the hepatic enzyme and DTT enhances deiodination in a dose dependent manner (Eales et al., 1984). However, the peripheral deiodination of thyroid hormones differs from that in the mammalian system in at least two respects. Triiodothyronine appears to be the only deiodination product formed: reverse T3 and subsequent deiodination products have not been detected either in trout plasma or liver homogenates (Eales et al., 1983; Pimlott and Eales, 1983). The percentage of T4 deiodinated to T3 is greater in trout than mammals (human). Eales in 1977 calculated from in vivo experiments that 59% to 69% of injected T4 is deiodinated to T3 compared to the human value of 43% (Surks et al., 1973).

The objective of this thesis has been to examine the T4 5'-deiodinase in the liver of Salmo gairdneri. This enzyme is strategically important in trout because it catalyzes the conversion of the prohormone T4 to the active T3 hormone. Deiodination, therefore, represents activation of the end products of the thyroid system. Previous studies in this laboratory (Pimlott and Eales, 1983; Eales et al., 1984) have focused on the supernatant from liver whole homogenate.

I hoped to extend information from these experiments to include the subcellular location of the enzyme and some aspects of enzyme regulation. Initial work involved optimizing the assay with regard to pH, time, and dependence on concentration of thiol and enzyme. Kinetic parameters Km and Vmax were calculated by varying the carrier T4 concentration in incubates from isolated microsomes. Various enzyme modifying agents, both inhibitors and activators were then tested to characterize the enzyme further. Finally, the effect of starvation on 5'-deiodination was examined by the use of livers isolated from starved and fed trout. From the above studies a comparison has been made between the trout and mammal T4 deiodinating systems.

LITERATURE REVIEW

The thyroid gland is believed to be the exclusive source for plasma T4. T3 may be both secreted by the thyroid and converted from T4 by extrathyroidal deiodination. It is estimated that in man 80% of circulating T3 arises from the reductive monodeiodination of T4 with thyroidal T3 secretion accounting for the remaining 20% (Engler and Burger, 1984). In this review I will summarize the pertinent literature on deiodination in mammals and other vertebrate groups including an overview of T4 5'-deiodination in fish.

A. Deiodination in mammals.

1. Sequential deiodination to T0.

T4 can be thought of as the starting molecule in the sequential deiodination of iodothyronines. Iodine can be removed from the 5' (outer-ring) or 5 (inner-ring) positions of T4 producing T3 and rT3 respectively. These molecules are in turn deiodinated in a series of enzymatic reactions to diiodothyronines (T2), monoiodothyronines (T1), and finally thyronine (T0). There are marked similarities among all the enzymes catalyzing outer-ring deiodination (both 3' and 5'). The possibility exists, therefore, that a single enzyme may be responsible for removal of iodine in the phenolic ring of iodothyronines. However, two T2 5'-deiodinases catalyzing the conversion of 3',5'-T2 to 3'-T1 have been isolated by Smallridge *et al*.(1981) lending support to a multi-enzyme

deiodination cascade. Until all the enzymes have been isolated and purified, it is probably best to assume that deiodination of iodothyronines proceeds via separate enzymes as represented in Fig. 3.

2. Type I T4 5'-deiodinase.

Rat liver and kidney have been the organs of choice for studying outer-ring (5'-deiodination) in mammals (Chiraseveenuprepund et al., 1978; Leonard and Rosenberg, 1978b; Visser et al., 1978; Fekkes et al., 1979; Maciel et al., 1979; Yoshida et al., 1983). The highest overall enzyme activity is found in these tissues with the kidney exhibiting a specific activity 1.5 to 2 times that of liver (Ferguson and Jennings, 1983). Other tissues in the rat have also been examined including heart, thyroid, pituitary, and brain (Rabinowitz and Hercker, 1971; Erikson et al., 1980; Kaplan, 1980; Visser et al., 1981,1982). Studies of 5'-deiodination have involved a cross-section of mammalian species. For example, bovine liver was used as an enzyme source for purification studies (Köhrle et al., 1980). The mechanism of diminished T3 production in the fetus was investigated in sheep (Chopra, 1978) and deiodinase activity has also been examined in the human thyroid (Ishii et al., 1982).

It is generally believed that the hepatic 5'-deiodinase is a membrane-bound enzyme of the ER. Fekkes et al. (1979), using discontinous gradient centrifugation to separate the subcellular fractions, found that deiodinase activity correlated positively with glucose-6-phosphatase, an ER

marker ($r>0.98$) and negatively with the plasma membrane marker 5'-nucleotidase ($-0.88<r<-0.97$). Using similar techniques, Maciel and coworkers (1979) concluded that the 5'-deiodinase is located in the plasma membrane of rat liver. This location would allow delivery of T3 to the nuclear receptor without the entry of T4 into the cytoplasm. There is a large body of literature, however, (Auf dem Brinke et al., 1979; Takaishi et al., 1979; Heinen et al., 1980; Fekkes et al., 1982a) that contradicts Maciel and subsequent work on rat liver has assumed the enzyme to be located in the ER.

The rat kidney's 5'-deiodinase, in contrast to the liver's, appears to reside in the plasma membrane (Leonard and Rosenberg, 1978a). Recent experiments on perfused kidney have demonstrated high T4 uptake and deiodination on the contraluminal membrane of the renal tubule (Ferguson and Jennings, 1983). In brain, the highest 5'-deiodinase is found in the purified plasma membrane fraction (Leonard et al., 1982). Finally, rat liver and kidney lysosomes deiodinate T4 or rT3, the enzyme having a marked preference for rT3 (Colquhoun and Thomson, 1984).

Solubilization or detergent emulsification of the enzyme has been attemped in several laboratories (Powell et al., 1982; Fekkes et al., 1983; Mol et al., 1983). Extraction of the 5'-deiodinase from the membrane involves a multi-step procedure. Specific activity of the recovered enzyme is 2 to 580 times the level measured in the microsomal membrane

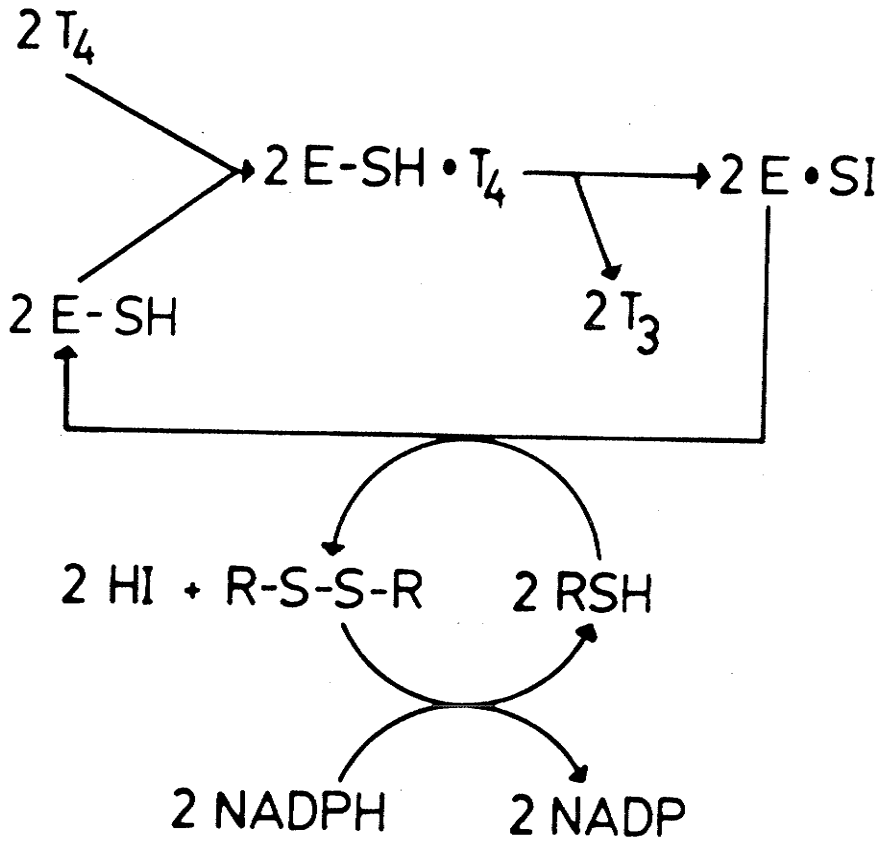
(Fekkes et al., 1980; Powell et al., 1982). Molecular mass estimates for the enzyme vary between 60 Kdaltons and 65 Kdaltons (Köhrle et al., 1980; Mol et al., 1983). Affinity labelling with N-bromoacetyl-3,3'-5-triiodothyronine, an inhibitor that selectively carboxymethylates catalytic SH groups, indicates that the enzyme is composed of two subunits (Mol et al., 1984). The ligand binding site has been tentatively described by the use of T4 and rT3 analogs. The enzyme prefers negatively charged side chains and bulky substituents in the 3 and/or 5 position to decrease rotation of the aromatic rings; it does not require iodine to be present in the 3' or 5' position for analog inhibition (Köhrle et al., 1984).

3. Mechanism of type I T4 5'-deiodinase.

The removal of iodine from the 5'-position of T4 and rT3 follows a ping-pong mechanism characterized by the transiodination and reduction of the sulfenyl iodide enzyme intermediate by a thiol cofactor (Fig. 4) (Visser, 1979; Leonard and Rosenberg, 1980). The nature of the enzyme mechanism is determined by examination of reciprocal plots of initial rate data. If the graph of velocity^{-1} versus substrate^{-1} in the presence of increasing exogenous thiol is a series of parallel lines, the reaction is said to follow the ping-pong mechanism (Goswami and Rosenberg, 1984).

Ozawa (1981) proposed a direct interaction between thiols and the thyroxine molecule. When T4 was preincubated with DTT, deiodination was markedly enhanced, possibly by

Figure 4. Bisubstrate or ping-pong mechanism of thyroxine monodeiodination (after Visser, 1979). RSH = one molecule of DTT or two molecules of GSH.


2 T4
2 E-SH • T4
2 E • SI
2 E-SH
2 T3
2 HI + R-S-S-R
2 RSH
2 NADPH
2 NADP

the donation of electrons to the phenolic ring of the molecule (Ozawa et al., 1981). This hypothesis has since been discounted in favor of a cosubstrate role for thiol compounds (Shulkin et al., 1983).

Apparent Km values for T4 in the liver and kidney vary between 0.9 μM and 5 μM (Hüfner et al., 1977; Chiraseveenuprapund et al., 1978; Ferguson and Jennings, 1983; Goswami and Rosenberg, 1984). The apparent Km for rT3 is 10 times lower than the Km measured with T4 as a substrate or in other words, rT3 is kinetically favored over T4 (Visser et al., 1982; Goswami and Rosenberg, 1984).

4. Identity of the endogenous enzyme cosubstrate.

Imai's group in 1980 suggested GSH may be the cofactor for T4 to T3 conversion. They observed that enhancement of T4 deiodination was saturated with millimolar amounts of GSH corresponding to the physiological concentration in the liver. They concluded that previous investigators had been unable to notice appreciable enhancement with GSH because they were adding to a system already saturated with the thiol (Imai et al., 1980).

Sato and Robbins (1981) contradicted Imai's work with results obtained from cultured rat hepatocytes. When hepatocytes were grown in a cystine-and/or methionine-deficient medium, total cellular GSH content decreased to less than 10% of control with no decline in deiodination.

GSH is formed by the reduction of GSSG by glutathione reductase, an NADPH-dependent enzyme. NADPH is itself reduced

from NADP via the pentose phosphate pathway (Sato et al., 1983). Sato et al. (1982) found greater enzyme activation with NADPH than with GSH and found that NADPH is more rate limiting than GSH in modulating T4 to T3 conversion during maturation. On the strength of these results, Sato and coworkers suggest that NADPH is in fact the endogenous cofactor that interacts directly with enzyme thiols rather than being simply a reducing agent for the putative endogenous cofactor GSH.

Dihydrolipoamide is a naturally occurring dithiol with 6 to 10 times the potency of DTT in enhancing 5'-deiodination in rat kidney (Goswami and Rosenberg, 1983). However, this compound is found only in the mitochondria, and is therfore prevented access to enzyme bound to the plasma membrane.

5. Type II T4 5'-deiodinase.

There is a second 5'-deiodinase designated as type II or PTU-insensitive. Most of the research has centered on the brain and anterior pituitary but recently placenta, brown adipose tissue, and kidney have also been examined as additional enzyme sources (Kaplan, 1980; Kaplan and Yaskoski, 1981; Visser et al., 1982; Leonard et al., 1983; Goswami and Rosenberg, 1984; Kaplan and Shaw, 1984). Unlike the type I 5'-deiodinase previously described, type II exhibits sequential reaction kinetics (Visser et al., 1983). Thiol, iodothyronine, and enzyme form a complex. The reaction proceeds by a series of intermediate steps and concludes with the release of the deiodinated hormone, oxidized thiol, and

regenerated enzyme. The relative PTU-insensitivity of the type II enzyme reflects a lack of participation of sulfhydryl groups in the mechanism (Visser et al., 1983). Km values for rT3 and T4 are three orders of magnitude lower for the type II than for the type I in the liver and kidney. T4 is the preferred substrate for the type II enzyme: the apparent Km for T4 is approximately one half the Km for rT3 (Visser et al., 1981,1982).

6. Contribution of type I and type II deiodination to the intracellular T3 pools.

In the pituitary and brain, any T3 produced by 5'-deiodination is either metabolized as T3 or deiodinated furhter. Intracellular T4 to T3 conversion contributes 50% of the nuclear T3 with the remaining 50% arising from the plasma (Schaison et al., 1984). Nuclear T3 in the pituitary, therefore, comes from two processes: transport of plasma T3 into the cell and transport of plasma T4 with subsequent intracellular conversion to T3. A dual T3 source explains why TSH secretion from the pituitary which depends on the level of T3 present, is a function of both T4 and T3 levels in the blood.

Type I deiodination is most active in the brain of euthyroid animals. However, under conditions of short-term hypothyroidism type II activity increases and type I activity declines . This switch from type I (high Km) to type II (low Km) facilitates optimal occupancy of brain nuclear receptors in the face of declining circulating hormone levels (Visser

et al., 1982).

7. Contribution of type II 5'-deiodinase to circulating T3 levels.

Euthyroid rats treated with maximal PTU doses for type I inhibition exhibited only a 60% reduction in peripheral T4 to T3 conversion (Silva et al., 1984). Silva et al. (1984) interpreted this as a significant contribution of the type II enzyme to circulating T3 levels. In the PTU-treated neonatal rat, peripheral T3 production declined by 25% and in hypothyroid animals PTU pretreatment was ineffective in blocking conversion suggesting that the type II pathway was the major source of T3 under these experimental conditions as well (Silva and Matthews, 1984).

Two tissues have been proposed as sources for plasma T3 generated by type II enzymes. Brown adipose tissue (BAT) is extensive and well vascularized and has the potential to produce 40 times more T3 via type II deiodination than the cerebral cortex (Leonard et al., 1983). Rat kidney microsomes contain 2 deiodinase systems. One has a high Km and the other a low Km 5'-deiodinase, the latter exhibiting similar properties to the type II of brain and BAT (Goswami and Rosenberg, 1984). This enzyme could contribute to extracellular T3 pools when low substrate conditions like hypothyroidism prevail.

8. T4 5' and 5-deiodinase; one enzyme or two?

Hepatic T4 5' and 5-deiodinases are similar in many respects and are thought to be the same enzyme. Both are

located in the endoplasmic reticulum, inhibited by thyroid hormone analogs, stimulated by thiols and indistinguishable by most chromatographic methods, and they comigrate on isoelectric focusing gels (Chopra and Teco, 1982; Fekkes et al., 1982a; Fekkes et al., 1983). There is some contrary evidence, however. Binding studies in rat liver generate hyperbolic curves that can only be explained on the basis of two specific binding sites (Heinen et al., 1980). Reverse T3 competes with T4 for the first site and T3 competes with T4 for the second. By implication, 5' and 5-deiodination could be mediated by two enzymes. A single enzyme catalyzing the production of the active hormone T3 and the metabolically inactive rT3 would have to be precisely regulated. Intracellular conditions, pH for example, could be subtly altered to switch from T3 to rT3 production, but a simpler system would be to have two separate enzymes; one for T4 to T3 conversion and a second for T4 to rT3 conversion. Complete purification of the microsomal deiodinase(s) should clarify this point.

