The function of the lake sturgeon (*Acipenser fulvescens*) thyroid system and dietary effects.

By

James Plohman

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

Department of Zoology

University of Manitoba

Winnipeg, Manitoba

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Abstract – The basic properties of the lake sturgeon (*Acipenser fulvescens*) thyroid system were studied to confirm and explain previously reported low plasma thyroid hormone (TH) levels. The blood plasma levels for both L-thyroxine (T4) (0.29 ng / ml) and 3,5,3'-triiodothyronine (T3) (0.19 ng / ml) were very low in accordance with previous data. Poor binding by plasma THs can explain the inability of the sturgeon plasma to hold greater quantities of hormone. The proportion of T4 in the red blood cells at equilibrium was greater and T3 and 3,5',5-triiodothyronine (rT3) lower than that reported for other fish species. This showed that the red blood cells (RBCs) do not contain unusually high proportions of the blood's thyroid hormones. The THs from a variety of sturgeon tissues were extracted and assayed to determine their concentrations. The brain and thyroid tissue contained the greatest concentrations of TH and all tissues contained much more T3 than T4. T3 levels were particularly high in the brain and thyroid. High brain T3 levels suggest the presence of a high-affinity TH binding protein like transthyretin with a higher affinity for T3 than T4. High thyroidal T3 levels suggest that T3 is the predominant form of the TH secreted by the thyroid. This theory was supported by significant activity of T4-outer ring deiodination producing T3 in the thyroid tissue. TH deiodinases similar to those in other vertebrates were most active in sturgeon liver and less so in the intestine. Enzymes deiodinating T4, T3, and rT3 were present in both these tissues. Once the basic principles of the sturgeon thyroid system were established, the ability of sturgeon to modify thyroid function in response to changes in dietary constituents was studied. Two diets, ocean plankton and Martin's trout pellets, were fed to two groups of sturgeon. The hepatic T3-inner-ring deiodination (T3-IRD) and rT3-outer-ring deiodination (rT3-ORD) activity were greater and plasma

T3 levels lower in the ocean plankton fed fish compared to the trout pellet-fed fish. The specific growth rate by length and by weight correlated positively with the plasma T3 levels and negatively with the hepatic and intestinal T3-IRD activity. This suggests a link between THs and growth in the sturgeon as in the salmonids. Finally the ability of the lake sturgeon to cope with a dietary induced T3-challenge was studied. Trout pellets containing 12 ppm T3 fed to 12 lake sturgeon, increased the plasma T3 concentration 2 X and decreased hepatic T4-outer-ring deiodination (T4-ORD) activity producing T3 without any significant effects on the other hepatic deiodinases or on the activity of lower intestine and brain deiodinases. In conclusion, the lake sturgeon thyroid system has several characteristics that differ from the teleost fish system. In contrast to teleosts, the sturgeon has much greater concentrations of T3 than T4 in the blood and tissues (especially the thyroid) and significant T3-inner-ring deiodination in the liver and intestine. This suggests that the lake sturgeon's thyroid system functions mainly by releasing TH as T3 from the thyroid gland and then inactivating the portion not required within the tissues by T3-IRD activity. This system differs from that in rainbow trout and it is concluded that the lake sturgeon's thyroid system functions most similarly to organisms that live in low iodide environments like the mammals.

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List of Abbreviations

ANOVA	Analysis of variance
ATG	Aurothioglucose
BSA	Bovine serum albumin
Ci	Curie
cDNA	Complimentary deoxyribonucleic acid
ddH_2O	double-distilled water
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetate
FT3	Free (unbound) T3 in plasma
FT4	Free (unbound) T4 in plasma
GH	Growth hormone
HPLC	High-pressure liquid chromatography
Hr	Hour
I^-	Free iodide
IAc	Iodoacetate
Kda	Kilodalton
K_m	Enzyme affinity (Michaelis-Menten Constant)
MMI	Methylmercaptoimidazole
MS222	Methane tricaine sulfonate
MTP	Martin's Trout Pellets
NSB	Non-specific binding
OP	Ocean plankton

Pmole	Picomole
Ppm	Parts per million
PTU	6-n-propyl-2-thiouracil
RBC	Red blood cell
RIA	Radioimmunoassay
Rpm	revolutions per minute
rT3	3,3',5'-triiodothyronine (reverse T3)
*rT3	^{125}I-radiolabelled rT3
rT3-ORD	rT3-outer-ring deiodination
SA	Specific activity
SEM	Standard error of the mean
SGR	Specific growth rate
3,3'-T2	3,3'-triiodothyronine
T3	3,5,3'-triiodothyronine
*T3	^{125}I-radiolabelled T3
T3-IRD	T3-inner-ring deiodination
T4	L-thyroxine, tetraiodothyronine
*T4	^{125}I-radiolabelled T4
T4-IRD	T4-inner-ring deiodination
T4-ORD	T4-outer-ring deiodination
TCR	Total count reference
TETRAC	Tetraiodothyroacetic acid
TFA	Trifluoroacetic acid

TGA	Thyroglobulin
TH	Thyroid hormone
*TH	^{125}I-radiolabelled thyroid hormone
TRIAC	Triiodothyroacetic acid
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
Tukey HSD	Tukey honestly significant difference
V_{max}	Maximum velocity of enzyme activity

Chapter 1

Introduction

Teleost Fish Thyroid

In teleost fish (bonyfish), the thyroid hormones (THs) are permissively acting hormones which stimulate growth, reproduction, and development. The thyroid system of fish has been reviewed by Eales and Brown (1993). The form of TH released from the teleost thyroid gland is thyroxine (T4) (Fig. 1-1) and the blood carries it to peripheral tissues (Fig. 1-2). These tissues absorb T4 for use in regulating protein transcription. However, T4 is not a very potent form of TH. 3,5,3'-triiodo-thyronine (T3), which contains one less iodine atom, has an approximately 10 X greater affinity than T4 for TH receptors. T3 is produced through the action of one or more types of deiodinase enzymes found within certain cells. Deiodinase enzymes interconvert the THs into their various forms by removing iodine atoms from either the outer or inner ring of their structure (Fig. 1-1). Removal of one iodide atom from the outer ring of T4 results in the formation of T3, the active form. T3 can either be utilized within the cell in which it was produced, or transferred back into the blood stream to be carried to other tissues that may absorb and use it. T4 can alternately be deiodinated to form 3,3',5'-triiodothyronine (rT3), a completely inactive form of TH, by removal of an inner-ring iodine. T3 and rT3 can in turn be further broken down by deiodinase enzymes (Fig. 1-1) to either inactivate T3 or to salvage the iodide for recycling back to the thyroid gland.

Within the tissue, T3 can bind to TH receptors to exert its effects, be deiodinated further, or be conjugated by the addition of a glucuronic acid or sulfate group to its 4'-OH

Figure 1-1: **Diagram showing the molecular structure of L-thyroxine (T4) and the deiodinase enzyme pathways present in teleost fish tissues. ORD = outer-ring deiodination, IRD = inner-ring deiodination, T3 = 3,3,5'-triiodothyronine, rT3 = 3,3',3'-triiodothyronine, 3,3-T2 = 3,3'-diiodothyronine.**

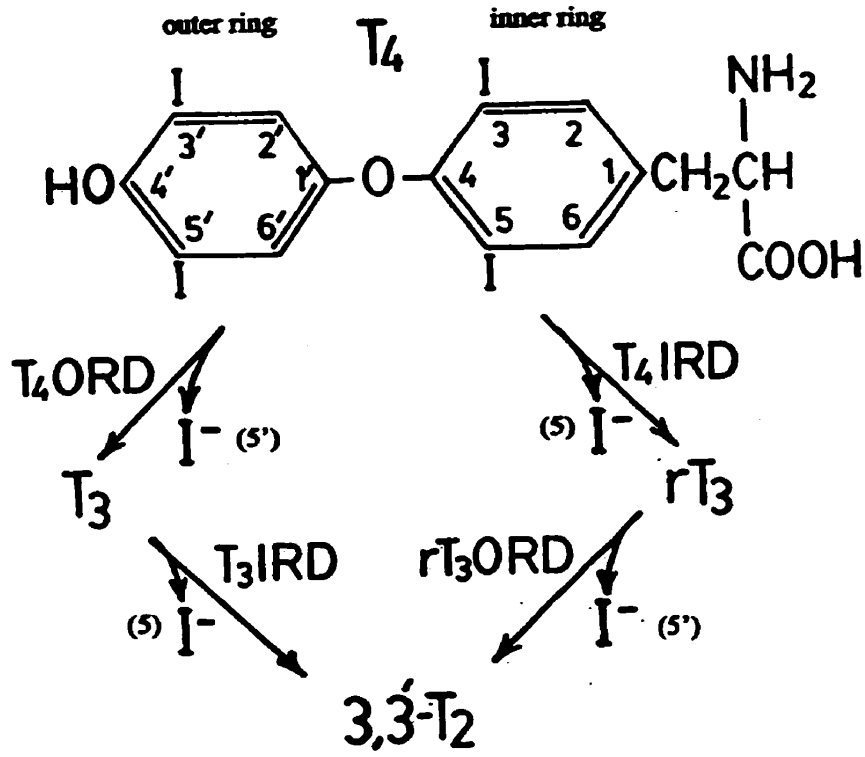
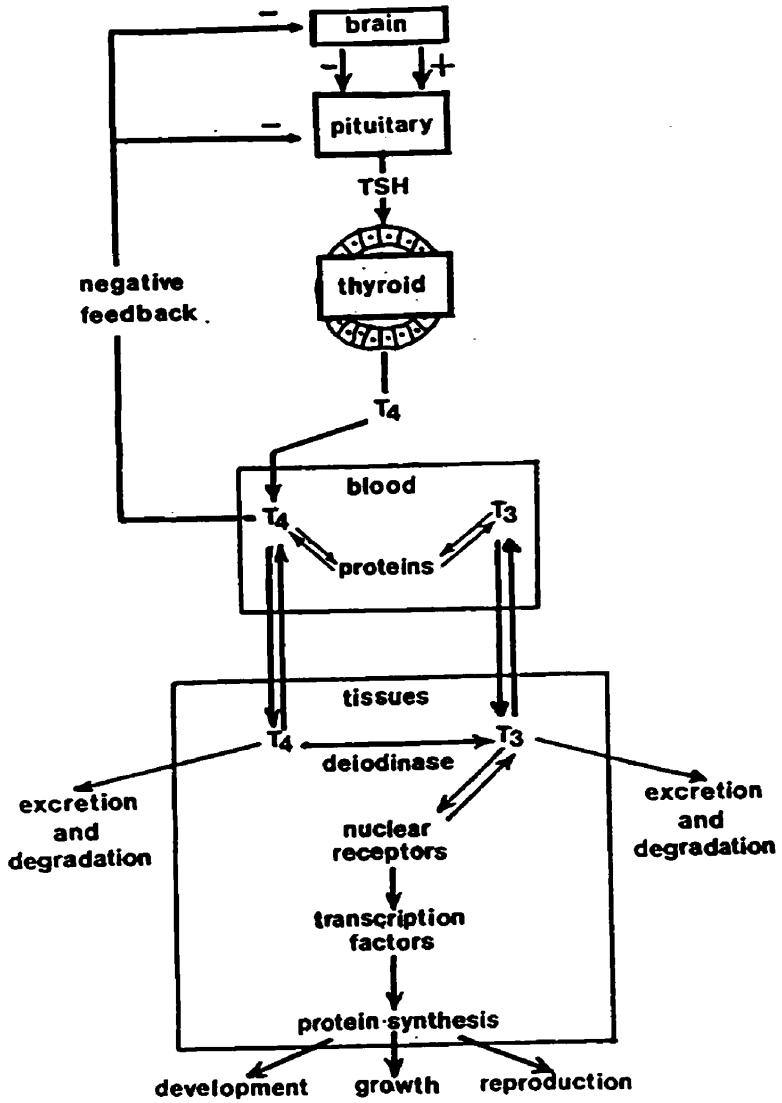

outer ring
T4
inner ring
I
I
NH2
3'
2'
3
2
HO
4'
1'
O
4
1
CH2CH
5'
6'
5
6
I
I
COOH
T4ORD
I- (5')
T4IRD
(5) I-
T3
rT3
T3IRD
rT3ORD
(5) I-
I- (5')
3,3'-T2

Figure 1-2: Diagram illustrating the pathway of thyroid hormone action from production in the thyroid gland to action at cellular receptors in teleosts.


brain
pituitary
TSH
thyroid
negative
feedback
T4
blood
T4
T3
proteins
tissues
T4
T3
deiodinase
excretion
and
degradation
nuclear
receptors
excretion
and
degradation
transcription
factors
protein synthesis
development
growth
reproduction

group. These reactions inactive the THs and make them more water soluble for easier excretion from the system in bile and urine (Visser, 1990). Both sulfation (Finnson and Eales,

1998) and glucuronidation (Finnson and Eales, 1997) of THs occur in the rainbow trout (*Oncorhynchus mykiss*).

Within the blood, THs may be reversibly bound to plasma proteins, or remain free within the plasma. Plasma proteins in the blood bind the majority of both T4 and T3 preventing their loss from the blood. However, the red blood cells (RBCs) also contain a store of THs, which they exchange with the plasma. The RBCs of rainbow trout contain approximately 20% of the total blood T3 (McLeese et al, 1998). The quantities and affinities of proteins in the plasma and in the red blood cells are important factors that influence the total amount of TH found within the blood.

The TH within the blood and tissues together forms the total body TH pool. The distribution and quantity of the various active and inactive forms of TH within this pool are important factors that determine the level of activity that the hormone manifests in the body.

These dynamics are sensitive to environmental influences. The composition of the diet is one factor that affects the status of the thyroid system and growth of fish. Brook trout starved for 12 days significantly decreased the deiodination of T4 to T3 (Higgs and Eales, 1977).

Thyroid hormones taken into the body in the diet can also effect the function of the thyroid system as the fish modifies (autoregulates) its system to counteract the rise in

body TH levels. Rainbow trout, fed T3-supplemented food decrease their deiodination of T4 to T3 in response to the increased levels of T3 in their system (Eales et al, 1990).

Sturgeon Thyroid System

The lake sturgeon (*Acipenser fulvescens*) is a member of the subclass Actinopterygii, infraclass Chondrostei, order Acipenseriformes and family Acipenseridae (Moyle and Cech, 1996). The sturgeon evolutionary line has been separated from that of the teleosts, also Actinopterygians (ray-finned fish), for over 200 million years. The lake sturgeon is found in the lakes and rivers of north central North America. It is the only primarily freshwater *Acipenser* species found in North America. At the turn of the century, the arrival of immigrants to North America rapidly depleted the lake sturgeon numbers as it was seen as a nuisance fish. It was not until much later that the value of the sturgeon in caviar and smoked flesh was realized. Attempts to recover the numbers of this species have recently been attempted.

The thyroid physiology of the Acipenseridae has been little studied. Plasma TH levels in white sturgeon (*Acipenser transmontanus*) measured over an annual period have been reported (T4 = 0 - 3.6 ng / ml, T3 = 0 – 6.9 ng / ml), (McEnroe and Cech, 1994). The only other published data are from a few Russian publications relating plasma TH levels to onset of female maturation in Russian sturgeon species (Detlaf and Davydova, 1974). No publications concerning the thyroid physiology of lake sturgeon are available.

The lake sturgeon is slow growing and very slow to mature compared to most fish. Unpublished data (S. B. Brown) indicate that the levels of T3 and T4 in the plasma of laboratory-raised lake sturgeon were below the detection limit of the assay (< 0.15 ng / ml). This is unexpected, as T3 is the presumed active form of the TH. Low T3 levels, if

consistently true, could help to explain the slow growth of this species. Low blood T3 levels may be related to the activities of the deiodinase enzymes present in the peripheral tissues, and which are responsible for T3 formation and degradation. Poor binding by plasma TH-binding proteins may also explain the low plasma TH levels, a low rate of secretion of TH from the thyroid gland, or rapid plasma clearance of the hormone may also explain the low plasma TH levels. Understanding the thyroid physiology of the lake sturgeon is important since, as in teleosts, it may be involved in the growth and maturation of the species. Therefore, determining if the status of the thyroid system is correlated with growth may help to establish a link between THs and growth in this species.

Objectives

The first objective of this study was to confirm the low plasma T3 and T4 levels in laboratory-raised and wild lake sturgeon plasma samples and determine whether the low TH levels are due to the conditions of captivity (Chapter 3).

The second objective was to examine the blood properties to determine if they could account for the low plasma TH levels (Chapter 3). Plasma proteins, which bind a majority of the TH found in the plasma, were examined. This provided an indication of the ability of proteins found within the blood plasma to bind and hold T4 and T3 within the blood. However, even if the blood plasma does not contain significant amounts of T4 and T3, there is still the possibility that the red blood cells may contain significant amounts of hormone. Therefore, the red blood cells were also analyzed to determine the percentage of whole blood TH contained in these cells at equilibrium. Analysis of the

plasma proteins and red blood cell hormone affinities together will determine the capacity of sturgeon blood for TH.

However, low TH levels in the blood do not necessarily indicate that there are not significant quantities of TH in sturgeon tissues. Therefore the third objective was to analyze a variety of lake sturgeon tissues (liver, intestine, kidney, brain, thyroid, muscle, gill, and whole body samples) to determine if significant quantities of TH exist in any of them (Chapter 3).

With the TH distribution in the blood and tissues determined, the fourth objective was to assay a variety of sturgeon tissues for the presence of TH deiodination activities (Chapter 4). Deiodination activity is central to the thyroid system and the activity levels of these enzymes could also explain the low plasma T3 levels. Once the most prominent deiodinating tissues were identified, the characteristics (pH, dithiothreitol (DTT) cofactor requirement, effects of inhibitors, substrate specificity, substrate affinity [K_m] and reaction rate [V_{max}]) where studied to optimize the assays and to compare to other species.

Finally, the responses of the lake sturgeon thyroid system to dietary components were determined. This involved studying two aspects: a) changes that occur in the thyroid system to compensate for changes in diet quality that result in modified growth rates (Chapter 5), and b) response of the sturgeon to a dietary induced T3-challenge (Chapter 6). This will provide an insight into the mechanism employed to cope with TH brought into its body from outside sources (diet).

Chapter 2

Literature Review

Thyroid Cascade

The thyroid system of teleost fish has been reviewed by Eales and Brown (1993). In teleosts, TH is released into the blood stream from the thyroid gland mostly as T4 (Figure 1-2). Release of T4 is controlled by TSH (thyroid stimulating hormone) release from the pituitary, which is inhibited by innervation from the hypothalamus in the brain. Within the blood T4 is carried reversibly bound to proteins in the plasma, and within the red blood cells and a small percentage (≅1%) exists in the free form. Plasma free T4 feeds back negatively on the brain and pituitary decreasing the positive stimulation of these centres on T4 release from the thyroid. The unbound fraction can be taken up by various target tissues both actively and by passive diffusion. These peripheral tissues then convert T4 into various other forms through deiodination, sulfation, or glucuronidation. T3 formed by outer-ring deiodination of T4 is the active TH. It has a much greater affinity for TH receptors than T4. T4 binds to the TH receptors found in the cell nucleus, mitochondria or cytoplasm and activates the receptors. This allows it to function as a transcription factor regulating the transcription of genes that code for proteins relevant in development, growth and reproduction in fish.