9. Modifying agents.

The tested or potential agents modifying 5'-deiodination that have been used previously or were used in this thesis can be divided into 7 categories: 1) thyroid inhibitors, 2) thyroid hormone analogs, 3) GSH and diamide, 4) enzyme binding agents, 5) end-product inhibitors, 6) chelating agents, and 7) hormones.

Thyroid inhibitors act at 3 sites to depress thyroid

hormone synthesis. They can interact with iodoperoxidase, or with the iodoperoxidase antagonist catalase, or compete with iodide for transport into the gland itself (Werner and Ingbar, 1978).

The analogs listed in Table I are characterized by having part or all of the molecule resembling T4 or rT3. Multi-ring structures of aurones, phenolpthalein dyes, blockers, and amiodarone are similar to the phenolic and tyrosyl rings of thyroid hormones. Iopanoic acid possesses both a ring structure and iodine atoms making it a potent competitive inhibitor of type I and type II 5'-deiodination (see Appendix Fig. 2 for the molecular structures of iopanoic acid, ANS, sodium salicylate, PTU, glutathione (GSH), and DTT). Salicylate and ANS displace thyroid hormones from plasma proteins and inhibit T4 to T3 conversion implying that the binding site for thyroid hormones on plasma proteins and the active site on the enzyme are similar (Chiraseveenuprapund et al., 1978).

The role of GSH as a cofactor was investigated with diamide, a specific GSH inhibitor (Table II) (Kaplan, 1979).

The fourth class of modifiers bind to the deiodinase enzyme itself (Table II). Two types of thiouracils have been used to study 5'-deiodination. One group inhibits thyroid hormone formation by blocking intrathyroidal iodoperoxidase and also depress peripheral conversion of T4 by interacting with the enzyme, forming a mixed disulfide (Chopra et al., 1982). Other thiouracil analogs exhibit no thyroid blocking

TABLE I.

Competitive inhibition by T4 analogs on T4 5'- deiodination in mammals.

Compound	Example	Reference
aurone[1]	3', (4',4,6)-tetra (tri)-hydroxyaurone	Auf'mkolk et al., 1983
β-adrenergic antagonist[2]	D,L propranolol	Shulkin et al., 1984
phenolphthalein[3]	bromophenol blue	Fekkes et al., 1982b
amiodarone[4]	---	Lindenmeyer et al., 1984; Pekary et al., 1984
radiographic contrast agents[5]	iopanoic acid/ipodate	Leonard et al., 1983; Laurberg and Boye, 1984
sodium salicylate[6]	---	Chopra et al., 1980
ANS[6]	---	Chopra et al., 1980

[1] 2° plant metabolite

[2] blocks β-adrenergic receptors

[3] dye agent

[4] anti-arrhythmic drug

[5] gall bladder visualization

[6] displaces thyroid hormones from plasma proteins

TABLE II.

Enzyme and cofactor modifiers of T4 5'-deiodination.

Compound	Inhibitor/Activator	Mechanism	Reference
GSH	A/I[+]	enzyme cofactor	Imai et al., 1980; Eales et al., 1984
diamide[1]	I/A*	---	Kaplan, 1979 Eales et al., 1984
thiouracil analogs[2]	I	interacts with enzyme sulfhydryls to form a mixed disulfide	Chopra et al., 1982
thiouracil analogs[3]	I	same as above	Nogimori et al., 1984
iodoacetate	I	binds irreversibly to SH residues on enzyme	Leonard and Visser, 1984
N-alkyl maleimide	I	S-carboxymethylates SH residues on enzyme	Leonard and Rosenberg, 1980

*activates T4 deiodination in trout

+inhibits T4 deiodination in trout

[1]GSH inhibitor

[2]blocks thyroid

[3]no thyroid blocking activity

activity but still inhibit T3 production by the mechanism outlined above (Nogimori et al., 1984). Iodoacetate and N-alkyl maleimides bind irreversibly to SH residues. Radioiodinated iodoacetate has been used to characterize the active centre of the enzyme (Leonard and Visser, 1984).

Iodide may act as an end-product inhibitor of T4 5'-deiodination, depressing the formation of T3. However, the inhibitory actions of iodide have not yet been tested.

Chelating agents, EDTA for example, form complexes with ions. EDTA has a marked stimulatory action on 5'-deiodination in young rats (Sato et al., 1983). There are correspondingly high levels of the deiodinase inhibitors Cu^{2+} and Zn^{2+} in these animals and EDTA could be complexing these ions, thus removing their inhibitory effect (Sato et al., 1983).

The seventh class of modifiers includes hormones such as TSH, insulin, adrenalin, and cortisol (glucocortoids) (Table III). Addition of TSH to thyroid preparations elevates T4 and T3 release and increases deiodination by inducing intrathyroidal deiodinase synthesis (Wu et al., 1982). Insulin stimulates glucose transport into the liver and indirectly activates deiodination by providing the glucose substrate for NADPH production. The hormone also stimulates enzyme synthesis directly (Grau et al., 1983). Adrenalin exerts two effects depending on the receptor site. Inhibition may be α-receptor mediated and activation involves binding to a β-receptor (Nauman, 1984b). Glucocorticoids inhibit T3 production by decreasing the tissue content of the deiodinase

TABLE III.

Influence of hormones on T4 5'-deiodination in mammals.

Compound	Inhibitor/Activator	Mechanism	Reference
TSH[1]	A	increases enzyme synthesis in thyroid	Wu et al., 1982
insulin2	A	stimulates de novo synthesis of the enzyme	Grau et al., 1983
adrenaline[3]	I/A	unknown but I may be α-receptor mediated	Nauman et al., 1984 a and b
		A may be β-receptor mediated	
glucocorticoids[4]	I	decreases tissue content of the enzyme	Heyma and Larkins, 1982; Cavalieri et al., 1984

[1] stimulates T3 and T4 release from thyroid

[2] regulates carbohydrate utilization

[3] elevates heart rate and metabolic rate

[4] regulates carbohydrate, fat and protein utilization

enzyme (Cavalieri et al., 1984).

10. Effect of starvation on deiodination.

Starvation results in decreased serum T4 and T3 levels and reduced T4 to T3 conversion. The mechanism of action has not been established conclusively and three separate theories are therefore presented.

Harris et al. (1979) noted a decline in non-protein sulfhydryl (NPSH) concentration, specifically glutathione (GSH), in livers from rats starved for two days. In vitro T3 production was completely restored to levels measured in fed animals upon the addition of the exogenous thiol DTT. Moreover, the depressed hepatic T3 generation was correlated with the level of NPSH rather than the quantity of 5'-deiodinase present in the tissue (Harris et al., 1979). Harris and coworkers suggested that depressed deiodination during starvation was the result of reduced amounts of sulfhydryl group cofactor.

A decrease in the amount of 5'-deiodinase present in the liver in addition to lower NPSH levels has also been observed in starved rats (Balsam et al., 1979; Gavin et al., 1980). Addition of glucose, not thiol compounds to isolated livers from starved animals fuuly restored deiodinase activity to levels measured in fed rats (Gavin and Cavalieri, 1980). Glucose feeding increased the tissue content of the enzyme as evidenced by the elevated Vmax and unchanged Km (Gavin et al., 1981). The effect of carbohydrates may be mediated by insulin rather than as a direct effect. Serum insulin is

greater in carbohydrate infused animals (Harris et al., 1978) and the insulin elevates T3 production by increasing the quantity of 5'-deiodinase present (Grau et al., 1983).

Reduced concentration of T4 in the serum or reduced transport of T4 from the blood into the liver in starved animals could also result in diminished T3 production (Heinen et al., 1981). Studies on perfused rat liver (Jennings et al., 1979) have shown that intracellular deiodination depended on T4 uptake from the blood. During fasting, T4 uptake declined to 42% of control but the intrahepatic conversion of T4 to T3 remained unchanged (Jennings et al., 1979). When the Km for T4 deiodination in the kidney (3 x 10^{-6} M) was compared to the serum FT4 levels (2 to 3 ng/dl) it became apparent that T4 uptake could be rate limiting at physiological FT4 concentrations (Ferguson and Jennings, 1983).

11. Effect of hypothyroidism on peripheral T3 generation.

Depressed T3 generation in hypothyroid animals results partially from a reduction in the tissue content of the type I 5'-deiodinase enzyme (Harris et al., 1979). Kaplan (1979) observed that the maximum velocity for T4 5'-deiodination in hypothyroid rats was smaller than Vmax values for euthyroid animals substantiating a reduced enzyme concentration in hypothyroidism. It is also possible that reduced substrate (T4) may have a role in the reduced T4 deiodination.

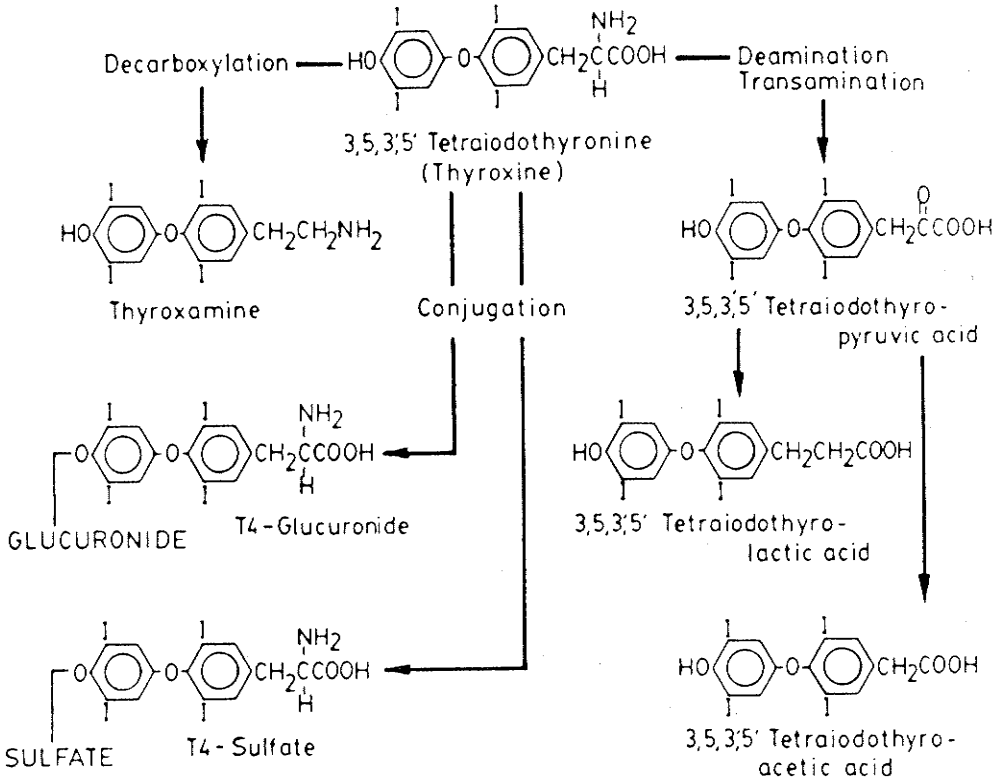
12. Sulfate conjugation.

Sulfate conjugation represents one of several pathways, apart from deiodination, for the degradation or transformation of iodothyronines (Fig. 5). Sulfate conjugation facilitates subsequent degradation of iodothyronines and until recently was considered separate from deiodination. It is now known, however, that while T3 and 3,3'-T2 are poor substrates for deiodination in the hepatic microsome fraction they are readily sulfate conjugated and then rapidly deiodinated in cultured hepatocytes (Otten et al., 1983; Visser et al., 1983). The rate of inner-ring deiodination (IRD) of sulfate-conjugated T3 (T3S) is two orders of magnitude faster than that of T3; by the use of Vmax/Km ratios as an indicator of enzyme efficiency, sulfate-conjugated T4 (T4S) IRD is 210 times more efficient than T4 IRD alone (Otten et al., 1983; Mol and Visser, 1984). Sulfation preferentially facilitates the production of inactive metabolites at the expense of T3 generation providing a rapid means to degrade iodothyronines and possibly salvage iodine.

B. Deiodination in non-mammalian tetrapods.

There is a paucity of knowledge about deiodination in birds, reptiles, and amphibians. Chickens exhibit a high rate of T4 to T3 conversion correlated with higher metabolic activity or faster exchange of T4 between cell and plasma (Rudas and Pethes, 1984). Although undetected in mammals, there is a PTU-insensitive deiodinase in the liver of some birds (McNabb et al., MS). Ontogenetic maturation of the

Figure 5. Pathways of T4 metabolism (excluding deiodination) (Eales, 1985).


Decarboxylation
Deamination
Transamination
3,5,3',5' Tetraiodothyronine
(Thyroxine)
Thyroxamine
Conjugation
3,5,3',5' Tetraiodothyro-
pyruvic acid
GLUCURONIDE
T4-Glucuronide
3,5,3',5' Tetraiodothyro-
lactic acid
SULFATE
T4-Sulfate
3,5,3',5' Tetraiodothyro-
acetic acid

5'-deiodinase differs between precocial and altricial birds. Enzyme activity peaks at hatching for the precocial japanese quail but is still rising at this developmental stage in the altricial ring dove (McNabb and Hughes, 1984). Two separate studies (Premanchandra et al., 1977; Rudas and Pethes, 1984) have failed to detect rT3 in avian plasma possibly because this inactive metabolite is cleared rapidly from the blood.

The reptilian thyroid system is the least understood. The major sites of peripheral deiodination, enzyme characteristics and regulation have not been investigated.

Experiments on amphibians have focused on the role of T4 5'-deiodination during metamorphosis. T3 production from T4 first develops in premetamorphic tadpoles and increases to metamorphic climax (Leloup and Buscaglia, 1980; Galton and Munck, 1981). Enzyme kinetics (Km, Vmax), pH dependency, and effects of most standard modifying agents remain to be examined.

C. T4 5'-deiodination in teleosts.

1. Characteristics of T4 5'-deiodination.

The deiodination pathway in trout involves the removal of a single iodine atom from the outer ring of T4 to produce T3. Law and Eales (1973) incubated brook trout homogenates with radiothyroxine (*T4) in the absence of DTT and examined the products formed using thin-layer chromatography. Deiodination was observed in gill, stomach, intestine, kidney, and muscle but not in the liver (Law and Eales, 1973). Leatherland (1981) using a radioimmunoassay procedure

and no added thiols demonstrated T4 to T3 conversion in hepatic and renal homogenates of rainbow trout. The reaction was enzymatic with a pH optimum of 7.0 to 8.0 in the liver (Leatherland, 1981).

Sephadex G-25 minicolumns (40x13 mm) separate iodothyronines from $*I^-$. Using this method, Pimlott and Eales (1983) measured $*I^-$ generation in rainbow trout liver homogenates incubated with *T4. The number of pmoles T4 converted depended on incubation time, and on the amount of enzyme present, required exogenous thiols for significant activity, and had a pH optimum between 6.8 and 7.4 (Pimlott and Eales, 1983). Tracer T3/carrier T3 underwent negligible deiodination and rT3 was not detected as product from *T4 by G-25 Sephadex chromatography (Pimlott and Eales, 1983). The apparent Km value (12°C) was 3.7×10^{-9} M (Pimlott and Eales, 1983), similar to the Km calculated for the type II or PTU-insensitive enzyme in brain and BAT.

2. Measurement of peripheral deiodination *in vivo*.

In vivo deiodination of T4 can be measured by comparing plasma radioiodide following the injection of $^{131}I^-$ and *T4 (Eales, 1977; Eales *et al.*, 1984). Trout are amenable to this procedure for two reasons. First, they have a large plasma iodide pool with a slow turnover rate; thus $*I^-$ generated by deiodination will not be recycled in the thyroid. Secondly, trout metabolize injected T4 quickly (Eales, 1977). The influence of various parameters such as starvation, refeeding and chemical agents on deiodination in

the intact fish can be examined by this method.