Iodide Requirements and Use by Fish

Iodide is necessary for the production of thyroid hormones. Iodide metabolism in fish has been reviewed by Leloup (1970). It is obtained by the fish mostly from the water by uptake across the gills, but some is also obtained from their diet. Branchial uptake

involves iodoperoxidase present in the external gill surface which oxidizes I^- into I_2. Inside the

branchial cells the iodine is reduced to iodide before diffusing down its electrochemical gradient into the blood. Within the blood of clupeiformes, iodide is carried predominantly bound to an albumin-like plasma protein called iodurophorine. Iodide carried to the thyroid tissue in the blood stream is transported into the follicles where it is incorporated into iodotyrosines (iodotyrosyls) which will be used to produce thyroid hormone.

Thyroid Hormone Production

The probable mechanism of thyroid hormone synthesis in teleost fish has been reviewed by Eales et al (1999). Thyroid tissue consists of follicles of cells surrounding a colloid filled lumen. The colloid consists mainly of thyroglobulin protein from which thyroxine is formed. Iodoperoxidase in the apical membrane of the follicle cells catalyzes the formation of thyroxine by oxidizing I^- which it uses to replace hydrogen at the 3, and 5 positions of tyrosyls present in the thyroglobulin structure. The end product is iodotyrosyl. Pairs of iodotyrosyls are then coupled producing a tetraiodothyronine which is still covalently incorporated in thyroglobulin. Colloid is brought into the follicle cells by endocytosis and combines with lyzozymes which contain cathepsins that digest the thyroglobulin releasing the tetraiodothyronine (T4). The TH is then available for release into the blood stream as predominantly T4 in teleosts (Sefkow et al, 1996).

Control of Thyroid Hormone Release from The Thyroid Gland

In teleosts, TH is secreted primarily as T4 from the thyroid gland into the circulatory system (Eales and Brown, 1993). The quantity of T4 released from the

thyroid gland may be controlled by other hormones in the system (Eales and Brown, 1993). TRH (thyrotropin releasing hormone) and TSH are involved in plasma TH homeostasis in mammals. TRH affects TH release in fish but there is not any evidence TRH is endogenously produced. Release of T4 from the thyroid gland is induced by injection of TRH in rainbow trout and Arctic charr (*Salvelinus alpinus*) (Eales and Himick, 1988). This action appears to be directly on the thyroid as TRH does not affect TSH release from the pituitary of coho salmon (*Oncorhynchus kistuch*) (Larsen et al, 1998). TSH (thyroid stimulating hormone) also stimulates the release of T4 from the thyroid of coho salmon (Specker and Richman III, 1984), prometamorphic flounder larvae (Inui et al, 1989), tilapia (Melamed et al, 1995), brook trout (*Salvelinus fontinalis*) (Chan and Eales, 1976; Leatherland and Flett, 1988), and Indian major carp (*Cirrhinus mrigala*) (Bandyopadhyay and Bhattacharya, 1993). T4 in the blood plasma in turn negatively feeds back on the pituitary reducing TSH release (Eales and Brown, 1993). This allows a set concentration of T4 to be maintained in the blood.

Thyroid Hormone in Blood

Plasma T4 and T3 levels range from less than 1 to not more than 100 ng / ml in all organisms studied (Table 2-1). Plasma rT3 levels have been little studied but are below the detection limit of the assay (< 40 pg / ml) in rainbow trout (Eales et al, 1983).

Within the blood, THs are transported primarily bound to plasma TH-binding proteins. Only a small fraction (<1%) remains free in the plasma at equilibrium. There are at least three types of proteins in fish blood plasma that bind TH (albumin and prealbumin (transthyretin), and B-globulin). These proteins are not all present in all vertebrates and their relative abundance varies between species (Tanabe et al, 1969). In

Table 2-1: Blood plasma (or serum) thyroid hormone (TH) concentrations in various vertebrates

Species	Plasma TH Concentrations T4 (ng/ml)	T3 (ng/ml)	Reference
Fish			
Oncorhynchus kisutch	4 - 14	4 - 8	Redding et al, 1984
	4.2	2.1	Specker et al, 1992
	9.16 - 9.82	5.60 - 6.02	Plisetskaya & Sullivan, 1989
Oncorhynchus mykiss	1.1 - 4.0	–	Eales et al, 1982
starved	1.15	1.42	Eales et al, 1981
Salmo salar (parr)	10 - 20	–	Lindahl et al, 1983
Tilapia nilotica	2.59	–	Byamungu et al, 1990
Reptiles			
Lizard			
(Trachydosaurus rugosus,	1.15 - 2.04	0.09 - 0.18	Hulbert & Williams, 1988
Tortoise			
(Chelodina longicollis)	0.43 - 0.54	0.18 - 0.20	Hulbert & Williams, 1988
Turtle			
Pseudemys scripta	69.6 - 137	< 1	Licht et al, 1990
Crocodile			
(Crocodylus johnstoni)	2.52	0.33	Hulbert & Williams, 1988
Mammals			
Human	60	1.1	Stock et al, 1978
Rat	40 - 51	–	Keiffer et al, 1976
	–	0.58	Nejad et al, 1975
Young pigs	37.0 - 46.0	0.38 - 0.62	Slebodzinski et al, 1982
Birds			
Immature broiler hens			
(Gallus domesticus)	14.0 - 28.2	2.17 - 4.30	Sharp et al, 1984

brook trout, T4 and T3 bind prealbumin (transthyretin), albumin, and B-globulin-like proteins (Falkner and Eales, 1973).

Binding of THs by plasma proteins is reversible and bound TH rapidly exchanges with the unbound TH in the plasma. Two high-affinity low-capacity binding sites, one exclusively binding T3 and the other binding predominantly T4 but also T3, as well as two low-affinity high-capacity sites, one binding just T4 and one predominantly binding T3 have been demonstrated in the rainbow trout (Eales, 1987). The high affinity T4 site has been identified as a 55 kDa protein and the T4 low-affinity site is a 150 kDa protein (Cyr and Eales, 1992). A 1500 kDa lipoprotein binding T4 has also been identified in the rainbow trout. This protein, as well as the low-affinity protein binding T4, bind a higher proportion of the T4 relative to the high-affinity protein, when the fish are treated with estradiol (Cyr and Eales, 1992). Two weeks starvation, bleeding of the fish 24 hr earlier, or hemoglobin contamination of the plasma samples had no effect on the proportion of TH bound by the proteins (Eales, 1987).

Binding of TH to prealbumin (transthyretin) occurs in the brook trout (Falkner and Eales, 1973). Recently transthyretin has been identified and sequenced in the liver of the sea bream (*Sparus aurata*); (Santos and Power, 1999) and the blood serum of masu salmon (*Oncorhynchus masou masou*) (Yamauchi et al, 1999). The salmon transthyretin has a much higher affinity for T3 than T4 (Yamauchi et al, 1999) in contrast to mammalian transthyretin which has higher T4 than T3 affinity (Dickson et al, 1985; Schreiber et al, 1990).

Red blood cells (RBCs) carry a portion of the TH in the blood. In rainbow trout, 5-11% of T4, 14-23 % of T3, and 23-24 % of rT3 whole blood TH is contained within the

RBCs (McLeese et al, 1998). The hormone is reversibly bound to proteins within the red blood cells. The degree of binding is influenced by the plasma proteins outside the cells as well as the degree of oxygenation and pH inside the cells (McLeese et al, 1998).

There are several proteins that bind and hold TH within RBCs. Their identity has not been determined in fish. However, they are well studied in mammals and may be similar to proteins present in fish RBCs. Hemoglobin binds both T4 and T3 in human red blood cells (Davis and Osawa, 1983). The bond is formed between the hormone and heme and is believed to be covalent as it is not freely dissociable and occurs progressively with time as the protein ages (Davis and Osawa, 1983). Hemoglobin does not likely bind TH in fish as T4 and T3 are dissociable from trout RBC proteins (McLeese et al, 1998). There are also several other cytosolic T3 binding proteins present in the human red blood cells. They are cystolic thyroid hormone binding proteins I, II, III, and IV (Fanjul and Farias, 1991) which differ in affinities for the hormone, size, and optimum binding conditions. RBC membranes in rats (Botta et al, 1983) and humans (Holm and Jacquemin, 1979) also bind TH with a particularly high affinity for T3.

Uptake of Thyroid Hormone by Peripheral Tissues

The TH is transported in the blood compartments to extrathyroidal tissues which actively absorb the hormone by an energy-dependant, carrier-mediated endocytotic process that has been studied in rainbow trout hepatocytes (Riley and Eales, 1993a, 1994). In the rainbow trout, (Fok et al, 1990) and rat (van Doorn et al, 1985; Obregon et al, 1978), the liver, kidney, and intestine contain the greatest concentrations of hormone outside of the thyroid. However, only the liver, kidney, heart, stomach, intestine, muscle and plasma were analyzed in the trout.

Once inside the tissue, THs may be metabolized by conjugation and deiodination, which convert them into the various active and inactive forms.

Conjugation and Excretion of TH

Within trout tissues, TH may be deiodinated by removal of one or more iodines from its structure, or it may be conjugated by phenol sulfotransferases which add a sulfate group to its structure (Finnson and Eales, 1998) or by uridine-diphosphoglucuronosyl transferases which add a glucuronic acid (Finnson and Eales, 1997). Conjugation of THs increases their solubility in water and loss from the body in urine and bile (Visser, 1990). Sulfate conjugation enhances iodothyronine deiodination and glucuronidated iodothyronines undergo enterohepatic cycling.

Deiodinase Enzyme Characteristics

Mammals

There are three main deiodinase types found in the mammalian tissue that have been studied extensively and reviewed by Leonard and Visser (1986). The deiodinases are always found associated with the membranes in the cell and there is evidence suggesting their presence in the membrane of the endoplasmic reticulum as well as in the plasma membrane (Leonard and Visser, 1986). The mammalian deiodinases have been used as models for comparison to deiodinases in other vertebrate classes. The three deiodinases are classifed as types I, II, and III (Table 1). Type I deiodinase catalyzes both ORD and IRD, has a high K_m for T4 (2.3 μM), has the greatest affinity for rT3 and sulfate conjugates of TH, and requires a thiol cofactor in a ping-pong kinetics reaction mechanism with enzyme and hormone substrate. It increases in activity with a T3-challenge and is sensitive to selenocysteine deiodinase inhibitors due to the presence of a

selenocysteine at the active site of the molecule. Type II deiodinase is a solely ORD, has a low K_m for T4 (0.5–2.0 nM), prefers T4 as a substrate, requires a thiol as a cofactor in a sequential reaction mechanism, is decreased in response to a T3-challenge and is less sensitive than type I to selenocysteine inhibitors. This seems to suggest a lack of selenocysteine at the active site of the hormone. However, more recent studies have shown the presence of selenocysteine at the active site of type II deiodinase and knock-out studies replacing selenocysteine with other amino acids removes activity of the enzyme (Berry et al, 1991a). Type III deiodinase found in brain, catalyzes exclusively inner-ring deiodination with T3 as the preferred substrate, has a K_m of about 40 nM, and is relatively insensitive to inhibition by selenocysteine inhibitors despite the presence of selenocysteine in its structure (Leonard and Visser, 1986; St. Germain et al, 1994; Larsen and Berry, 1995; Croteau et al, 1995).

Fish

Deiodinase enzymes are also found in the tissues of fish and exhibit many similarities to those of the mammalian enzymes. Tilapia (*Oreochromis aureus*) tissues contain deiodinases similar to all three mammalian enzyme types (Mol et al, 1993; Mol et al, 1997). A high-K_m rT3-ORD in tilapia kidney is similar in certain respects to mammalian type I deiodinase except that it is not as sensitive to selenocysteine inhibitors as its mammalian counterpart. A type II similar deiodinase is in the liver and a type III similar enzyme is in the brain and gill of the fish.

T4-outer ring deiodination in rainbow trout liver does not completely resemble either type I or type II deiodinase isozymes (Sweeting and Eales, 1992a; MacLatchy and Eales, 1992; Frith and Eales, 1996). It is similar to type II deiodinase in exhibiting a low-

Km for T4 which is its preferred substrate and being inhibited by a T3 challenge. It is similar to type I deiodinase in showing some inhibition by selenocysteine inhibitors, and undergoing ping-pong kinetics with DTT cofactor as opposed to a sequential reaction mechanism in type II deiodinase. An hepatic rT3-ORD in rainbow trout resembles type 1 mammalian deiodinase in some respects (Finnson et al, 1999).

Inner-ring deiodinase of T4 and T3 is similar to type III mammalian deiodinase except the K_m is much lower (4.9 nM) than the value of 10-40 nM reported for the mammalian type III enzyme and the trout enzyme is more sensitive to inhibition by the inhibitor, ATG (Frith and Eales, 1996). Both inner ring deiodinase pathways also occur in the brain and liver of Atlantic salmon, (*Salmo salar*) (Morin et al, 1993; Eales et al, 1993) and in the coho salmon liver (Sweeting et al, 1994).

The sea lamprey (*Petromyzon marinus*), has a type-II-like deiodinase in its liver and intestine (Eales et al, 1997) that resembles the T4-ORD found in rainbow trout liver and brain (Frith and Eales, 1996). Inner-ring deiodination is present only in upstream-migrant lamprey and not larval stage lamprey and is strongly induced in the intestine during metamorphosis. This enzyme is similar to mammalian type III deiodinase except the K_m for the lamprey T3-IRD is lower (1.3 nM) (Eales et al, 1997).

Selenocysteine

The deiodinases in several vertebrates have been sequenced and the important functional groups have been determined. One important group at the active site of the enzymes is selenocysteine. Many studies have been done to determine its role in activity and whether it exists and is important in all deiodinase isozymes.

Selenocysteine is a rare amino acid found in the protein structure of some enzymes including glutathione peroxidase, formate dehydrogenase, and thyroid hormone deiodinases (Bermano et al, 1995). The amino acid is formed by the addition of a dietary selenolate anion to a cysteine within animal cells. Selenocysteine is encoded by a TGA codon in tilapia (*Oreochromis niloticus*) (Sanders et al, 1997), *Fundulus heteroclitus* (Valverde et al, 1997) and rats (Buettner et al, 1998); or UGA triplet in the rat (Berry et al, 1991b). A selenocysteine is encoded in the cDNA of mammalian types I, II, and III deiodinases (Berry et al, 1991c; Buettner et al, 1998; Davey et al, 1995; Croteau et al, 1995). It is located at the active site of the molecule and is essential for proper enzyme function. Substition of selenocysteine by cysteine reduces the activity 100 fold, while substitution by an unrelated amino acid like glycine removes enzyme activity almost completely (Berry et al, 1991b).

The selenolate anion in selenocysteine is the iodine acceptor in the active site of the enzyme. It reacts with TH removing an iodine. The selenolyl iodine form of the enzyme then reacts with thiol cofactor releasing iodide (Berry and Larsen, 1992). This mechanism of enzyme reaction with substrate followed by cofactor and back to substrate again is know as "ping-pong" kinetics. In nature this thiol cofactor could be glutathione, dihydrolipoamide, glutaredoxin, or thioredoxin (Berry and Larsen, 1992). A synthetic dithiol called dithiothreitol (DTT) is used to study *in vitro* deiodination in the lab.

Enzyme inhibitors that act by competing for binding to selenocysteine at the active site of the enzyme include propylthiouracil (PTU), iodoacetate (Iac), and aurothioglucose (ATG) (Berry and Larsen, 1992). PTU inhibits deiodinase activity by competing with thiol for the selenolyl iodine form of the enzyme producing an inactive

selenolyl-thiol complex. The gold in ATG acts by competitive inhibition binding to the selenolate anion in the free enzyme to form the inactive selenolyl-gold complex. These inhibitors do not work on all deiodinases tested. Mammalian type II deoidinase is not affect by ATG. Mammalian type I deiodinase is sensitive to PTU but a deiodinase in tilapia (Sanders et al, 1997) and rainbow trout (Finnson et al, 1999) resembling mammalian type I is not sensitive to PTU.

Due to the important role of selenocysteine in deiodinase function, a deficiency of selenium in the diet would be expected to affect the proper production and expression of deiodinase enzymes. Most studies concerning dietary deficiencies of selenium have been done on rats. In all cases, T3 production decreased in liver, kidney and brain, with plasma T4 levels increasing and T3 levels decreasing (Beech et al, 1995; Veronikis et al, 1996; Beckett et al, 1989; Bermano et al, 1995; Beckett et al, 1987; Chanoine et al, 1992). In addition, Beech et al (1995) reported that thyroidal type I deiodination activity in the thyroid increased by 42 %. The reason for the discrepancy in reaction between tissues was due to the greater efficiency of the thyroid tissue at retaining selenium during dietary deficiency.

Effects of thyroid hormone challenge

Teleosts

Most studies concerning the effects of added TH on thyroid system function in fish have been done on salmonids. TH is introduced into the body of the fish either by submersion in a TH-containing medium, by intraperitoneal injection, or most commonly by dietary supplementation.

T3 Challenge

A T3 challenge induced by dietary supplementation increases plasma T3 levels and decreases hepatic deiodinative production of T3 from T4 (T4-ORD) in all salmonids studied, including coho salmon (Darling et al, 1982), rainbow trout (Eales et al, 1990) (Sweeting and Eales, 1992b) (Eales and Finnson, 1991) and Atlantic salmon (Saunders et al, 1985). Deiodinative pathways diverting T4 to rT3 (T4-IRD) and degrading T3 (T3-IRD) also increase in rainbow trout liver but not brain (Fines et al, 1999). This counteracts the elevated plasma T3 levels caused by the T3 challenge and removes T4 from the tissue to prevent its conversion to T3. The effect on plasma T4 levels is inconsistent and varies with the age and species of fish. Sometimes plasma T4 levels increase (Sweeting and Eales, 1992b), decrease (Rivas et al, 1982) or do not change (Eales et al, 1990). However, in all cases, plasma T4 is not greatly effected. This suggests that T3 does not feed back on the thyroid to modify the set-point of hormone released from the thyroid gland.

Subjecting salmonids to a T3 challenge through immersion in T3-containing water produces the same effects as the T3 feeding studies. Coho salmon immersed in T3 containing water decreased dramatically their T4 to T3 conversion capability in liver (Darling et al, 1982).

T4 Challenge

T4 challenges studies have also been done through dietary supplementation. Dietary levels of up to 48 ppm had no significant effect on low-Km T4-ORD activity in the liver or gill (Sweeting and Eales, 1992b) of rainbow trout, but significantly decreased the activity of a high-Km T4-ORD in liver and kidney (MacLatchy and Eales, 1993).

Lack of effects of T4 may be a result of its poor absorption from the gut (Whitaker and Eales, 1993) as plasma T4 levels were elevated negligibly to moderately.

T4 injected intraperitoneally increases plasma T4 levels but does not lead to a change in T3 plasma levels in trout (Fok and Eales, 1984). The proportion of T4 deiodinated is decreased to prevent T3 plasma elevation as the metabolic clearance rate of T4 and T3 is unchanged.

A T4 challenge in Atlantic salmon induced by immersion in T4-containing water elevated plasma T4 to 15 ng / ml and decreased plasma T3 due to a reduction in hepatic and brain T4-ORD activity as well as an increase in T4-IRD and T3-IRD activity (Morin et al, 1995).

Mammals

In contrast to what occurs in fish, rats respond to a T4 injection by *increasing* hepatic type 1 deiodination of T4 and rT3 (Kaplan and Utiger, 1978; Smallridge et al, 1982). The increased activity is due to an increased Vmax (enzyme quantity). In contrast, type II deiodination in neuroblastoma cells (St. Germain, 1986) and rat glial cell cultures (Leonard et al, 1990) is markedly reduced when T4 or T3 is added to the cell cultures.

Dietary Effects on Thyroid Function

Teleosts

Salmonid teleosts have been most extensively studied to observe the effects of starvation, refeeding a single meal, diet quality and ration level on the thyroid system of fish.