3. Effect of starvation on peripheral deiodination.

Starvation for 3 days (rainbow trout) and 12 days (brook trout) alters thyroid hormone metabolism (Higgs and Eales, 1977; Eales, 1979b). The metabolic clearance rate (MCR) of T4 and T3 and extrathyroidal conversion of T4 to T3 are depressed during starvation, and the starvation-induced inhibition of deiodination is removed upon food presentation (Higgs and Eales, 1977). Thyroid hormone levels (T4 and T3) are significantly lower in samples from starved trout than fed trout (Flood and Eales, 1983). In fish bled 4 hours after food presentation both plasma T4 and T3 levels are elevated $*I^-$ and plasma *T3 levels of recently fed *T4-injected starved trout also increase, indicating that the elevated T3 could be partly a consequence of deiodination (Flood and Eales, 1983). Preliminary *in vitro* experiments on coho salmon show depressed hepatic T4 to T3 conversion in fish starved for 18 days (Leatherland, 1982).

4. Role of GSH.

The addition of GSH increases T4 deiodination *in vivo* and *in vitro* (Eales *et al.*, 1984). Injected GSH increased deiodination and plasma clearance of *T3 and *T4 in both starved and fed trout. However, a dose-dependent relationship was not shown (Eales *et al.*, 1984). Low concentrations (up to 0.5 mM) of GSH enhanced deiodination in trout liver *in vitro* but higher amounts markedly inhibited the reaction (Eales *et al.*, 1984). If diamide and GSH were added to the

homogenate, the inhibitory action of GSH was reversed and diamide by itself activated $*I^-$ production (Eales et al., 1984). Incubation under N_2 with NADPH, conditions that maintain reduced thiols or promote thiol synthesis, was without effect. Thus there is little evidence that GSH regulates of T4 5'-deiodination in rainbow trout (Eales et al., 1984).

DTT in vitro increased hepatic T4 to T3 conversion in rainbow trout in a dose-dependent manner up to the maximum concentration used (20 mM) (Eales et al., 1984). In contrast, Darling et al. (1982) observed a biphasic response in DTT's enhancement of deiodination in coho salmon liver homogenates. Low levels (5 mM) activated deiodination while at high concentrations (20 mM) inactivation occurred.

5. Effect of inhibitors of deiodination.

Several agents (5 mM concentration) were tested (Eales et al., 1984). The T4 analog ANS was the most potent inhibitor followed by, in decreasing order of potency: PTU, $HgCl_2$, diphenylhydantoin, and salicylate.

6. Regulation of T4 5'-deiodination.

The hypothalamic-pituitary axis may have a role in the regulation of deiodination in teleosts (Fok and Eales, 1984). Indirect evidence for this hypothesis comes from results obtained from T4-challenged rainbow trout. A dose of T4 (69 ng/g) that elevates plasma T4 50-fold 24 hours after injection had no effect on plasma T3 levels (Fok and Eales, 1984). Furthermore, metabolic clearance rates of T3 and T4

were unaffected as was radiothyronine uptake by the liver, gall bladder, and small intestine (Fok and Eales, 1984). Fok and Eales (1984), concluded that stable levels of T3 in the face of T4 challenge were achieved by a reduction in the proportion of the peripheral T4 undergoing deiodination. Hypophysectomy depressed deiodination in rainbow trout (Levin and Eales, 1982). Also, Ng and coworkers (1982) suggest that a pituitary glycoprotein may act as a deiodination activator. Regulation of T4 to T3 conversion, therefore, could involve the monitoring of plasma T3 levels in the hypothalamus and subsequent activation or inhibition of peripheral deiodination by the systemic release of pituitary hormones (Fok and Eales, 1984).

MATERIALS AND METHODS

A. Experimental animals.

Rainbow trout were obtained from the Rockwood Hatchery, Balmoral, Manitoba. One-to four-year-old trout (Idaho, Sundalsora, and Manx stock) ranging in weight from 175 g to 1000 g were used. Separate stocks were held in 2.3 Kl fiberglass tanks supplied with flowing aerated and dechlorinated Winnipeg city water at 12°C. All fish were fed Ewos trout grower pellets (Rundle Feed Mill, Palmerston, Ontario) corresponding to 1-2% body weight once daily and were maintained on a 12 h L : 12 h D photoperiod (light 0700-1900 h).

B. Subcellular fraction preparation.

At the beginning of each preparation fish were anesthetized by placing them in plastic tubs containing 1.2 g MS222 (tricaine methane sulfonate, Syndell Laboratories Ltd., Vancouver) dissolved in 18 l of aerated isothermal water.

The trout were sacrificed by concussion and the livers quickly freed from the gallbladder, removed and rinsed in ice-cold buffer. Two buffers were used in the preparation of subcellular fractions. Buffer A, containing 0.25 M sucrose, and 3 mM DTT, 10 mM Tris-HCl, pH 7.4, was used for the first set of experiments. This was replaced later with buffer B containing 0.1 M KH_2PO_4/Na_2HPO_4, 0.25 M sucrose, and 3 mM DTT, 5mM EDTA at pH 7.2. For the GSH-diamide experiments,

DTT-free buffer B was used.

After being weighed, livers from several fish were pooled and added into 4 volumes of buffer A or buffer B. The livers were minced with a scalpel and dispensed into either a 10-ml or 55-ml Wheaton homogenizing vessel. Tissue was initially treated with a Polytron homogenizer (Brinkman Instruments) set at setting 6 for 4 seconds, followed by 2 strokes with a motorized pestle (Tri-R Stir-R, Tri-R Instruments Inc., New York). The resulting crude homogenate was filtered through two layers of gauze and diluted with 2 volumes of ice-cold buffer (A or B) to a final dilution of 1:8 w/v.

The centrifugation procedure was modified from Fekkes et al. (1979). Aliquots of homogenate were transferred into plastic centrifuge tubes and spun for 20 minutes at 730 x g in a Clinicool centrifige (Damon/IEC Division, Needham Heights, Mass.). The supernatant was decanted from the crude nuclear pellet and spun in a SW 40.2 rotor (Beckman Instruments, Palo Alto, Cal.) at 25,200 x g for 10 minutes in a Beckman L8 ultracentrifuge to obtain the crude mitochondria/lysosome pellet. The supernatant was decanted as before and spun at 110,000 x g for one hour in a SW 40.2 rotor (Beckman) to separate the microsomal fraction from the cell sap. Depending on the assay, the microsome pellet was resuspended in either buffer A or B or sucrose-EDTA buffer and 1-2 ml of the suspension was dispensed into plastic vials and covered with parafilm. When other fractions (homogenate,

crude nuclei, mitochondria/lysosomes) were prepared, pellets were resuspended in identical volumes of the appropriate buffer and the homogenate was stored undiluted. Purified rainbow trout hepatic nuclei were a gift from O. Bres. All subcellular fractions were stored at -70°C until use.

In the preparation of large quantities of microsomes approximately 4 times the original amount of liver was homogenized. A 50.2 Ti fixed-angle rotor (Beckman) was substituted for the SW 40.2 rotor in subsequent centrifugation steps. The final protein concentration was approximately 10 mg/ml (range 5.2-15.6 mg/ml).

C. T4 5'-deiodinase assay.

Assay procedure followed the method of Pimlott and Eales (1983) except for the substitution of different subcellular fractions. Microsomes (or other fractions) were thawed and diluted to the appropriate concentration with ice-cold buffer (A or B). If DTT was added to the assay it was first dissolved in a few drops of buffer from the stock assay tubes and then pipetted back into the 125 x 16 mm glass incubation tubes (final assay volume 1.0 ml). Parallel control tubes containing buffer plus any reagents added to the experimental tubes, but no subcellular fractions, were run for each assay.

The substrate for the reaction consisted of 20 µl 0.1 N NaOH containing approximately 100,000 cpm *T4 (s.a. 750 µCi/µg; radioiodide content 4-8%; Industrial Nuclear, MO.) and carrier T4 at a final concentration of 50 ng/ml, defined

as the *T4/T4 standard . Three to five replicates of control and experimental tubes were equilibrated for 30 minutes in the dark in a shaker bath set at 12°C and 150 rpm. At time zero, 20 µl of the *T4/T4 mixture was pipetted into the tubes and shaken. After a 20-minute incubation period the first tube was removed from the shaker bath and 20 µl of KI (2×10^{-3} M) was added to displace any $*I^-$ bound to the walls of the tube.

The $*I^-$ content was determined in duplicate by adding 100 µl of each incubate onto Sephadex G-25 minicolumns (Pharmacia Chemicals, Uppsala, Sweden; bed volume 40 x 13 mm) previously layered with 250 µl 0.1 N NaOH. Any iodoprotein (labeled iodide covalently incorporated into protein) was eluted off the columns with 2.25 ml barbital buffer (7.4×10^{-2} M; pH 8.6) Initial experiments showed this fraction to contain 30-40 cpm and in later experiments it was routinely eluted to waste. A second elution with 2.25 ml barbital removed the iodide fraction. The iodide and total counts reference (TCR = initial *T4 radioactivity added to each incubate) were counted in a Beckman 8000 gamma counter (Beckman). Columns were regenerated with 7.5 ml of diluted outdated human plasma, diluted 1:9 with either barbital buffer pH 8.6 or deionized distilled water. The proteins in the human plasma bind, and hence remove residual thyroid hormones on the Sephadex. The hormone bound to the plasma was then eluted off with 10 ml of deionized distilled water and the columns were capped and stored in 0.1 N NaOH.

Protein was assayed with the Bio-Rad protein kit (Bradford, 1976).

D. Calculation of T4 deiodination.

Four assumptions were made in calculating the T4 deiodination rate: T4 was labeled exclusively in the outer-ring (3' and/or 5' positions), only one of the two available iodines was labeled at the specific activity of the labeled T4, any T3 formed underwent negligible outer-ring deiodination, and iodide formed by deiodination was unreactive (Fig. 6). The assumptions are supported by previous in vivo (Eales, 1977) and in vitro work (Pimlott and Eales, 1983) and by studies in this thesis. The T4 deiodination rate, expressed as pmol T4 converted $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$ was calculated as the product of the fraction of *T4 deiodinated and the total T4 added to the incubate divided by both the incubation time and protein content of the incubate (see Appendix I for detailed explanation and sample calculation).

E. Variations in standard assay procedure.

1. Time study.

In these experiments 10, 20, 40 and 60 minute incubations were used.

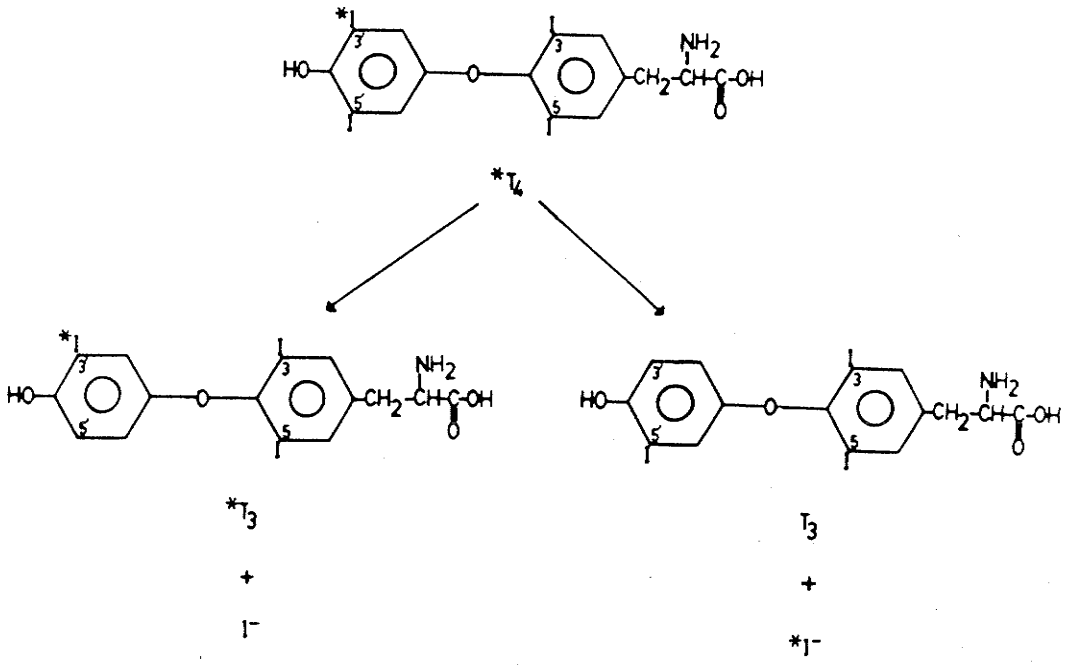
2. pH profile.

Microsomes were assayed in buffer B over a pH range of 6.0 to 8.0.

3. Heat treatment.

Microsomes were pretreated for one hour in a water bath

Figure 6. Products formed from *T4 5'-deiodination in trout.


*I
3
HO
5
O
I
3
5
NH2
CH2-CH-C-OH
O
*T4
*I
3
HO
5
O
I
3
5
NH2
CH2-CH-C-OH
O
*T3
+
I-
I
3
HO
5
O
I
3
5
NH2
CH2-CH-C-OH
O
T3
+
*I-

set at 60°C and then assayed as usual.

4. T3 deiodination.

Standard procedure was followed except that T3 carrier (83.4 ng/ml) and *T3 (100,000 cpm; s.a. 550 μCi/μg; Industrial Nuclear, MO.) were used in place of the *T4/T4 substrate (see Appendix II for calculation of T3 deiodination rate).

5. Substrate concentration.

Standard assay procedure was followed except that different concentrations of T4 carrier were used. Calculation of pmol T4 converted with varying substrate is outlined in Appendix III.

6. Modifying agents.

All of the agents were dissolved into buffer B and the pH of the solution adjusted if necessary. This stock solution was diluted with buffer to yield the concentration used in the assay. For the GSH and diamide experiments, modified DTT-free buffer B was used and no DTT was added to the incubate.

F. Long-column chromatography.

To investigate the *T4 products, incubated samples were chromatographed using a Sephadex G-25 column (fine mesh; 27.8 cm x 1.5 cm). The procedure was a modification of that of Green (1972) and Rudolph et al. (1978), involving an automated system consisting of an LKB 2152 HPLC controller, LKB 2211 fraction collector and LKB 2132 peristalic pump. The column was equilibrated in eluent I (0.1 N NaOH-0.01 M NaCl

with 3.6 g/l $Na_2S_2O_5$).

Microsomes were assayed as per standard procedure. A 500 µl aliquot was removed from the incubation tube and diluted 1:1 (v/v) with equilibration solution (0.1 N NaOH-1 M NaCl with 3.6 g/l $Na_2S_2O_5$) prior to storage at -70°C. At the beginning of each run, 0.3-0.4 ml of sample (experimental or control incubate or *T4/*T3 standard) was injected onto the column with a 1-ml syringe The tubing was flushed with 3 ml of equilibration solution and the fractions eluted with eluent I at a flow rate of 1 ml/min. Total fraction volume was 4 ml/tube. After 25 fractions were collected eluent II (0.15 N NaOH-0.01 M NaCl with 3.6 g/l $Na_2S_2O_5$) was passed through the column and a further 55 fractions collected. A continuous gradient was run from fractions 80 to 85 with decreasing proportions of eluent I and increasing proportions of equilibration fluid. A total of 110 fractions was collected and counted. To identify the T4 and T3 peaks on the chromatograph, *T4 and *T3 were cochromatographed under identical experimental conditions. Although not chromatographed in isolation to confirm its identity, the peak of radioactivity in fraction 15 was assumed to be $*I^-$ on the basis of previous experiments by Pimlott and Eales (1983).

G. Effect of starvation on T4 5'-deiodination.

Rainbow trout (domestic Nisqually stock) were transferred from a 2.3 K1 tank into four 200 1 tanks, each tank holding 29 fish. All fish were acclimated for one week

in running 12°C dechlorinated, aerated city water on a 12 h L : 12 h D light cycle and were fed Ewos trout pellets once each day (1100-1130) at a ration of approximately 1% body weight /day.