Starvation followed by single-meal refeeding

Starvation suppresses the salmonid fish thyroid system. Brook trout starved for 12 days had decreased plasma T3 levels (Higgs and Eales, 1977, 1978). Refeeding increased plasma T3 levels back to normal. Starved rainbow trout decreased hepatic T4-ORD activity (Shields and Eales, 1986) and responded to refeeding with a temporary elevation in plasma T4 levels (Himick and Eales, 1990). In another study, refeeding increased plasma T3 levels (Flood and Eales, 1983).

Acute Effects

Carbohydrates and glucose are the most important dietary components acutely affecting thyroid status. Increased quantities of carbohydrate in the diet (Himick et al, 1991), as well as glucose injections (Himick and Eales, 1990) serve to increase by 4 hours the plasma T4 levels in previously starved rainbow trout.

Chronic Effects

Chronically, protein appears to be the most influential dietary component affecting thyroidal status. High protein diets increase hepatic T4-ORD activity in rainbow trout (Eales et al, 1992) with glycine being the amino acid with the greatest stimulatory affect (Riley et al, 1993b). Brook trout fed a low protein, low calorie diet have reduced conversion of T4 to T3 and increased plasma T4 levels (Higgs and Eales, 1979).

Carbohydrates and lipids have less chronic effects on thyroid status than protein. A reduction in dietary content of carbohydrates and lipids did not suppress the thyroid system in rainbow trout in one study (Eales et al, 1990) where the fish were held at 6.5 °C. However, increased dietary lipid levels did cause decreased plasma T3 and increased

plasma T4 levels when the rainbow trout were held at 5 °C but not 17 °C in another study (Leatherland et al, 1984). This indicates a relationship between temperature and lipid metabolism in the rainbow trout. Differences in the strain of fish, dietary rations, and other experimental conditions may have resulted in the effects seen in one study but not the other.

Chronically, carbohydrates do not have a marked consistent effect on thyroid status. Rainbow trout fed on diets of increasing carbohydrate content showed a trend towards increased plasma T3 levels (Leatherland et al, 1984) with no effect on plasma T4 levels. No chronic effects of carbohydrates were observed in another study on trout (Eales et al, 1990)

Ration Effects

Increased dietary rations are correlated with elevated T3 plasma levels in Arctic charr (Eales and Shostak, 1985a) and increased conversion of T4 to T3 in brook trout (Higgs and Eales, 1978). Rainbow trout respond to increasing ration level (0 to 3% body weight) with increased hepatic T4-ORD activity, plasma T3 levels and weight gain (Sweeting and Eales, 1993a). Elevations in plasma T4 and T3 also occurred in juvenile Atlantic salmon fed increasing rations (0 to 1.6 % body weight) (McCormick and Saunders, 1990).

Positive correlations between ration size, plasma T3 levels, and growth rate have been established in Arctic charr (Eales and Shostak, 1985a). However, in rainbow trout, ration and plasma TH levels are correlated without any correlation to growth rate (Leatherland et al, 1984). Increased T4 to T3 conversion is the likely cause of the elevated hormone levels in response to increasing ration (Higgs and Eales, 1977).

Mammals

In mammals, the thyroid system is also modified by food quantity and quality. Starvation in mammals leads to significant reductions in blood plasma levels of T3 and elevations in T4 concentrations due to decreased deiodination of T4 to T3 and a reduced metabolic clearance rate of T4 (Ingbar and Galton, 1975; Eisenstein et al, 1978; Slebodzinski et al, 1982). Van Der Heyden et al (1986) additionally observed decreased transport of T4 into the tissues reducing the availability of T4 for deiodination to T3. The overall result is a reduction in whole body content of T3 lowering the metabolic stimulating effects of the active thyroid hormone.

Similar effects on thyroid status to those observed during starvation occur when mammals are subjected to a carbohydrate deficient diet. Carbohydrates stimulate the production of deiodinase enzyme, which increases the production of T3 in the liver (Glick et al, 1985; Gavin et al, 1981). Sufficient quantities of protein are also required in the diet of rats as they increase the availability of enzyme cofactor in the tissues (Gavin et al, 1981). So in mammals, both carbohydrates and proteins are important in stimulating thyroid hormone deiodination activity.

Thyroid Hormone Action at the Receptor

T3 exerts its effects by binding to thyroid hormone alpha or beta receptors in the nucleus of cells. Binding activates the receptors as transcription factors which then act at the promoter of many different genes to increase transcription levels. Thyroid hormone receptors can either act as a dimer with other receptors in their family like retinoic acid receptors or as monomers.

Proteins whose transcription is enhanced by thyroid hormones have effects on development, growth and reproduction in fish. Developmental effects include induction of parr-smolt transportation in coho salmon, metamorphosis of flounder larvae (Inui and Miwa, 1985) dorsal fin-ray resorption during this metamorphosis (DeJesus et al, 1990), and enhanced survival of striped bass fingerlings (Brown et al, 1989). Effects on reproduction include amplification of gonadotropin stimulated release of estradiol from rainbow trout ovarian follicles (Cyr and Eales, 1988a), enhanced ovarian mitochondrial steroidogenesis in perch (Kaul and Bhattacharya, 1988), and enhanced gonadotropin ovarian maturation in rainbow trout (Cyr and Eales, 1988b).

The active thyroid hormone, T3 is correlated with growth rate in fish. T3 supplemented in the diet of underyearling coho salmon significantly improved food conversion to useable body substances and enhanced growth (Higgs et al, 1979). Correlations between specific growth rate and plasma T3 levels have also been reported for Arctic charr (Eales and Shostak, 1985a) and rainbow trout (Sweeting and Eales, 1993).

In contrast, T4 has no effects on growth of fish when administered in the diet. T4-supplementation in the diet of coho salmon did not influence either growth or food conversion (Higgs et al, 1979). The fact that T4 is not the metabolically active form of the hormone and that it is poorly absorbed from the gut may explain its lack of effects.

Link Between TH and Growth Hormone

The TH's involvement in growth in fish is supported by the ability of growth hormone (GH) to effect thyroid status. Human GH (MacLatchy and Eales, 1990) and Pacific salmon GH (MacLatchy et al, 1992) administered *in vivo* increase plasma T3

levels and deiodination of T4 to T3 in the rainbow trout liver. However, an increase in deiodination does not occur when isolated rainbow trout hepatocytes are exposed to human growth hormone *in vitro* (Sweeting and Eales, 1992c) suggesting that growth hormone does not act directly on hepatocytes to increase deiodination or that the hepatocytes lose their responsiveness *in vitro*. Starvation of rainbow trout decreases the effect of GH on TH deiodination (Leatherland and Farbridge, 1992).

CHAPTER 3

Thyroid Hormone Distribution in Lake Sturgeon Blood and Tissues

Introduction

In mammals, the TH is secreted mostly as T4 with significant amounts of T3. In the teleosts studied to date, T4 is secreted with very little T3 (Eales and Brown, 1993). Teleosts, therefore, must rely on extrathyroidal sites to produce the majority of the active form of the thyroid hormone (T3) used in the body. The form of the hormone produced and secreted by the lake sturgeon thyroid is unknown.

In teleost fish, the TH is released into the blood where it is found mostly within the plasma with a majority reversibly bound to plasma proteins. There is also a significant quantity of exchangeable TH present in the red blood cells (McLeese et al, 1998). Within these blood compartments, the TH is carried to peripheral tissues, which actively take up the free hormone. The amount of TH found within each tissue type depends on the quantity and affinity of TH binding proteins and the number of thyroid hormone transporters and their affinity for the hormone (Riley and Eales, 1994). Large quantities of hormone within a tissue may indicate the tissue requires a lot of hormone for proper function or it may be a storage tissue in which TH is sequestered until it is needed by other parts of the body (Eales and Brown, 1993).

In this chapter, the concentration of T4 and T3 carried within the blood plasma of lake sturgeon was investigated to confirm previous unpublished data (S. B. Brown) suggesting a very low blood plasma content of TH. The binding capacity of the plasma proteins was then investigated to determine if the low plasma TH levels were related to low binding by the proteins. RBCs were also analyzed to determine if TH is sequestered

in these cells within the blood possibly accounting for the majority of the bloods TH pool.

The form of TH released from the thyroid of lake sturgeon is not known, nor is its distribution in the peripheral tissues. Therefore, the thyroid region as well as many other tissues and whole body extracts, were analyzed to determine the distribution of the hormone within the various body tissues.

Materials and Methods

Fish Maintenance

Laboratory-raised lake sturgeon approximately 2 years in age weighing 307.9 ± 18.9 g and measuring 43.5 ± 0.8 cm total length were used for these experiments. They were held in fiberglass tanks in running dechlorinated Winnipeg City water at 12 °C in a 12 hour light / 12 hour dark photo cycle and fed twice daily at 10:00 and 16:00 a 1.0 % body weight ration of Martin's trout pellets each feeding. To obtain blood and tissue samples the fish were first anesthetized with tricaine methane sulfonate (MS222, 0.083 g/l) (Syndel Laboratories LTD.) and as much blood as could be acquired was withdrawn from the caudal artery with a heparinized hypodermic syringe. Treatment of the blood from this point on varied with the experiment. The fish were killed by concussion to the head and the tissues were removed for immediate use in extraction of TH.

Wild lake sturgeon blood serum samples were obtained from fish caught with gill nets from Nutimik Lake, South-East Manitoba, on June 7, 1997 by Dr. T. A. Dick, Department of Zoology, University of Manitoba. After being removed from the gill nets the sturgeon were weighed, measured, and blood samples were taken before releasing the sturgeon back into the lake. The wild sturgeon had an average age of 25 ± 2 years (mean

± SEM), weighed 10 ± 3 kg, and had a total length of 116 ± 4 cm. The blood was taken to the laboratory where it was placed into 1.5-ml Eppendorf tubes and spun at 17,000 g for 5 minutes in a centrifuge to separate the blood serum from the RBCs. The RBCs were discarded and the serum was placed into 5-ml cryovials and frozen at –78 °C until analysis of plasma or serum TH levels by radioimmunoassay (RIA) and the extent of TH binding to plasma proteins.

Radioimmunoassay

General Theory

The RIA uses the principle of competitive binding between TH containing a radiolabelled iodide (*TH) and non-radiolabelled TH competing for binding sites on antibody for either T4 or T3. Varying known quantities of a TH (T4 or T3) are placed onto mini-columns containing G-25 Sephadex gel. Then a constant small quantity of radiolabelled hormone is added to each column. The labelled and unlabelled hormone is allowed to bind to the gel before a constant quantity of antibody for the hormone is placed onto each column. The antibody competes with the gel to bind the TH. The greater the quantity of TH retained on the column, the lower the proportion of radiolabelled hormone that is bound by the antibody. After an incubation period, buffer is used to elute the antibody with its bound hormone off of the column while the sephadex-bound hormone remains on the column. The eluent is counted in a gamma counter, which determines the quantity of antibody-bound radiolabelled TH.

Detailed procedure

Blood plasma or serum T4 and T3 levels were determined by RIA in comparison to plasma standards containing known quantities of hormone. Plasma standards were

prepared as follows. The plasma is separated from the RBCs in whole blood by centrifuging fresh blood at 17,000 g for 5 minutes to pellet the RBCs. The standards were made by adding known concentrations of TH to TH-free sturgeon plasma, which was prepared by using charcoal to remove the endogenous hormone present in the plasma. This was done by mixing 1 gram of washed charcoal into 5 ml of sturgeon plasma. The mixture was allowed to stand over night and then centrifuged in plastic centrifuge tubes for 20 minutes at 20,000 rpm X 2. The supernatant was collected and filtered through Whatman number 1 filter paper to remove any remaining charcoal. Varying concentrations of T4 or T3 were then dissolved in aliquots of the hormone-stripped plasma to produce the plasma standards used in the assay.

The mini-columns used in the assay were prepared by combining 0.3 g of G-25 Sephadex gel to every 5 ml of double distilled water (ddH_20) in a large beaker. The mixture was allowed to stand for 3 hours and then stirred using a stir bar to suspend the gel throughout the water. While mixing, 5ml aliquots were withdrawn using a 5ml pipet and placed into clean mini-columns. The Sephadex was then allowed to pack before a plastic filter disc was placed on its top surface.

The radioimmunoassay was performed according to the procedures of Brown and Eales (1977). Mini-columns containing G-25 Sephadex were used in triplicate for each standard concentration (0, 0.15, 0.31, 0.62, 1.25, 2.5, 5.0, 10, 20 ng/ml) and duplicate columns were used for each sample analyzed. Two sets of columns were used, one for T4, and one for T3 analysis. The columns were stored containing 0.1 N NaOH. For the assay, the NaOH solution was drained from all columns by removing the tips blocking the bottom of the columns. The tips of each column were then replaced to prevent added

solution from running through the column in the following steps. A volume of 100 µl of 0.1 N NaOH containing 5,000 to 10,000 cpm of either *T4 or *T3 was dispensed onto each column. Then 100 ul of each standard or sample was added to the appropriate columns. The columns were swirled to mix the radiolabelled hormone with the standard or sample plasma before draining by removing the tips. A volume of 3.5 ml of barbital buffer (15.6 g / L sodium barbituate, pH 8.6) was then added to each of the T4 containing columns and an equal volume of phosphate buffer was added to the T3 columns. The eluent from three of the columns was collected for counting in the gamma counter to determine the radioiodide contamination of the *T4 or *T3 added to the columns. The columns were then transferred onto clean collecting test tubes. Each column then received 0.5 ml of barbital buffer containing a quantity of lyophylized rabbit antisera to T4 linked to human serum albumin (T4 antibody) or 0.75 ml of phosphate buffer (26.81 g $Na_2HPO_4 \cdot 7H_2O$, 11.17 g Na_2EDTA, pH 7.4) containing a comparably generated antibody to T3. The concentration of antibodies used was equivalent to the amount required to bind 40-60 % of added radiohormone in absence of unlabelled hormone. This concentration was determined by pre-assay trial and error and varied slightly between antibody bottles. For each assay, an equivalent amount of buffer was added to three columns that did not receive antibody to determine the non-specific binding (NSB). The buffer was allowed to drain from the columns and then the columns were allowed to incubate for at least 5 hours. After this period, all columns were eluted with 3.5 ml of barbital buffer for the T4 columns or an equivalent amount of phosphate buffer for the T3 columns. The eluents were collected in test tubes and then counted in the gamma counter to determine the percentage of radiolabelled hormone bound by antibody on each

column. The T4 and T3 concentrations of the samples were then determined by interpolation from a graph relating the percentage of hormone bound by antibody to the concentration of standards.

The detection limit varied between assays and was determined as follows: Detection limit = standard deviation of y intercept / regression slope. (Brown and Eales, 1977). The coefficient of variation is between 4.9 and 16.9 % for the T4 RIA and 5.1 to 15.9 % for the T3 RIA (Brown and Eales, 1977).

Determination of the proportion of free T4 or T3 in plasma

The properties of the plasma thyroid-hormone-binding proteins of the sturgeon were analyzed by two methods according to the procedures employed by Eales and Shostak (1985b). One method involving equilibrium dialysis and the other involved partition of free and bound hormone on G-25 Sephadex columns.

Equilibrium Dialysis

General Theory

In this technique, radiolabelled TH is added to a dialysis sac containing diluted sturgeon plasma, which in turn is immersed in a plastic tube containing 20 ml of buffer. The radiolabelled TH redistributes itself throughout this system and eventually comes to equilibrium. The sac then contains TH bound to plasma proteins as well as unbound TH free in the plasma, whereas the saline outside the sac contains only unbound TH. At equilibrium, the concentration of unbound radiolabelled hormone inside the sac is equivalent to the concentration outside the sac. Therefore, the proportion of TH bound by plasma proteins inside the sac can be determined by subtracting the quantity of TH

unbound from the quantity of hormone inside the sac. This represents an index of the proportion of free TH in plasma.

Detailed Procedure

Three ml of sturgeon plasma diluted 1: 9 in HEPES buffer (pH = 7.4) inside the sac was dialyzed against 20 ml of HEPES buffer (11.99 g HEPES / 500 ml ddH2O) outside the sac for 16 hours at 12 °C. The sac was made of Spectrapor 2 membrane (Molecular weight cut-off of 12,000 – 14,000) tubing tied off at one end to form a sac. 100,000 cpm of radiolabelled T4 or T3 in 15 µl of 0.1 N NaOH was added to each dialysis sac at the start. The apparatus was allowed to dialyze for 16 hours. At the completion of the equilibration period, duplicate 100-µl samples of fluid were pipetted from each side of the membrane and placed onto miniature G-25 Sephadex columns to separate labelled TH from any contaminant radioiodide. The sample was allowed to drain onto the column and the radioiodide was then eluted with 3 ml of phosphate buffer. The radiohormone was then eluted from the column with two 4-ml washes of 0.1 N NaOH. The two NaOH washes were collected and counted in a gamma counter to determine the quantity of the radiolabelled hormone inside or outside the dialysis sac. The % free hormone was calculated as follows:

$$\% \text{ free hormone} = \left[\left(\frac{\text{cpm outside}}{\text{cpm inside} - \text{cpm outside}} \right) \Big/ 10 \right] \times 100$$

The percentage of hormone bound to plasma proteins was then equal to 100 - % free hormone.

Equilibration on G-25 Sephadex columns

General Theory

In this method, equilibration of TH binding to plasma proteins occurred between the proteins and G-25 Sephadex gel in mini-columns. The greater the concentration and affinity of the plasma proteins for TH, the less hormone is weakly bound to the Sephadex gel and the more TH is eluted from the column. This method provides a relative index of thyroid hormone binding to plasma proteins and is highly correlated in rainbow trout with the dialysis procedure (Eales and Shostak, 1985b)

Detailed Procedure

All G-25 columns containing 0.3grams Sephadex gel stored in 0.1 N NaOH were drained by removing the caps and tips on the columns. A 100-µl volume of 0.1 N NaOH containing 10,000 cpm of radiolabelled TH was then added to each column. All columns were eluted with 3.5 ml of phosphate buffer (pH=7.2, 8.8 mM KH_2PO_4, 30.4 mM $Na_2HPO_4{\cdot}H_2O$, 1mM NaN_3). Three of these elutions were collected to obtain the $^{125}I^-$-contamination of the labelled hormone. All columns were then retipped and 0.75 ml of blood plasma (15 % dilution with phosphate buffer) was added to each. This was allowed to incubate at room temperature for 15 minutes before all columns were eluted with 3.5 ml of phosphate buffer. Each eluate were collected and counted in the gamma counter for 10 minutes. The count obtained was expressed as the percentage of added radiolabelled hormone eluted from the column. This value represents the percentage of the hormone in the diluted plasma that is bound by plasma hormone binding proteins

Tissue Hormone Content

The TH content of the sturgeon tissues was determined by performing RIAs following extraction of the TH from digested tissue samples. Weighed samples of various sturgeon tissues were suspended in enough phosphate buffer ($Na_2HPO_4 \cdot 7H_2O$, 28.6 g/L, 1 mM PTU, pH 7.5) containing 0.0025 g / ml Pronase (Sigma) to completely submerse each sample. The tissues were allowed to digest while submersed in a 37 °C water bath until the tissue broke up easily upon stirring (about 18 hours). Digested tissues were then extracted by adding 5 ml ethanolic ammonia (99:1, vol/vol) to the digestion tubes. The mixture was stirred and then allowed to sit in the fridge for 24 hours. The tubes containing the digested tissue were then centrifuged at 1420 g for 5 minutes to pellet remaining particulate. The supernatant was removed and placed into a test tube before another 5 ml of ethanolic ammonia was added, the mixture stirred, followed by centrifugation and removal of the supernant to the same test tube in which the first supernant volume was placed. This procedure was repeated once more. The extract collected in the three pooled supernant volumes from each sample were evaporated to reduce the volume to about 10 ml, which was then added to a 60-ml separatory funnel with 45 ml of chloroform and briefly shaken to mix. The chloroform removed the lipids from the extract (lipids bind thyroid hormone preventing their detection in the RIA). A 15-ml volume of 2N NH_4OH was then added to extract hormone from the lipids being drawn into the chloroform. The funnels were shaken for 5 minutes using a Burrel Wrist-action Shaker (Burrell Corp. Pittsburgh, PA) to mix the chloroform and aqueous fractions. The funnels were then allowed to sit for 1 hour to allow the chloroform and aqueous components to separate. The lower chloroform layer

was drained off and discarded while the upper aqueous layer was collected into test tubes to be evaporated in a water bath under a fumehood at 37 °C. The final extract was then resuspended in 1 ml of 0.1 N NaOH. The hormone content of the extract was then determined using an RIA with the standards constituted in 0.1 N NaOH. 50,000 cpm of T4 and T3 were each added to 2 whole body extracts at the beginning of the extraction steps to determine the percentage of hormone recovered at the end of the procedure.