For the two-week experimental period two tanks of fish were fed a ration of approximately 1% body weight at the same time each day (1100-1130). The fish in the other two tanks were starved. At the conclusion of the experimental period the fed and starved fish were weighed and sacrificed and the livers from each group processed in pools of five. All the liver tissue was processed identically and stored at -70°C until analyzed. From the average fish weights for each tank, the exact food rations were calculated to be 0.81% body weight/day for the first tank and 0.96% body weight/day for the second tank.

H. Glucose-6-phosphatase assay.

The activity of the ER marker enzyme glucose-6-phosphatase was assayed according to Baginski (1974). Briefly, the fraction to be analyzed (homogenate, nuclei, mitochondria/lysosomes, microsomes) was resuspended in ice-cold sucrose-EDTA buffer (0.25 M sucrose, 1 mM EDTA, pH 7.0) and kept on ice until use. Volumes of 100 µl each of sucrose-EDTA, 0.1 M glucose-6-phosphate and 0.1 M cacodylate buffer (pH 6.5) were pipetted into each test tube. This mixture was vortexed and equilibrated in the dark at 12°C for 30 minutes in a shaker bath set at 100 rpm. At the beginning of the assay, 100 µl of sucrose-EDTA (blank), phosphate

standard (1.5 mM) or sample were added to the corresponding tubes at 30 second intervals and incubated. The reaction was stopped with 2 ml of a mixture of ascorbic acid and trichloroacetic acid (TCA) (2% w/v ascorbic acid; 10% w/v TCA). Control tubes were also run with the sample added after the ascorbic acid/TCA. All of the tubes were then removed from the bath, vortexed and spun at 3000 x g for 3 minutes in either a Sorvall GLC-1 bench top or Beckman J2-21M centrifuge. One ml of the supernatant was pipetted into a clean test tube followed by 500 µl of ammonium molybdate (1% w/v) and vortexed. Finally, 1 ml of an arsenite-citrate mixture (2% w/v arsenite; 2% w/v citrate) was added, vortexed and allowed to stand for 15 minutes. The solution was read at 700 nm. A sample calculation of phosphatase activity and the phosphate standard curve are given in Appendix IV and Appendix Fig. 1.

I. Statistical analysis.

Linear regressions (including Lineweaver-Burk plots) were calculated with the least squares method. Differences between the means of two groups of data were analyzed with an unpaired t-test. Prior to analysis, the homogeneity of the variances was confirmed to be insignificant ($p<0.05$) with a two-tailed F-test. The statistical techniques employed in this study are outlined in Mendenhall (1979), Snedecor and Cochran (1971), and Sokol and Rohlf (1969).

RESULTS

A. Optimization of assay.

1. Influence of enzyme concentration on T4 5'-deiodination.

Pelleted microsomes were resuspended in buffer A (pH 7.4) and serially diluted to determine the effect of enzyme concentration on T4 5'-deiodination. Enzyme activity increased linearly with increasing protein concentration up to 0.5 mg/ml (Fig. 7).

2. pH.

Microsomes were resuspended in buffer B at pH 6.0, 6.5, 7.0, 7.5, and 8.0. In two trials, maximum deiodination was noted at pH 7.0 (Fig. 8).

3. Effect of DTT.

DTT was dissolved into the incubate in varying concentrations to examine the effect of exogenous thiols on deiodination. DTT elevated enzyme activity up to a thiol concentration of 11 mM (Fig. 9). Although zero DTT wasn't employed here, later experiments with GSH showed no deiodination without the addition of DTT.

4. Incubation time.

Microsomal suspensions were incubated for 10, 20, 40, and 60 minutes. The number of pmoles T4 converted increased with increasing incubation time up to the maximum time employed (Fig. 10).

Figure 7. Influence of enzyme concentration (mg/ml of protein in incubate tube) on T4 5'-deiodination. Each point represents a mean (± SEM) of 5 separate incubates.

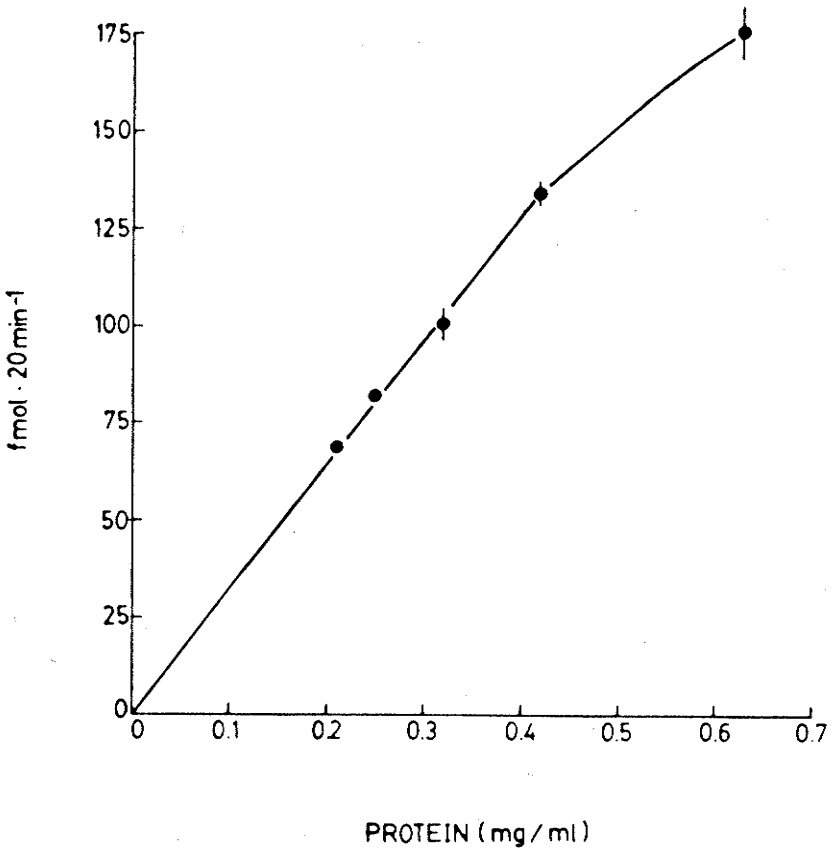

175
150
125
100
75
50
25
0
fmol · 20 min-1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
PROTEIN (mg / ml)

Figure 8. Influence of pH of assay mixture on T4 5'-deiodination. Each point represents a mean (± SEM) of 5 separate incubates. ● trial 1, O trial 2.

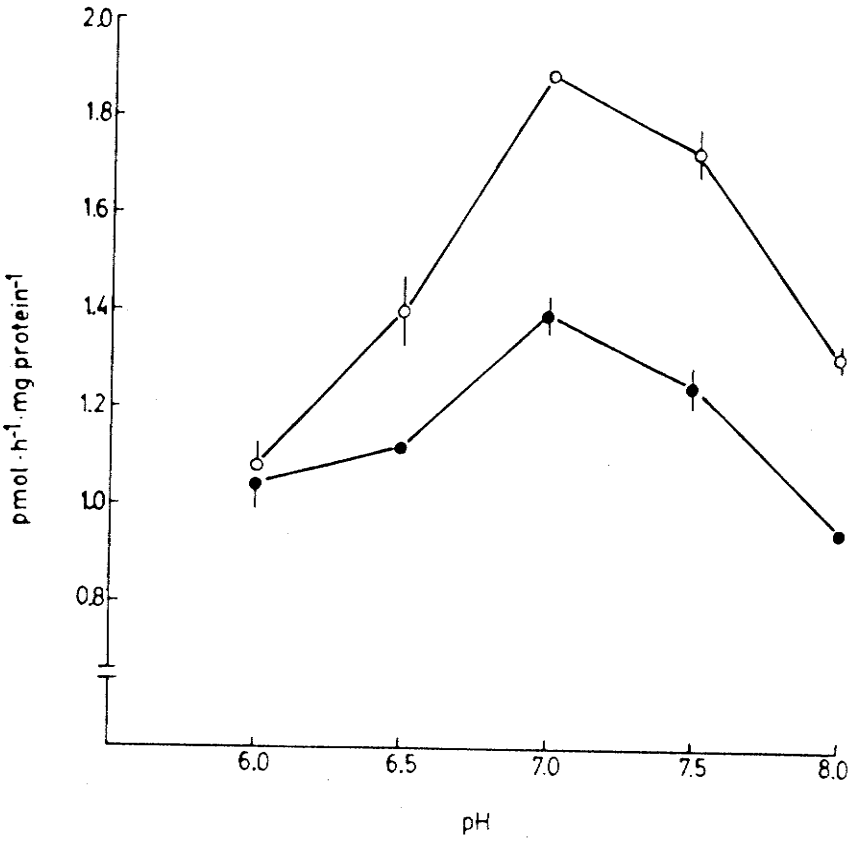

2.0
1.8
1.6
1.4
1.2
1.0
0.8
pmol · h⁻¹ · mg protein⁻¹
6.0
6.5
7.0
7.5
8.0
pH

Figure 9. Effect of DTT on T4 5'-deiodination. Each point represents a mean (± SEM) of 3 separate incubates. Point at origin derived from Figure 16.

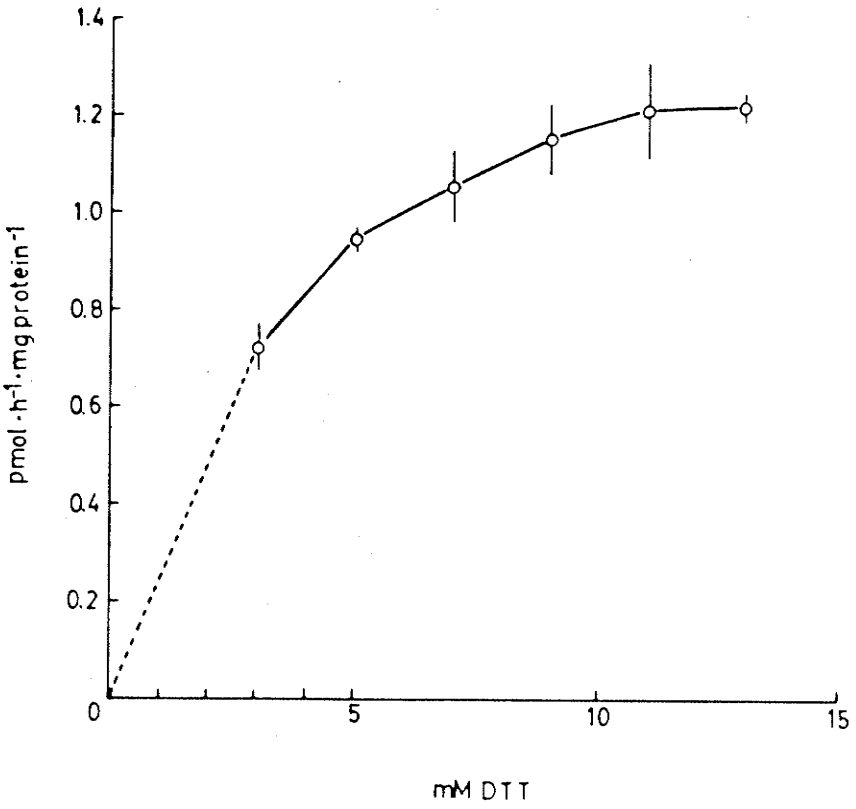
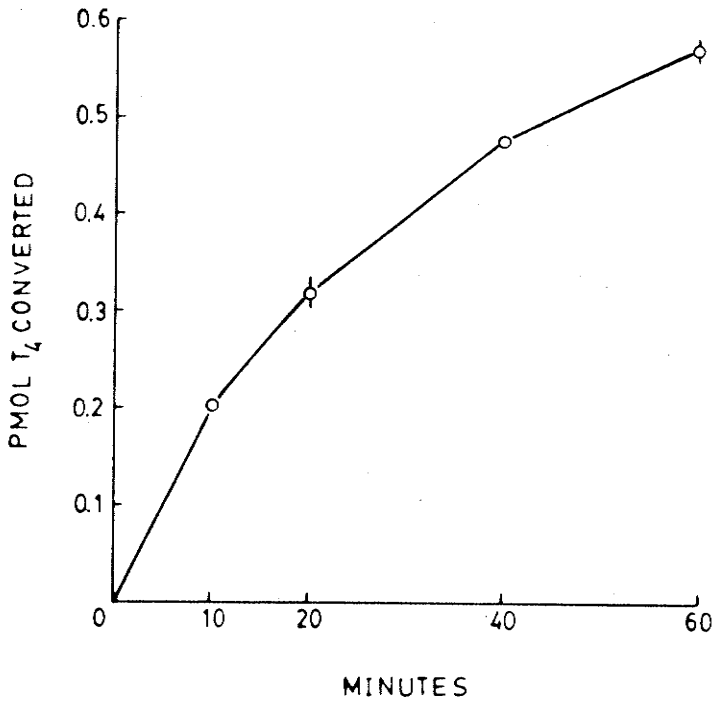

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
pmol · h-1 · mg protein-1
mM DTT

Figure 10. Influence of incubation time on T4 5'-deiodination. Each point represents a mean (± SEM) of 3 separate incubates.


0.6
0.5
0.4
0.3
0.2
0.1
0
10
20
40
60
PMOL T_4 CONVERTED
MINUTES

5. Comparison of buffer A with buffer B.

Microsomes were processed in either buffer A or buffer B and T4 deiodinase activities compared. $*I^-$ production was significantly higher ($p<0.01$) in buffer B (Table IV).

6. Heat treatment.

One set of incubation tubes (designated heat-treated) was preheated at 60°C for one hour and assayed for deiodinase activity in tandem with a second set of tubes (designated control) that were kept on ice for one hour prior to the assay. Preheated microsomes showed significantly less activity ($p<0.01$) than incubates previously held at 4°C (Table V).

On the basis of information obtained from these studies, the following assay conditions were employed for all subsequent experiments: 12°C (acclimation temperature of fish), buffer B at pH 7.2, 5 mM DTT, 20 minute incubation, protein concentration not exceeding 0.5 mg/ml.

B. Long-column chromatography.

Chromatographic analysis of the *T3/*T4 standard revealed distinct *T3 and *T4 peaks in fractions 32 and 58 respectively (Fig. 11A). The $*I^-$ contamination of the *T3 and *T4 standards appeared in fraction 15. Analysis of the control incubate (identical to sample but without microsomes) showed three peaks corresponding to $*I^-$, *T3 and *T4 (Fig. 11B). The radioactivity peaks from the duplicate samples containing the microsomal fraction were symmetrical, demonstrating clean separation of major iodomaterials (Fig.

TABLE IV.

Comparison of T4 5'-deiodination in replicates from a single trout microsome fraction using assay buffers A or B.

	pmol T4 converted $\cdot h^{-1} \cdot protein^{-1}$	
	Buffer A	Buffer B
	0.265	0.461
	0.275	0.362
	0.310	0.330
	0.263	0.397
	0.271	0.413
$\bar{X}$	0.277	0.392[+]
S.D.	0.019	0.050

[+] Significantly different ($p < 0.01$) from buffer A values as determined by two-tailed t-test.