Red Blood Cell Steady State Hormone Distribution

General Theory

Thyroid hormone free in the plasma is in equilibrium with binding to plasma proteins, and uptake by the red blood cells. Therefore, determination of the proportion of THs in the red blood cells at equilibrium will determine if these cells contain a significant quantity of whole blood TH. The proportion of blood TH in red blood cells at steady state was determined by equilibration of added *T4, *T3, or *rT3 between the red blood cells and plasma (McLeese and Eales, 1998). Since TH can adhere to the outside of RBC and since the exchange of TH across the RBC membrane can be rapid, the partition of labelled TH between the RBC and plasma was achieved by rapid centrifugation of the RBCs through oil and an alkaline aqueous medium (McLeese and Eales, 1998).

Detailed Procedure

Blood was obtained from a sturgeon and the hematocrit was determined using a micropipet and hematocrit scale. The micropipet was dipped into a well-mixed blood sample and blood drawn into the pipet. The end was then sealed with putty and the micropipet spun in a centrifuge to separate the red blood cells from the plasma. A

hematocrit scale was then used to determine the percentage of blood constituting RBCs (hematocrit).

Two-ml blood samples were put into a number of 16 x 100-mm polycarbonated tubes and equilibrated in a shaking water bath at 12 °C. 250 µl of suspension buffer (124 mM NaCl, 3.4 mM KCl, 0.9 mM $CaCl_2$, 0.9 mM $MgSO_4$, 2.56 mM NaH_2PO_4, 10 mM HEPES, 2.8 mM glucose, 4.0 mM Na-pyruvate, 30 mM Na_2CO_3, 0.1 mg / ml ammonium heparin, pH=7.5) containing 0.1 µCi of thyroid hormone was added to each tube. The T3 added samples were then incubated for 40 minutes, and the T4 and rT3 samples for 120 minutes, as in rainbow trout the later two THs take a longer time to come to equilibrium. After incubation, duplicate 200-µl samples of blood from each tube were layered above 350-µl of silicone oil, which was layered above 150-µl of alkaline glycine buffer (pH=10) in a 1.5-ml microcentrifuge tube. Each microcentrifuge tube was then immediately centrifuged at 17,000 g and room temperature to pellet the red blood cells. The supernatant was removed from above the red blood cell pellet and Q-tips were used to remove residual supernatant adhering to the red blood cell pellet or tube wall. The red blood cell fractions was counted and supernatants including the Q-tips were then counted in a gamma counter to determine the percentage of hormone inside and outside the red blood cells at equilibrium.

Statistics

Significant differences between means for the plasma TH levels and plasma protein binding analysis were determined using a two-sample t-test assuming unequal variances.

Results

Plasma T4 and T3 levels were both very low in laboratory-raised sturgeon (T4 = 0.29 ± 0.05 ng / ml; T3 = 0.19 ± 0.01 ng / ml) and in wild sturgeon (T4 = 0.83 ± 0.85 ng / ml; T3 = 1.31 ± 1.64 ng / ml) (Table 3-1). The wild sturgeon values for T4 were significantly greater than those measured in the laboratory-raised sturgeon ($p < 0.05$). However, there was no significant difference in the plasma T3 levels between the wild and laboratory sturgeon ($p > 0.05$).

The extent of binding of plasma THs by plasma proteins was compared between rainbow trout and sturgeon using equilibrium dialysis (Fig. 3-1). Higher percentages of TH were free in plasma of sturgeon (T4 = 1.1 ± 0.3 %; T3 = 0.44 ± 0.04 %; rT3 = 3.04 ± 0.37 %) than in trout plasma (T4 = 0.18 ± 0.01 %; T3 = 0.08 ± 0.01 %; rT3 = 0.72 ± 0.09 %) ($P < 0.05$). In both species, plasma proteins bound a greater proportion of plasma T3 than T4 or rT3. Using these results the concentration of plasma unbound T4 (FT4) and unbound T3 (FT3) was determined to be 0.033 ng / ml plasma for T4 and 0.004 ng / ml plasma for T3.

The extent of binding of thyroid hormones by plasma proteins was also compared between the sturgeon and trout using the Sephadex G-25 column method. Binding of T4, T3, and rT3 was again much weaker in the sturgeon plasma than in trout plasma for all three hormones at all of the 5 dilutions of plasma tested (Fig 3-2). T3 was again bound to plasma proteins more strongly than either T4 or rT3.

The red blood cells contained a greater percentage of whole blood T4 (19.5 ± 0.5 %) than rT3 (6.9 ± 0.7 %) or T3 (6.1 ± 0.3 %); (Fig. 3-3). The hematocrit of these blood samples was 21 ± 1.9 % (n = 2).

Table 3-1 - The concentrations of TH in the plasma of laboratory-raised lake sturgeon compared to wild sturgeon samples determined by radioimmunoassay.

	Thyroid Hormone Levels						
	T4(ng/ml)			T3 (ng/ml)			
	X	SEM	Range	X	SEM	Range	n
Laboratory	0.29*	0.05	< 0.12 - 0.86	0.19	0.01	< 0.02 - 0.40	18
Wild	0.83	0.85	< 0.04 - 2.90	1.31	1.64	< 0.18 - 9.65	14

* Indicates a significant difference between the wild and laboratory-raised sturgeon using an independant sample t-test assuming unequal variances ($p<0.05$)

Wild sturgeon samples were blood serum whereas the laboratory-raised samples were plasma.

Laboratory sturgeon were raised at 15 C with two daily feedings (10:00 & 16:00) of Martin's trout pellets and sampled on May 7, 1997.

Wild sturgeon blood samples were obtained from gill-netted fish from Lake Nutimik on on June 7, 1997.

Figure 3-1: Percentage of plasma thyroid hormones free in the blood plasma of lake sturgeon as determined by equilibrium dialysis. Triplicate measurements were made of a pool of 6 lake sturgeon plasma samples and a pool of 2 rainbow trout samples (mean ± SEM). An asterick (*) indicates significant differences between the trout and sturgeon

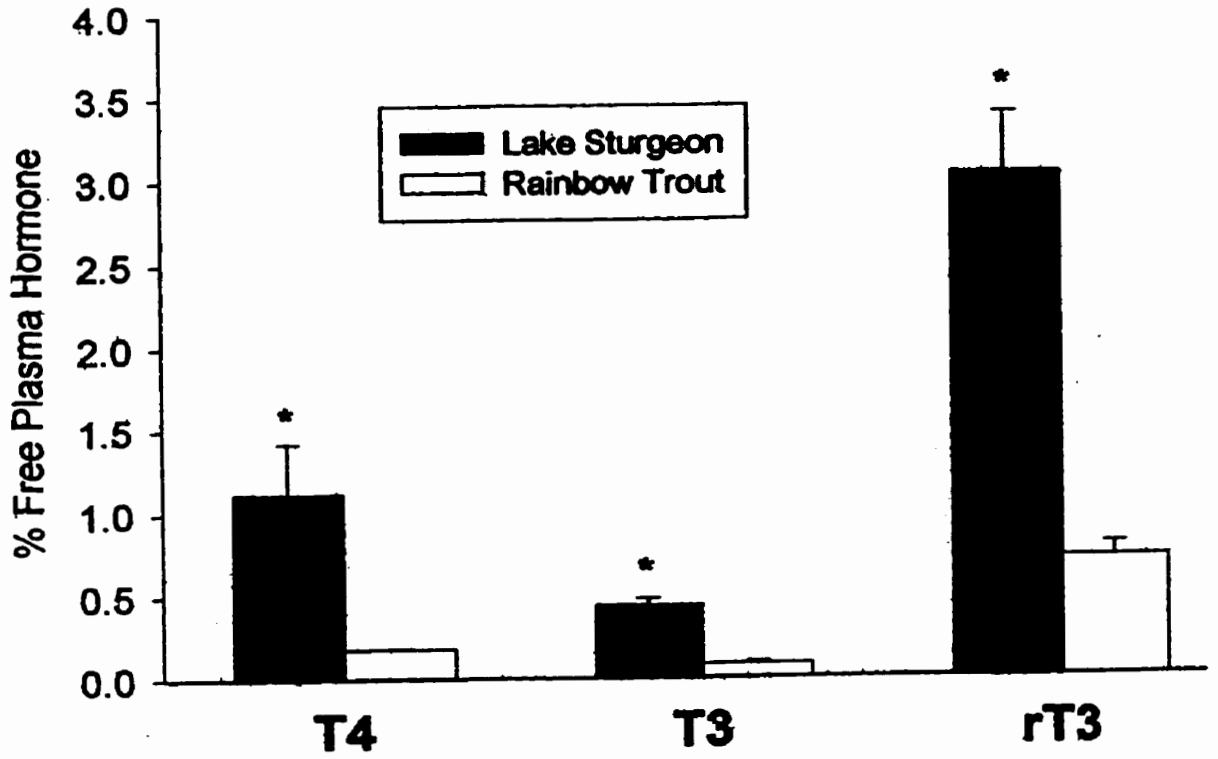

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
% Free Plasma Hormone
Lake Sturgeon
Rainbow Trout
*
*
*
T4
T3
rT3

Figure 3-2: Percentage of plasma thyroid hormones (T4, T3, and rT3) bound to lake sturgeon and rainbow trout plasma proteins as determined by equilibration of plasma with radiolabelled hormone on G-25 Sephadex columns. The plasma was diluted and equilibrated in triplicate at 0.62, 1.25, 2.5, 5.0, and 10 % plasma in phosphate buffer (mean ± SEM).