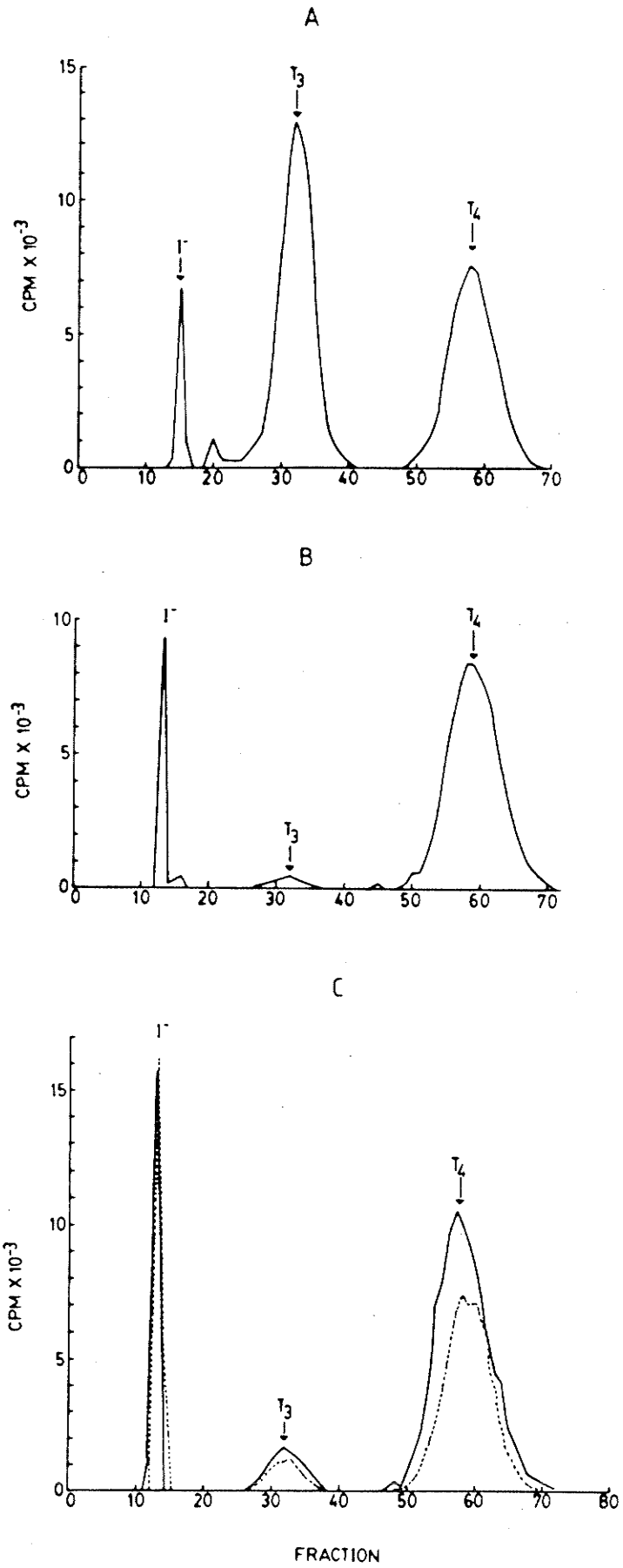
TABLE V.

Influence of heat-treatment (H) on microsomal T4 5'-deiodinase activity in separate determinations. C = control microsomes

	pmol T4 converted · h^{-1} · mg protein $^{-1}$	
	H	C
	0.124	1.46
	0.031	1.34
	0.047	1.48
	0.007	1.59
	0.015	1.43
$\overline{X}$	.045+	1.46
S.D.	0.047	0.09

+ Significantly different ($p < 0.01$) from control microsomes as determined by two-tailed t-test.

Figure 11. Elution profiles of (A) *T4/*T3 standard, (B) control (no microsomes), and (C) sample (containing microsomes). G-25 Sephadex chromatography (fine mesh; 27.8 x 1.5 cm). (-----), trial 1; (_____), trial 2.


A
B
C
CPM X 10⁻³
I⁻
T3
T4
FRACTION

11C). Approximately equal production of *T3 and $*I^-$ from *T4 were noted in trial I (715 cpm $*I^-$, 504 cpm *T3) and trial II (887 cpm $*I^-$, 741 cpm *T3) confirming that iodide is being removed from the outer ring of *T4 and indicating negligible outer ring deiodination of *T3. Detailed calculations of the relative amounts of $*I^-$ and *T3 generated from *T4 are outlined in Appendix V.

C. Investigation of enzyme substrate.

1. T3 deiodination.

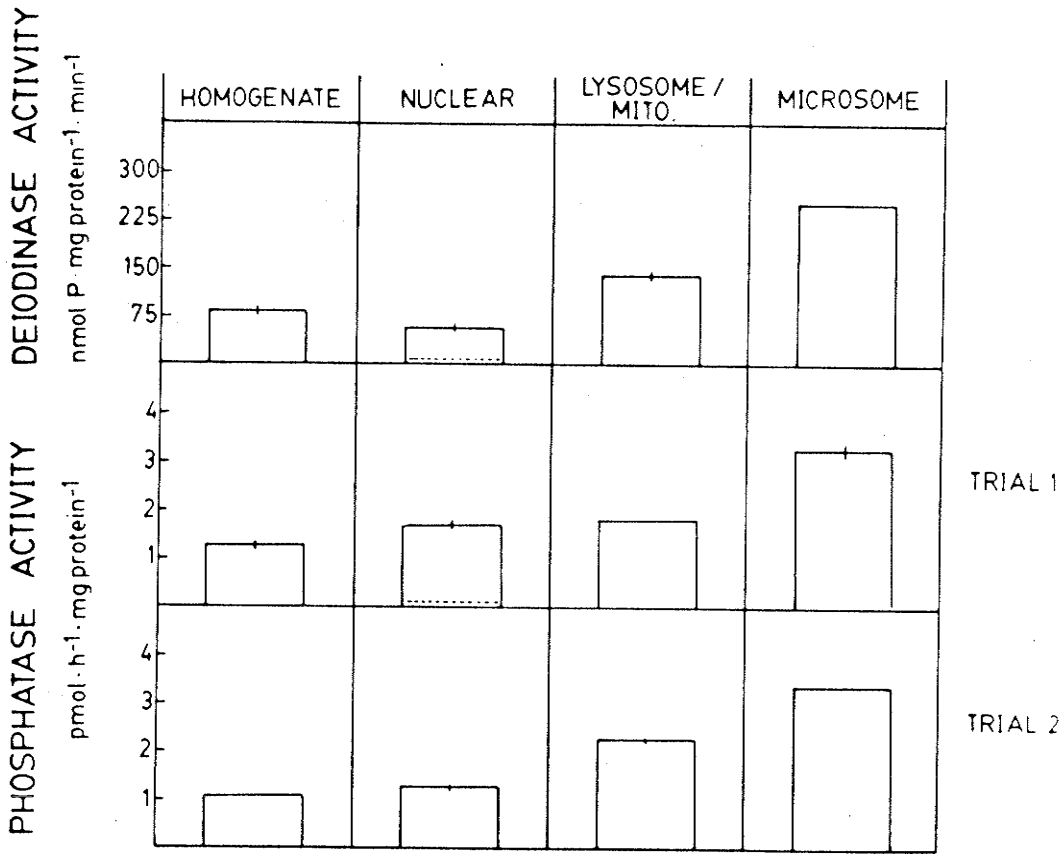
Cross reactivity of the enzyme for T3 was examined by substituting 100,000 cpm *T3/carrier T3 (final concentration 83.4 ng/ml) for *T4/T4 carrier in the incubate. T3's conversion rate was approximately 3% of T4's as calculated from parallel incubates.

D. Subcellular localization.

Four crude and one pure subcellular fractions were analyzed for glucose-6-phosphatase, an ER marker enzyme. Glucose-6-phosphatase activity was highest in the microsomal fraction and progressively lower in the lysosomal-mitochrondrial fraction, whole homogenate, crude nuclear fraction, and purified nuclear fraction (Fig. 12).

T4 5'-deiodination was also assessed in corresponding fractions prepared from two pools of liver. In both trials, the microsomal fraction exhibited the largest T4 deiodinase activity (Fig. 12). The presence of high levels of glucose-6-phosphatase and deiodinase activities in the microsome fraction implies that the T4 5'-deiodinase is

Figure 12. Subcellular distribution pattern of T4 5'-deiodinase and glucose-6-phosphatase in fractions containing crude whole homogenate, crude and purified (----) nuclei, lysosome/ mitochondria and microsomes.


DEIODINASE ACTIVITY
nmol P · mg protein⁻¹ · min⁻¹
HOMOGENATE
NUCLEAR
LYSOSOME / MITO.
MICROSOME
300
225
150
75
PHOSPHATASE ACTIVITY
pmol · h⁻¹ · mg protein⁻¹
4
3
2
1
TRIAL 1
TRIAL 2

located in the ER.

E. Enzyme kinetics.

The effect of substrate concentration on deiodination was determined by the measurement of changes in enzyme activity with varying amounts of carrier T4. Intermediate carrier concentrations (70-1125 nM) were employed in Km and Vmax determinations in fed and starved trout. The Km value for the hepatic T4 5'-deiodinase was 1.9 x 10aa M and the maximum velocity (Vmax) was 3.4 pmol T4 converted $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$ (Fig. 13).

F. Agents modifying deiodination.

The modifying agents tested in this study encompass 6 of the 7 categories discussed in the literature review including: 1) thyroid inhibitors, 2) thyroid hormone analogs, 3) GSH and diamide, 4) enzyme sulfhydryl binding agents, 5) end product inhibitors, and 6) chelating agents.

Thyroid inhibitors were defined as compounds that block iodoperoxidase (e.g. thiourea), catalase (e.g. sodium azide) or compete with iodide for thyroid uptake (e.g. thiocyanate). None of the three agents had any effect on hepatic deiodination (Fig. 14A, 14B, 14C).

The T4 analog ANS was the most potent inhibitor. Inhibition was detectable at 2 uM ANS and virtually no deiodinase activity (<4%) could be detected at 0.1 mM concentrations (Fig. 15). In contrast, the addition of 10 mM salicylate, a second analog, depressed deiodination by only 60% (Fig. 15).

Figure 13. Lineweaver-Burk plot of T4 5'-deiodinase kinetic data. Km = 1.9×10^{-7} M; Vmax = 3.4 pmol T4 converted $\cdot h^{-1} \cdot$ mg protein^{-1}. Each point represents a mean of 3 replicates.

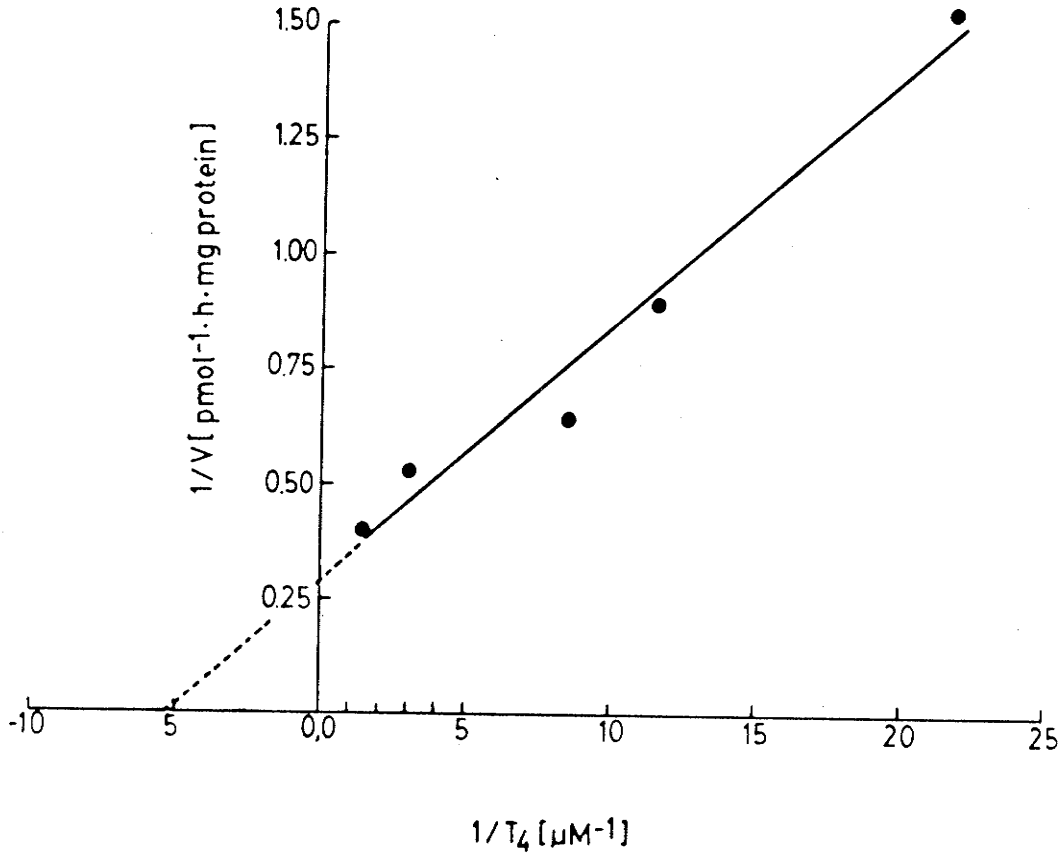

1.50
1.25
1.00
0.75
0.50
0.25
1/V[pmol-1·h·mg protein]
-10
5
0,0
5
10
15
20
25
1/T4 [µM-1]

Figure 14. Influence of thyroid inhibitors on T4 5'-deiodination. The mean value of 3 control (no inhibitor or activator) incubates represents 100% activity. Percentage activity of the modifiers is calculated as:

$$\frac{\text{activity of 5'-D (+ activator or inhibitor)}}{\text{activity of 5'-D (control)}} \times 100$$

Data are plotted as mean percentage activity (± SEM). A) sodium azide. B) thiourea; ● trial 1, O trial 2. C) thiocyanate; ● trial 1, O trial 2.

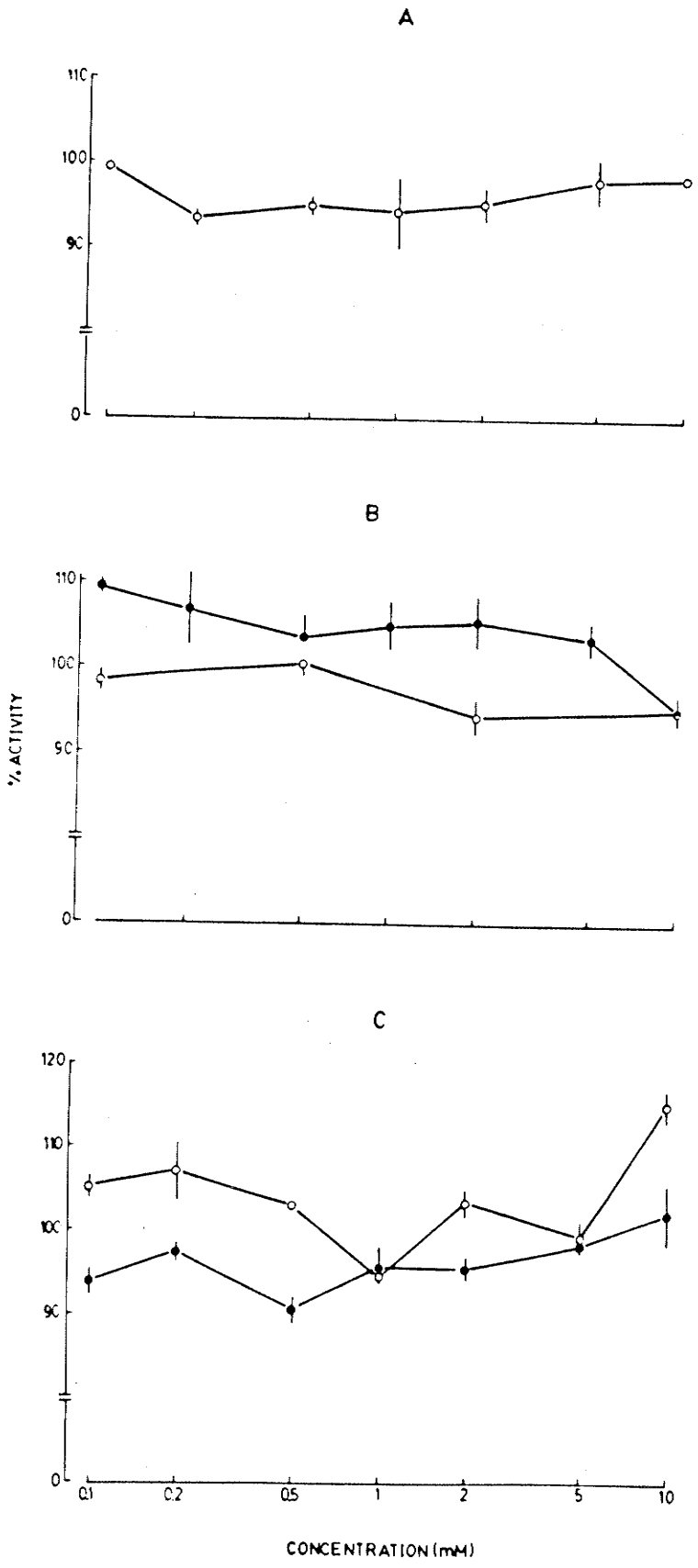
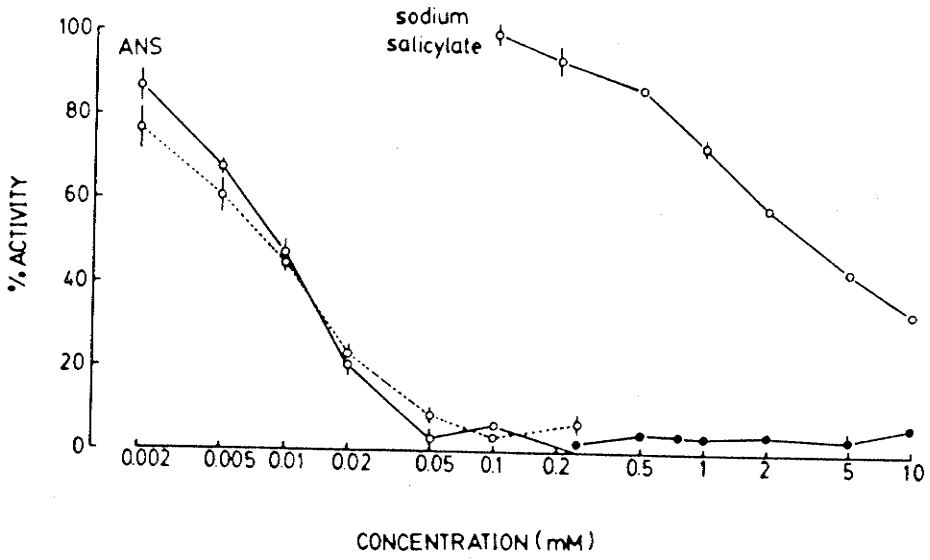

A
110
100
90
0
B
110
100
90
0
% ACTIVITY
C
120
110
100
90
0
0.1
0.2
0.5
1
2
5
10
CONCENTRATION (mM)

Figure 15. Influence of T4 analogs ANS and sodium salicylate on T4 5'-deiodination. Agent potency is expressed as in Figure 14.


sodium
salicylate
ANS
100
80
60
40
20
0
% ACTIVITY
0.002
0.005
0.01
0.02
0.05
0.1
0.2
0.5
1
2
5
10
CONCENTRATION (mM)

GSH had little effect on deiodination either at low (<0.01 mM) or high (2 mM) concentrations (Fig. 16) while the specific GSH-blocking agent, diamide, enhanced deiodination (Fig. 16).

PTU and N-ethyl maleimide, two molecules that react with enzyme sulfhydryl groups in mammals, inhibited T4 deiodination (Fig. 17A, 17B).

According to the law of mass action, addition of large amounts of iodide (as KI) should inhibit the deiodination of T4. An 18% decline was noted with 100 mM KI (Fig. 18). The negative results with equimolar KCl confirmed the specific inhibition by iodide.

Finally, the role of divalent ions in deiodination was examined with the non-specific chelating agent EDTA and the Ca^{2+}-specific EGTA. Both molecules enhanced deiodination, EDTA being slightly more effective than EGTA (Fig. 19A, 19B).

G. Influence of starvation on T4 5'-deiodination.

Hepatic T4 deiodination was significantly depressed ($p<0.01$) in fish starved for 14 days when compared to fed fish (Table VI). Kinetic analysis revealed that the affinity of the enzyme for T4 (Km) and maximum capacity (Vmax) were depressed as was the Vmax to Km ratio (Fig. 20A, 20B; Table VII).

A portion of the T4 converted in livers from starved trout could be produced via a PTU-insensitive pathway. To test this, enzyme activity was measured in hepatic microsomes from starved and fed trout in the presence of 1 mM and 10 mM

Figure 16. Influence of GSH and diamide on T4 5'-deiodination. Zero GSH had no measurable activity. Each point represents a mean (± SEM). GSH (----); diamide, ● trial 1, O trial 2 (____).

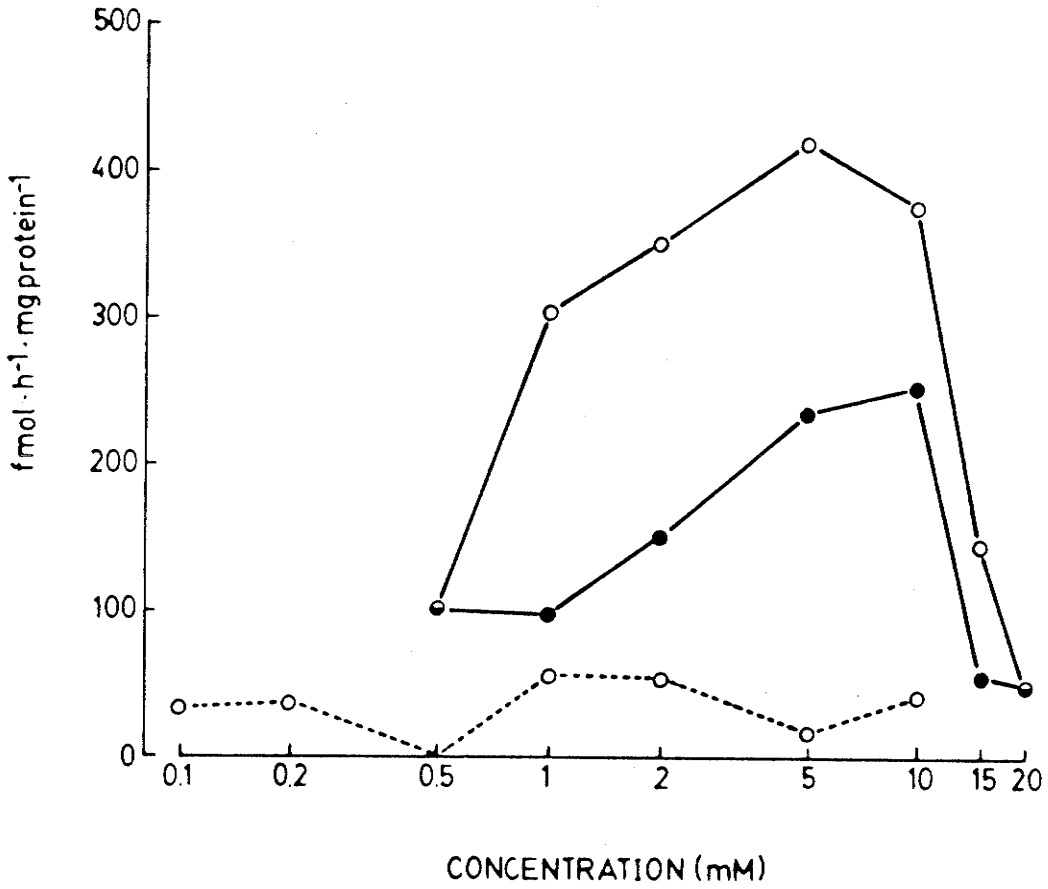

500
400
300
200
100
0
fmol·h⁻¹·mg protein⁻¹
0.1
0.2
0.5
1
2
5
10
15
20
CONCENTRATION (mM)

Figure 17. Influence on T4 5'-deiodination of inhibitors that bind to sulfhydryl groups. Inhibitor potency is expressed as in Figure 14. A) N-ethyl maleimide. B) PTU; ● trial 1, O trial 2.

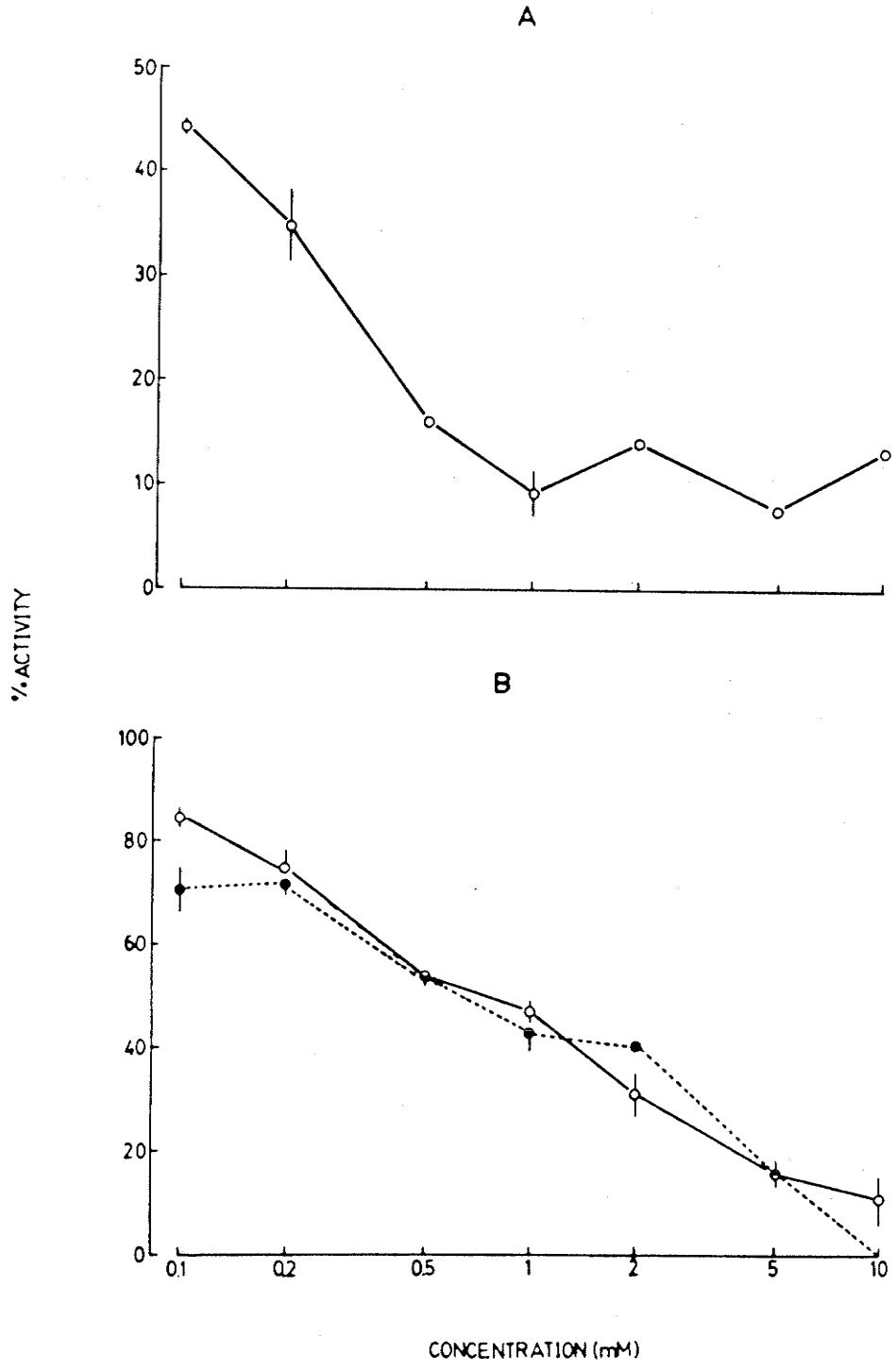

A
50
40
30
20
10
0
B
100
80
60
40
20
0
0.1
0.2
0.5
1
2
5
10
% ACTIVITY
CONCENTRATION (mM)

Figure 18. Influence of KI on T4 5'-deiodination. KI potency is expressed as in Figure 14. Enzyme activity in the presence of KCl is graphed for comparison. O KCl, ● KI.

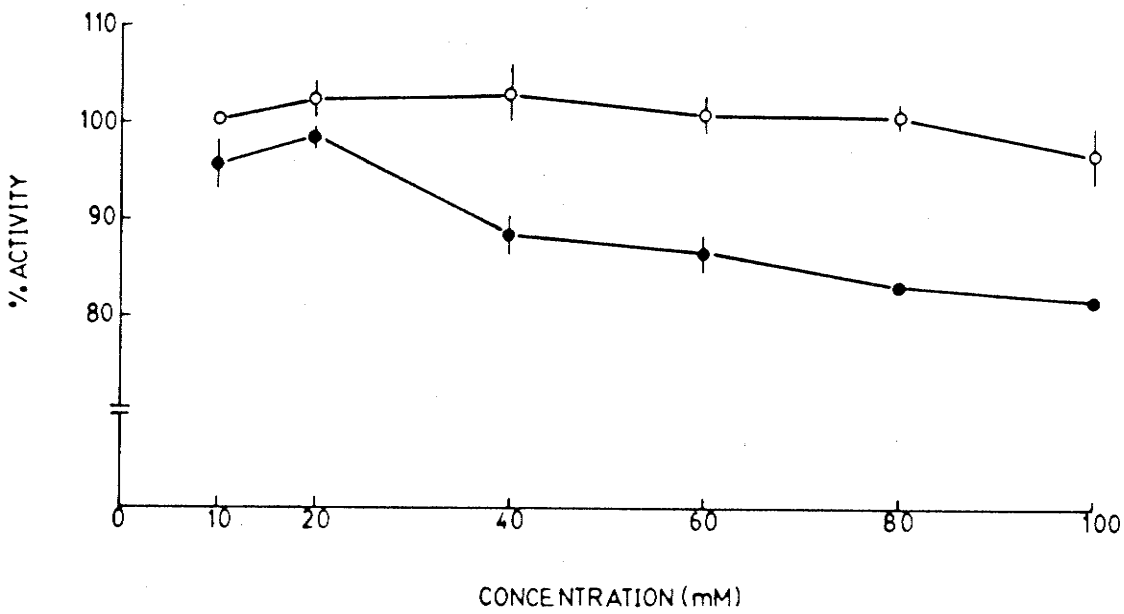

110
100
90
80
% ACTIVITY
0
10
20
40
60
80
100
CONCENTRATION (mM)

Figure 19. Influence of chelating agents on T4 5'-deiodination. Agent potency is expressed as in Figure 14. A) EGTA. B) EDTA; ● trial 1, O trial 2.

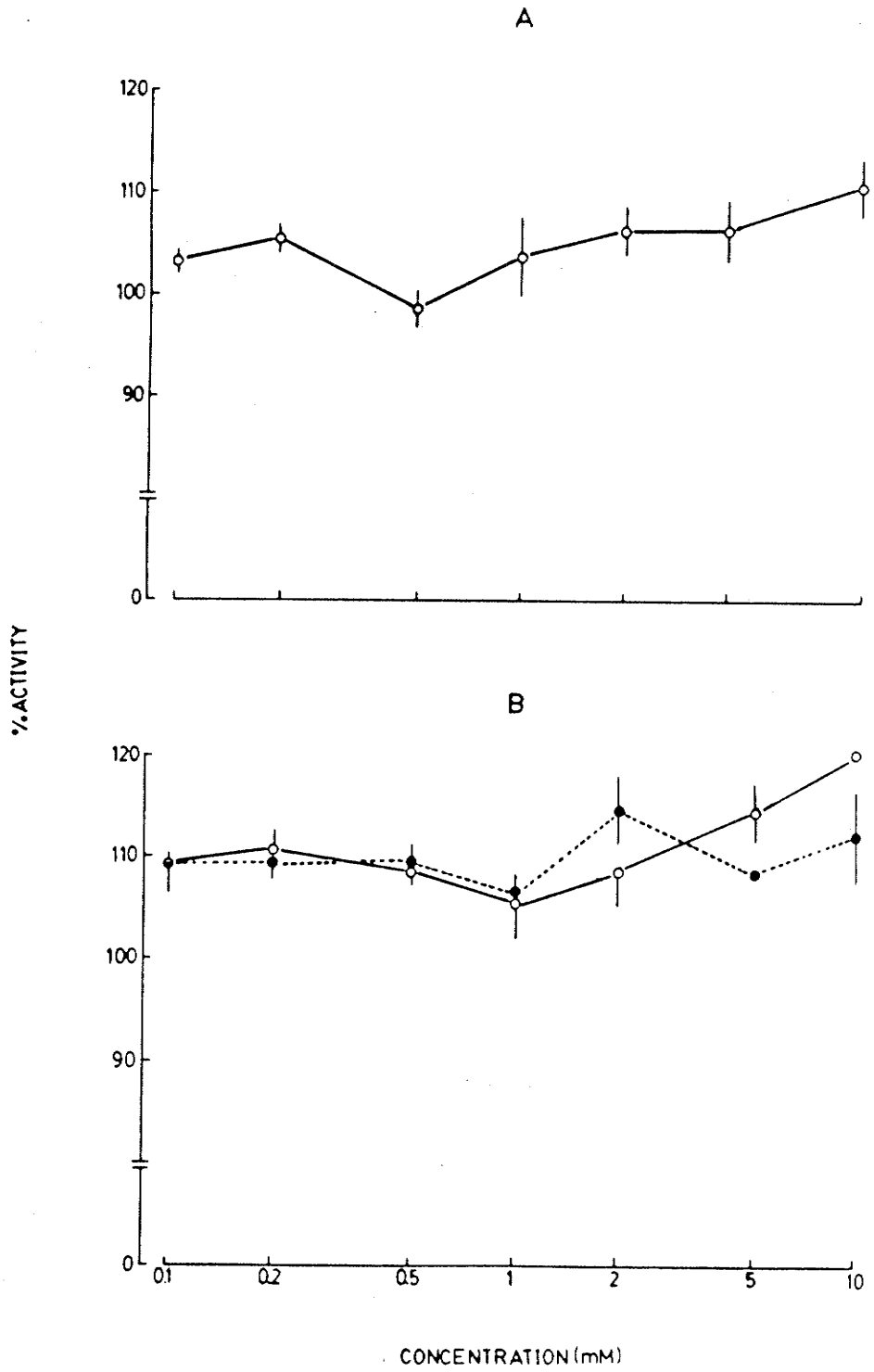

A
120
110
100
90
0
B
120
110
100
90
0
0.1
0.2
0.5
1
2
5
10
%ACTIVITY
CONCENTRATION (mM)

TABLE VI.

Comparison of T4 5'-deiodination in the hepatic microsome fraction of starved and fed trout.

	pmol T4 converted · h^{-1} · mg $protein^{-1}$	
	Starved	Fed
	0.601	1.72
	0.968	1.13
	0.657	2.90
	0.611	1.56
	1.04	1.58
	0.637	1.64
	0.944	2.98
	0.951	3.04
	0.864	1.85
	0.840	1.96
	1.91	1.43
	0.991	(5.29)*
$\overline{X}$	0.917	1.98+
S.D.	0.351	0.673

+ Significantly different ($p < 0.01$) from starved trout as determined by two-tailed t-test;

* (5.29) not included in $\overline{X}$ or S.D. calculation. All data were analyzed also with a White-Wilcoxon non parametric test. The fed group was again significantly ($p < 0.01$) higher than the starved group.

Figure 20. Lineweaver-Burk plots of T4 5'-deiodinase kinetic data from starved 0 and fed 0 trout. A) trial 1 starved fish, Km = 0.7 x 10^{-7} M, Vmax = 1.1 pmol; fed fish, Km = 1.5 x 10^{-7} M, Vmax = 4.1 pmol B) trial 2 starved fish, Km = 1.2 x 10^{-7} M, Vmax = 2.5 pmol; fed fish, Km = 1.7 x 10^{-7} M, Vmax = 8.3 pmol. Each point represents a mean of three replicates.

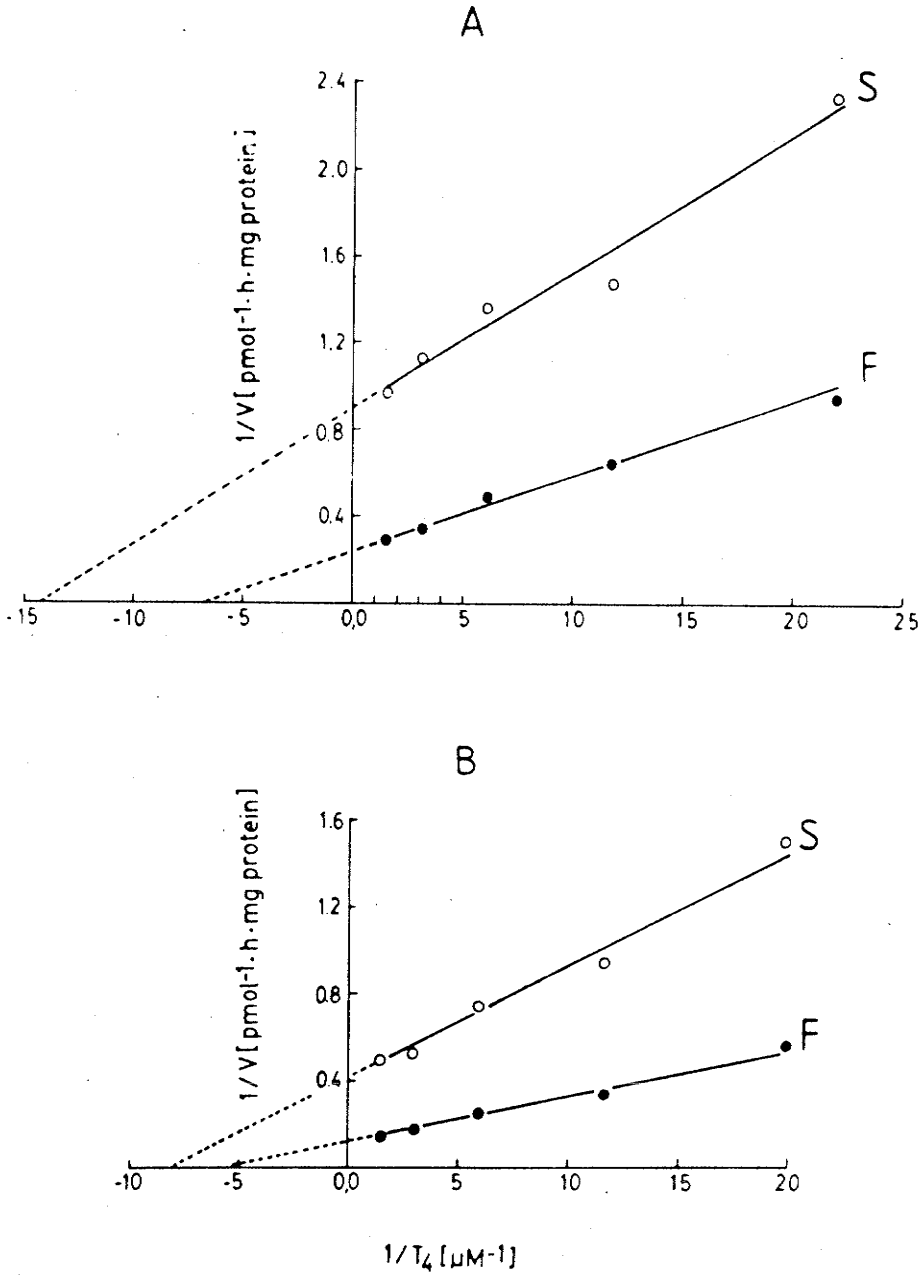

A
S
F
2.4
2.0
1.6
1.2
0.8
0.4
-15
-10
-5
0,0
5
10
15
20
25
B
S
F
1.6
1.2
0.8
0.4
-10
-5
0,0
5
10
15
20
1/T4 [µM-1]

TABLE VII.

Comparison of Vmax/Km ratios for fed and starved trout.

Trial	Condition	Vmax (pmol · h^{-1} · mg $protein^{-1}$)	Km (x 10^{-7})	Vmax / Km
1	Fed	4.1	1.5	2.7
	Starved	1.1	0.7	1.2
2	Fed	8.3	1.7	4.9
	Starved	2.5	1.2	2.1

PTU. No consistent trend was shown between the two groups and without a repeat experiment no conclusions could be made regarding the contribution of a type II enzyme in starvation (Table VIII).

TABLE VIII.

Influence of PTU on T4 5'-deiodination in the hepatic microsomal fraction of starved and fed trout. PTU potency is expressed as percent activity; 100% is assigned to activity with no PTU present.

PTU Concentration (mM)	Trial 1		Trial 2	
	Fed	Starved	Fed	Starved
0	100	100	100	100
1.0	35.9 ± 1.1	44.7 ± 2.3	35.7 ± 1.0	36.4 ± 1.8
10.0	6.7 ± 0.4	10.2 ± 1.0	6.9 ± 0.8	9.4 ± 3.1

DISCUSSION

The finding of substantial T4 5'-deiodination in the liver of rainbow trout confirmed previous work by Leatherland (1981) and Pimlott and Eales (1983). Enzyme activity depended on the addition of exogenous thiols (DTT) and was enhanced with increasing DTT concentrations. It was not possible to propose a mechanism for the reaction with DTT without the results of competitive inhibition experiments. A linear dependence between enzyme concentration and pmoles T4 converted was demonstrated. The pH optimum of 7.0 was in the range of values reported by Leatherland (1981) and Pimlott and Eales (1983). Increasing incubation times also elevated total deiodination, but preheating the enzyme to 60°C for one hour abolished deiodination.

T4 deiodination in trout is a one step process. The labeled deiodination products from *T4 are either $*I^-$ or *T3 depending on which of the outer ring iodines is labeled and which is removed (Fig. 6). Data from G-25 Sephadex chromatography on the long column substantiate this hypothesis. In microsomal incubates, $*I^-$ and *T3 were produced from *T4 in approximately equal amounts. Furthermore, there was negligible evidence of subsequent deiodination of *T3. When *T3/T3 was substituted for the standard *T4/T4 substrate in the microsomal incubate, T3 was deiodinated to only a small extent (3% of the T4 level).

Reverse T3 and sulfate conjugated iodothyronines were

not detected by chromatography as evidenced by a clean separation of the *I^-, *T3, and *T4 peaks. The absence of rT3 agrees with previous in vivo (Eales et al., 1983) and in vitro data (Pimlott and Eales, 1983) on trout. Mol and Visser (1984) suggested that in mammals the sulfation of T4 functioned to increase the concentration of inactive metabolites such as rT3. The conversion of T4 to inactive iodothyronines and deiodination of T3 itself may be unnecessary in trout because of ample iodine availability and a negligible requirement to salvage iodine (Eales et al., 1983).

Both glucose-6-phosphatase and T4 5'-deiodinase activity were greatest in the crude microsome fraction. However, the enzyme could only be tentatively located in the ER as opposed to the plasma membrane because the distribution of a plasma membrane marker such as 5'-nucleotidase was not assessed. Use of additional marker enzymes and the isolation of purified plasma membrane and ER would confirm the subcellular ER location.

There was an incomplete fractionation of ER membranes from lysosomes and mitochondria as indicated by the corresponding glucose-6-phosphatase activity. The crude nuclear pellet consisted of a broken cell preparation contaminated with whole cells, membrane fragments, and red blood cells trapped in the interior of the liver during homogenization. Unlike the mammalian erythrocyte, trout red blood cells possess a complete cell structure including ER.

These membranes together with the other contamination previously mentioned could contribute to the deiodinase and phosphatase activity measured in the crude nuclear fraction. Recent studies by Tseng and Latham (1984) have shown that rat hemoglobin catalyzes the oxidative deiodination of iodothyronines to iodide and intermediates. Production of $*I^-$ by hemoglobin in the crude nuclear fraction cannot be discounted. However, purified trout nuclei, free of ER, exhibited no deiodinase or phosphatase activity. Nuclei themselves probably do not contribute to intracellular T3 generation in the liver.

An average apparent Km of 1.7×10^{-7} M was calculated from three separate experiments on three pools of liver from fed fish. This value lies between the apparent Km of the mammalian hepatic enzyme and the value calculated for the liver whole homogenate (Pimlott and Eales, 1983). It is not possible to determine which Km (microsomal or whole homogenate) is more representative but some comparisons between kinetic parameters are valid. The Km and Vmax are clearly different between the two fractions but the ratio of Vmax to Km for each fraction differs by only a factor of 1.6. This ratio has been proposed by Mol and Visser (1984) as an index of enzyme efficiency reflecting increased production by the tissue as a whole rather than on a per molecule basis. Although the microsomal fraction was a more pure preparation, the enzyme in the trout whole homogenate was 1.6 times more efficient than in the microsome fraction.

Before the mechanism of reductive deiodination was understood, extrathyroidal T4 to T3 conversion was assumed to be the reverse of intrathyroidal tyrosine iodination. The latter reaction is oxidative, catalyzed by an iodoperoxidase with hydrogen peroxide serving as the hydrogen acceptor. The peroxidase antagonist catalase degrades hydrogen peroxide and is itself inhibited by sodium azide. Azide did not affect deiodination in trout liver microsomes suggesting that hepatic T4 to T3 conversion is not the reverse of iodination but a distinct reaction and that azide probably acts indirectly on the iodoperoxidase (via catalase). The lack of dependence of the hepatic system on iodoperoxidase was confirmed by the ineffectiveness of an iodoperoxidase inhibitor thiourea in blocking deiodination in either the trout liver microsome or homogenate fractions (Leatherland, 1981). The thyroid uptake mechanism is not specific for I^-. Other anions for example, thiocyanate and perchlorate compete successfully with iodide for uptake into the gland. The inhibitory role for thiocyanate in the liver relates to its similarity to iodide, a potential end-product inhibitor of peripheral deiodination. Given the small amount of inhibition noted with large amounts of KI the ineffectiveness of the analog thiocyanate was not surprising.

There are some similarities between the catalytic site of the mammalian T4 5'-deiodinase and the T4 plasma protein binding site (Chopra et al., 1980). ANS, a thyroid hormone analog, also competed with T4 for plasma binding sites in

trout (S. Golob, unpublished) and was the most potent inhibitor of T4 deiodination tested. The high potency of ANS as a deiodinase inhibitor was previously demonstrated in trout liver (Eales et al., 1984) and rat liver homogenates (Chiraseveenuprapund et al., 1978).

A second T4 analog, sodium salicylate, also displaced thyroid hormone from trout plasma proteins (S. Golob, unpublished) but was not as effective at inhibiting deiodination as ANS. For example, 50% inhibition was observed with 7 μM ANS but 3 mM salicylate was required to obtain comparable inhibition. The discrepancy can be explained by the greater similarity between ANS and the T4 molecule.

Reduced glutathione is a putative deiodinase activiting agent in mammals (Visser et al., 1978). Eales et al. (1984) observed a slight increase in $*I^-$ production with GSH concentrations below 1 mM, but little enhancement of deiodination with GSH was noted in the present study. In support of a previous study (Eales et al., 1984), addition of diamide (a GSH inhibitor) accelerated deiodination, providing indirect evidence that glutathione inhibits, rather than activates, T4 deiodination in trout.

Sulfhydryl groups in the enzyme catalytic site are involved in T4 to T3 conversion in mammals (Visser, 1979). Two agents that reacted with enzyme-SH residues were tested. N-ethyl maleimide irreversibly carboxymethylates sulfhydryls and in the presence of 5 mM DTT inhibited the reaction by 56% at concentrations of 0.1 mM. The reversible binding agent PTU

also blocked deiodination with 10% activity remaining at 10 mM PTU. The low (0-10%) residual activity with high levels of PTU could represent a PTU-insensitive, type II enzyme. Kinetic analysis in the presence of 10 mM PTU or addition of larger amounts of inhibitor would distinguish between the two enzyme activities.

Assuming the reductive deiodination of T4 proceeds with the production of iodide, the reaction should be end-product inhibited by excess iodide. Varying quantites of iodide (as KI) were added to sample incubates. As expected, inhibition did occur but only at high (100 mM) concentrations of exogenous iodide. In a second experiment, KCl added in identical amounts resulted in virtually no inhibition; the effect was iodide specific.

Results obtained from the addition of the chelating agents EDTA and EGTA, although not conclusive, point to a role for divalent metal ions in 5'-deiodinase regulation. High levels of Zn^{2+} were measured in young rats and exogenous Cu^{2+} and Zn^{2+} ions inhibited peripheral conversion in young animals (Sato et al., 1983). The potentiating effect of EDTA was noted in adult animals (Visser et al., 1978); 10 mM EDTA almost doubled T3 production in kidney homogenates (Chiraseveenuprapund et al., 1978). In this study, EDTA was more effective than EGTA in elevating T4 deiodination. Perhaps because EDTA is a nonspecific chelator it has the potential to complex a wider variety of inhibitory divalent ions than the Ca^{2+}-specific EGTA. In other words, other

ions as well as Ca^{2+} may have an inhibitory influence on deiodination.

In summary, the use of several modifying agents has provided valuable information about the characteristics of the T4 5'-deiodinase in the hepatic microsome fraction of rainbow trout. The enzyme is different from iodoperoxidase in the thyroid. There is preliminary evidence that the binding site for T4 on the plasma proteins resembles the active site of the deiodinase enzyme. Diamide is a moderate activator of deiodination suggesting GSH is not the endogenous thiol in this system. DTT activates T4 deiodination possibly by acting as a cofactor in the reaction. Addition of either PTU or N-ethyl maleimide to microsomal suspensions depresses deiodination. These molecules inhibit by binding to sulfhydryl groups (including SH residues at the catalytic site) making them unavailable to react with the T4 molecule. Finally, chelating agents enhance T3 production implying an inhibitory function for divalent ions in peripheral deiodination.

Starvation induces profound changes in the extrathyroidal metabolism of thyroid hormones. There is no question that T4 and T3 plasma levels decline in starved mammals and trout (Chopra et al., 1980; Flood and Eales, 1983). The difficulty lies in interpreting the relationship between depressed hormone levels and deiodination. T4 treatment over a period of 4 days fully restored in vitro deiodination in starved rats (Chopra et al., 1980. It

appears that under starvation conditions, the enzyme was deprived of substrate and unable to catalyze T4 to T3 conversion at a rate comparable to fed animals. Since the starved trout were not treated with T4, no conclusion could be made regarding the role of depressed plasma hormone levels in reduced hepatic deiodination. However, injection of T4 into starved fish followed by tissue processing and analysis would yield more information.

The 5'-deiodinase itself is altered in some way during food deprivation. There is general agreement in the mammalian literature that the Vmax and hence the level of active enzyme is reduced in starvation (Kaplan, 1979; Gavin et al., 1980; Heinen et al., 1981). Declining Vmax and Km values in starved trout suggest that enzyme synthesis is depressed and the affinity of the pre-existing enzyme is also altered. The Vmax to Km ratio is 2.1 times higher, that is the enzyme is more efficient, in fed fish than in starved fish. The Vmax value is changing more than the Km in other words, the depressed quantity of active enzyme is more relevant than the altered enzyme affinity.

Uptake of T4 from the plasma into the liver is an important intermediate step in the hepatic deiodination of circulating hormone. Previously considered to diffuse passively into the cell, T3 and T4 are now believed to be actively transported across the membrane involving a Na^+- K^+ ATPase (Krenning et al., 1978,1981). Any condition that influences the cellular level of ATP therefore could affect

the transport of iodothyronines into the hepatocyte. Krenning et al (1981) postulate that fasting may result in diminished intracellular ATP and subsequently reduce T4 uptake from the plasma.

Reduced availability of sulfhydryl groups as enzyme cofactors does not appear to account for depressed T3 production in starvation. Even with the addition of 5 mM DTT to incubates from starved and fed trout, significantly less activity was still noted in the livers of starved animals. These data are inconsistent with data of Harris et al. (1979) who observed complete restoration of activity when DTT was added to liver homogenates from starved rats.

In summary, diminished T4 deiodination in starved rainbow trout probably results from an interplay of several factors: declining plasma hormone levels, depressed T4 uptake into the hepatocyte, reduced enzyme synthesis, and alteration of the enzyme already present.

The mammalian and trout hepatic T4 5'-deiodinase are similar in many fundamental properties. Both are located in the ER membranes of the microsome fraction, are activated by thiols such as DTT and inhibited by the T4 analogs, ANS and salicylate. Enzyme sulfhydryls are important in both systems but the exact nature of the reaction mechanism (sequential or bisubstrate) in trout is unknown. Overall, the hepatic deiodination of T4 is simpler in fish than in mammals. Inner ring or 5-deiodination is absent in trout and T3 appears to be the end product of T4 deiodination, subsequent

metabolites such as 3,5-T2 being undetected by chromatography. The identity of the endogenous thiol in trout is unknown but it is probably not GSH, the putative thiol in mammals.

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APPENDIX

Appendix I.

Calculation of pmol T4 converted (h^{-1} · mg $protein^{-1}$); explanation of the equation and sample calculation.

pmoles T4 converted =

fraction of T4 deiodinated X total T4 added to the incubate

Fraction of T4 deiodinated

$$\frac{(\text{cpm enzyme}^{a} - \text{cpm control}) \times 10.4^{b} \times 2^{c}}{\text{cpm } *\text{T4}^{d}}$$

a) correction for $*I^-$ contamination in the tracer
b) correction for using 100 µl from a total volume of 1.04 ml in incubate
c) 2 iodine atoms in the outer ring available for labeling
d) total counts reference (TCR) of *T4 corrected for $*I^-$ contamination [TCR - (cpm control x 10.4)]

Total T4 added to the incubate

$$\left[\left(\frac{*\text{T4 cpm} \times 1.3^{f}}{(1.54 \times 10^6)^{e}}\right) + 1^{g}\right] \times 1.3^{h}$$

e) cpm in 1 µCi at 70% counting efficiency
f) ng T4 in 1 µCi calculated from s.a. of 750 µCi/µg
g) carrier T4 in ng
h) conversion factor for ng T4 to pmoles T4

Sample calculation

raw data: sample = 1818.4 cpm (mean of 2 values)
control = 976.66 cpm
TCR = 103430 cpm

pmoles T4 converted:

$$\frac{(1818.4 - 976.66) \times 10.4 \times 2}{93272.74^{*}} \times \left[\left(\frac{93272.74^{*} \times 1.3}{(1.54 \times 10^6)}\right) + 1\right] \times 1.3$$

* 103430 - (976.66 x 10.4)

Appendix I Continued.

pmoles T4 converted:

= 0.26 pmol T4

protein and time correction:

protein = 0.32 mg/ml
incubation time = 20 minutes

$$\frac{0.26 \times 3 \text{ (to convert to 1 h)}}{0.32}$$

= 2.43 pmol T4 converted . h^{-1} . mg $protein^{-1}$

note: see Appendix III for evaluation of carrier T4 present in the tracer.

Appendix II.

Calculation of pmol T3 converted (h^{-1} . mg $protein^{-1}$).

pmoles T3 converted =

fraction of T3 deiodinated X total T3 added to the incubate

Fraction of T3 deiodinated

$$\frac{(\text{cpm enzyme} - \text{cpm control}) \times 10.4}{\text{cpm } {}^{*}\text{T3}_{a'}}$$

a') total counts reference (TCR) of *T3 corrected for $*I^-$ contamination [TCR - (cpm control x 10.4)]

Total T3 added to the incubate

$$\frac{{}^{*}\text{T3 cpm} \times 1.8^{f'}}{(1.54 \times 10^6)} + 0.8^{g'} \times 1.5^{h'}$$

f') ng T3 in 1 µCi calculated from s.a. of 550 µCi/µg
g') carrier T3 in ng
h') conversion factor for ng T3 to pmoles T3

Appendix III.

Calculation of pmol T4 converted with varying amounts of carrier T4 (Bolger and Jorgenson, 1980).

Maximum specific activity of *T4 in the tracer

1 if all of the T4 in the tracer is labeled
$= 2.18 \times 10^{12}$ µCi
2 molecular weight of *T4 = 772 g/mole

$$\frac{2.18 \times 10^{12} \text{ µCi}}{7.72 \times 10^{8} \text{ µg}} = 2824 \text{ µCi/µg}$$

3 two iodine atoms in the outer ring of T4 can be labeled

$2824 \times 2 = 5648$ µCi/µg = maximum specific activity

s a. of the tracer used in this thesis = 750 µCi/µg

% labeled T4 in the tracer = $\frac{750}{5648} = 13.3\%$

% unlabeled T4 in the tracer = 100% - 13.3% = 86.7%

A = amount of labeled T4 in the tracer in ng

$$A = 0.133 \times \overset{a}{\text{decay factor}} \times \overset{b}{} \frac{\overset{c}{\text{cpm *T4}}}{\underset{d}{1.54 \times 10^{6}}} \times \overset{e}{\frac{1}{750}} \times \overset{f}{1000}$$

a) % labeled T4 in the tracer
b) correction factor for *T4 decay (from decay chart)
c) total counts reference (TCR) of *T4 corrected for $*I^-$ contamination [TCR - (cpm control x 10.4)]
d) cpm in 1 µCi at 70% counting efficiency
e) µg T4 in 1 µCi calculated from s.a. of 750 µCi/µg
f) conversion factor for µg T4 to ng T4

B = amount of unlabeled T4 in the tracer in ng

$$B = \overset{a'}{0.867} \times \frac{\text{cpm *T4}}{1.54 \times 10^{6}} \times \frac{1}{750} \times 1000$$

a') % unlabeled T4 in the tracer

Appendix III Continued.

Total T4 in the tracer (labeled and unlabeled) = A + B

pmoles T4 converted $\cdot h^{-1} \cdot$ mg protein^{-1} =

fraction of T4 deiodinated X total T4 added to the incubate

Fraction of T4 deiodinated

$$\frac{(\text{cpm enzyme} - \text{cpm control}) \times 10.4 \times 2}{\text{cpm } {}^{*}T4}$$

Total T4 added to the incubate

$$[A + B + \text{carrier T4 in ng}] \times 1.3^{a}$$

a) conversion factor for ng T4 to pmoles T4

Sample calculation

raw data: sample = 852.30 cpm (mean of 2 values)
control = 599.50 cpm
TCR = 101351.27 cpm
decay = 0.9549

$$A = 0.133 \times 0.9549 \times \frac{95116.47^{*}}{1.54 \times 10^{6}} \times \frac{1}{750} \times 1000$$

* 101351.27 - (599.5 x 10.4)

$= 1.1 \times 10^{-2}$ ng

$$B = 0.867 \times \frac{95116.47^{*}}{1.54 \times 10^{6}} \times \frac{1}{750} \times 1000$$

* 101351.27 - (599.5 x 10.4)

$= 7.1 \times 10^{-2}$ ng

$A + B = 8.2 \times 10^{-2}$ ng
carrier T4 = 2.582 ng
total T4 = 2.58 ng

pmoles T4 converted:

$$\frac{(852.30 - 599.50) \times 10.4 \times 2}{95116.47} \times 2.582 \times 1.3$$

= 0.18 pmoles T4

Appendix IV.

Sample calculation for glucose-6-phosphatase assay (Baginski *et al.*, 1974).

Phosphate (P) liberated from glucose-6-phosphate at $O.D._{700}$

$$= \frac{E_{sample} - E_{control}}{E_{standard}} \times 0.15 \text{ [µmole/assay mixture]}$$

E	
0.38	standard (1.5 mM phosphate)
0.68	sample (subcellular fraction)
0.03	control (fraction added after the reaction is stopped with ascorbic acid/TCA mixture)

$$P = \frac{0.68 - 0.03}{0.38} \times 0.15$$

$$= 0.26 \text{ µmol}$$

protein and time correction:

protein = 1.5 mg/ml
incubation time = 32 minutes

glucose-6-phosphatase activity =

$$\frac{0.25}{0.15 \text{ mg protein in incubate} \times 32}$$

$$= 0.05 \text{ µmol P} \cdot \text{mg protein}^{-1} \cdot \text{minute}^{-1}$$

Appendix V.

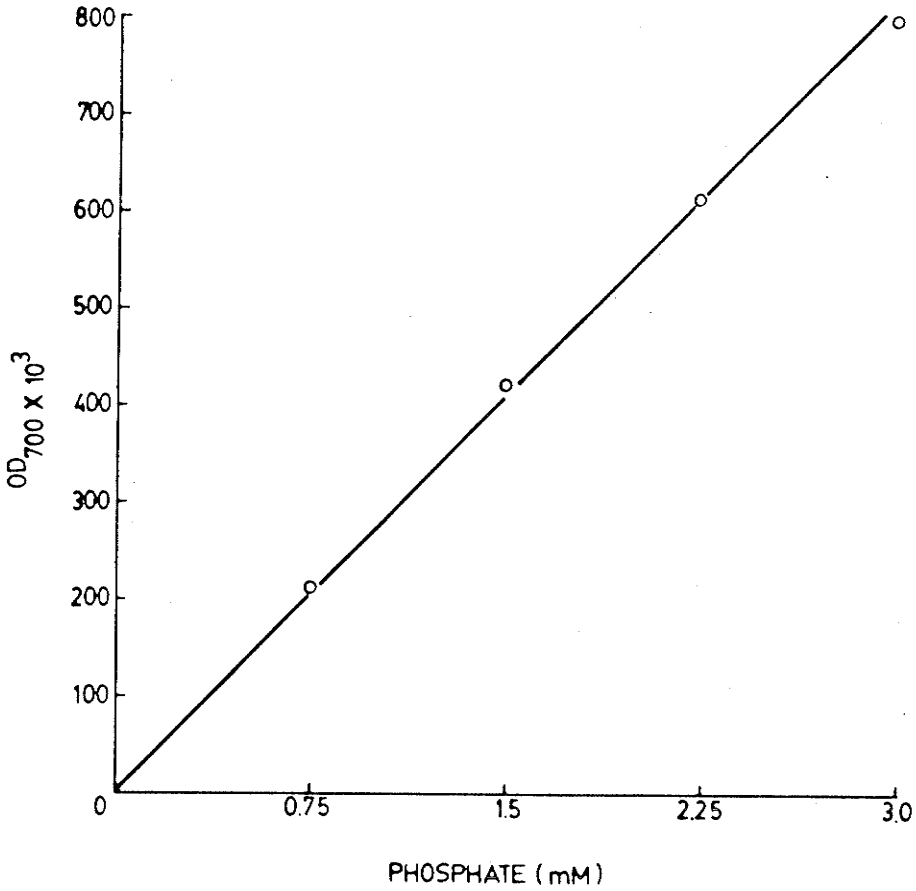
Relative amounts of $^{*}I^{-}$ and $^{*}T3$ produced from $^{*}T4$ in microsome incubates as measured by G-25 Sephadex chromatography (fine mesh; 27.8 x 1.5 cm). Both the $^{*}I^{-}$ and $^{*}T3$ cpm are corrected for $^{*}I^{-}$ and $^{*}T3$ contamination (10.1% and 2.3% respectively) of $^{*}T4$ incubated with no microsomal fraction.

		cpm			
Trial	Fraction	Total	Observed	Correction Factor	Corrected
1	$^{*}I^{-}$	9592	1684	967*	751
	$^{*}T3$		724	220+	504
2	$^{*}I^{-}$	12609	2161	1274	887
	$^{*}T3$		1031	290	741

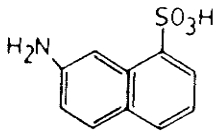
*0.101 x Total

+0.023 x Total

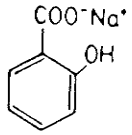
Appendix Figure 1. Standard curve of phosphate concentrations in glucose-6-phosphatase assay (1.5 mM concentration used in subsequent assays). Each point represents an average of 2 incubates.


800
700
600
500
400
300
200
100
0
$OD_{700} \times 10^3$
0.75
1.5
2.25
3.0
PHOSPHATE (mM)

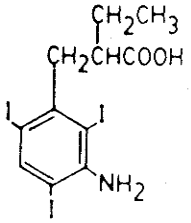
Appendix Figure 2. Molecular structures of ANS, sodium salicylate, PTU, iopanoic acid, GSH, and DTT (Cleland, 1964; Goodman Gilman *et al.*, 1980; Lehninger, 1982; Windholz, 1983).



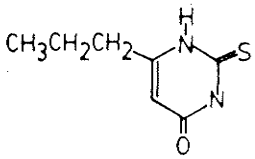
ANS



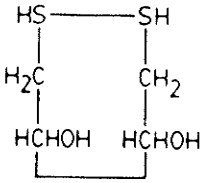
sodium salicylate



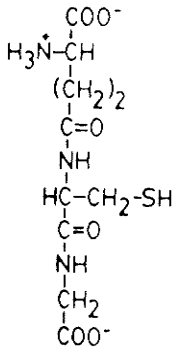
iopanoic acid



PTU



DTT



GSH