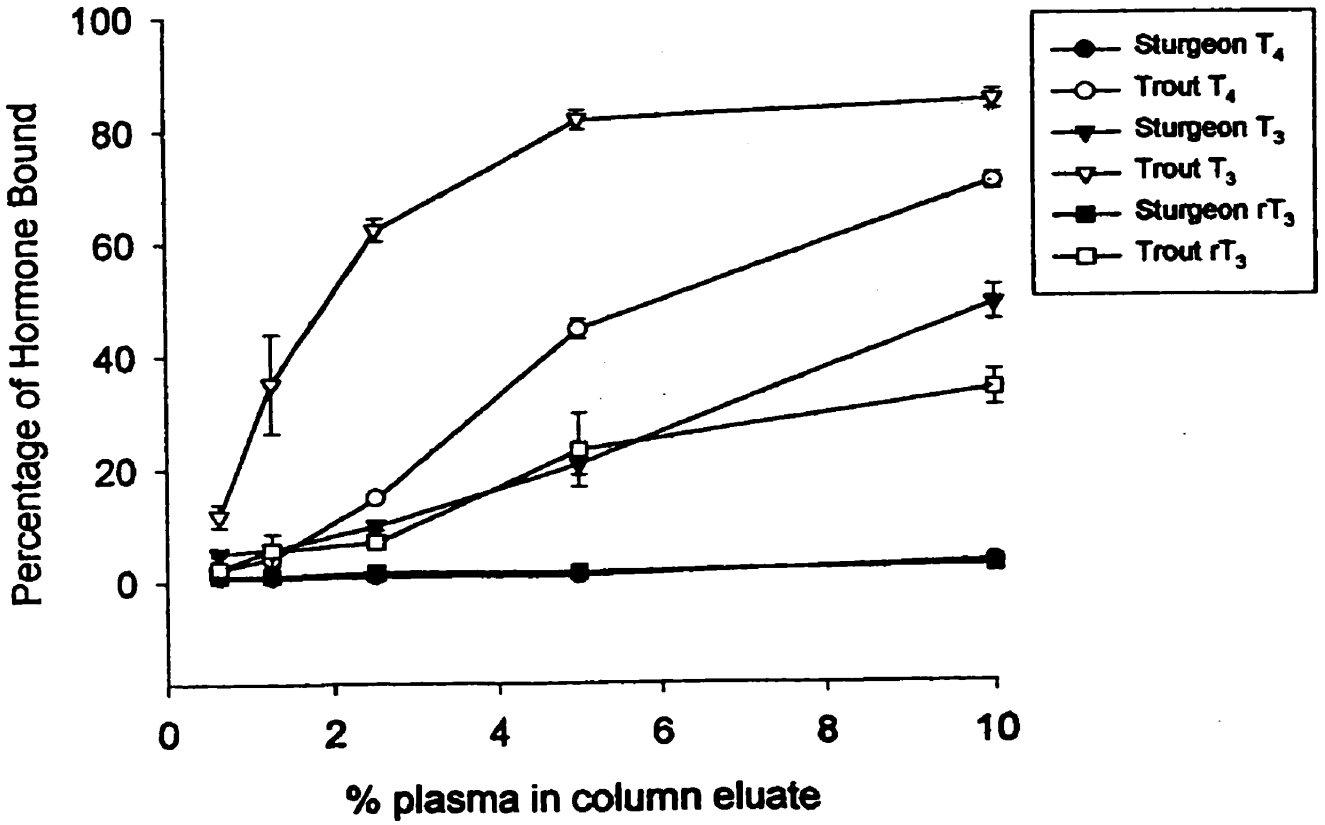
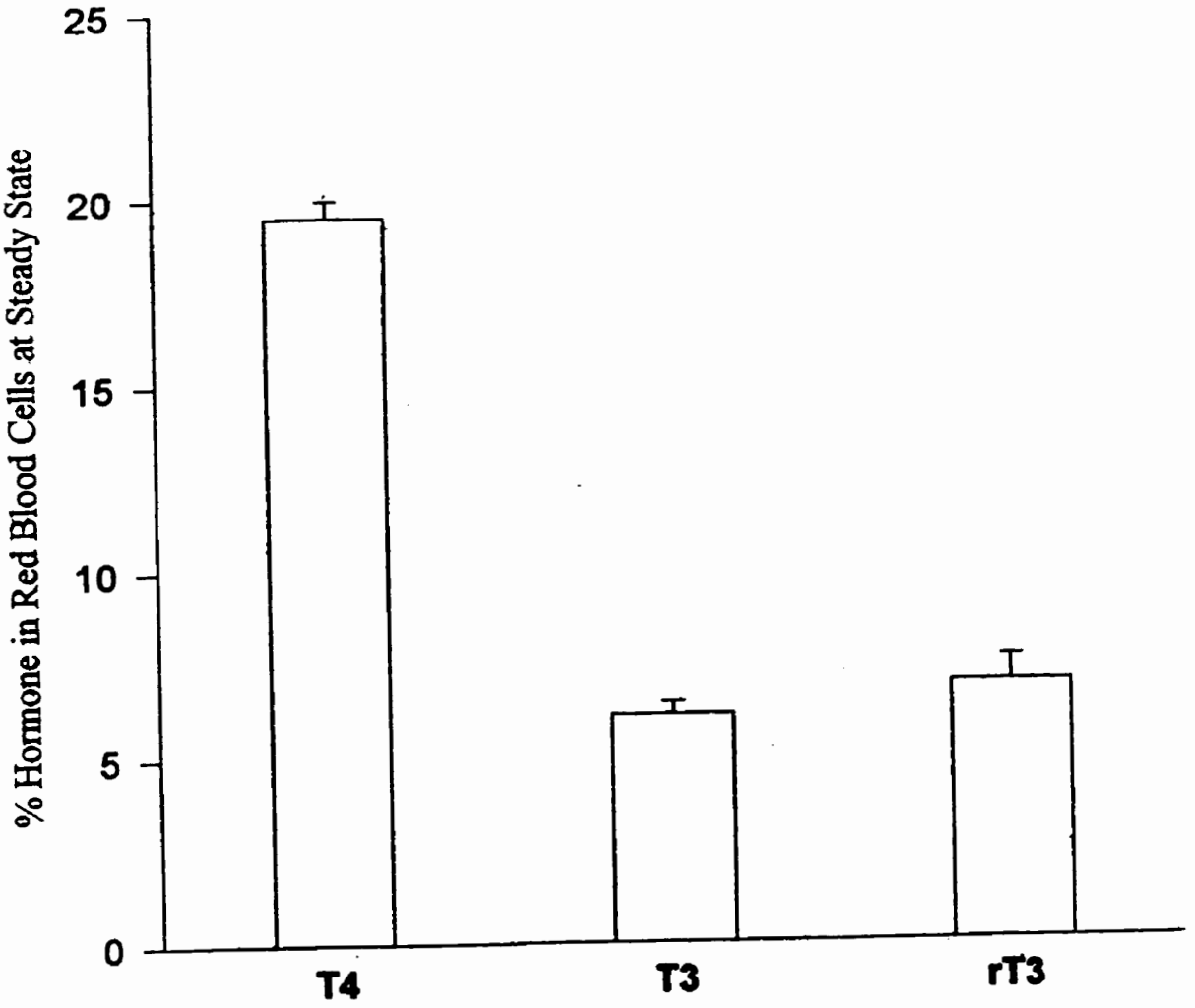

100
80
60
40
20
0
Percentage of Hormone Bound
0
2
4
6
8
10
% plasma in column eluate
Sturgeon T4
Trout T4
Sturgeon T3
Trout T3
Sturgeon rT3
Trout rT3

Figure 3-3: **Percentage of added radiolabelled thyroid hormones (T4, T3, rT3) in red** blood cells at **equilibrium in sturgeon whole blood (n = 12, mean ± SEM).**


% Hormone in Red Blood Cells at Steady State
25
20
15
10
5
0
T4
T3
rT3

The hormone contents of the tissue and whole body samples are summarized in Tables 3-2 and 3-3. The extraction efficiency was 57.5 % for T4, and 66.6 % for T3, and all reported values have been corrected for recovery. There were greater concentrations of T3 than T4 in most tissues analyzed and in the body as a whole. The gall bladder, skin and kidney were the only organs that contained more T4 than T3. The greatest concentration of T3 was in the thyroid, which contained 10.6 X more T3 than T4. The brain contained the second greatest concentration of T3, which was 11.3 X as much as the T4 content. The upper and lower intestine contained 0.77 and 0.54 ng T3 / gram of tissue respectively with the T4 levels at the detection limit. All other tissues analyzed contained quantities of TH at or near the detection limits of the assays. The detection limits of the assays were: Average T4 = 0.12 (0.02 - 0.19) ng / ml, Average T3 = 0.02 (0.006 – 0.035) ng / ml.

Sometimes a thick pigment was present in the tissue extracts which may have interfered with the RIA by preventing hormone from binding to the G-25 Sephadex gel. The interference of the pigment was ruled out by serially diluting two whole body extracts three times and then determining the T3 content of each by the RIA method. The slope of the line formed by the dilution of the extracts was similar to the slope of the line of the NaOH standards (Fig 3-4). This ruled out the possibility of interfering effects of the pigments contained in the extracts.

Discussion

Thyroid Hormones in Blood

The blood is the major vehicle that carries THs from the thyroid follicles to the peripheral body tissues, between peripheral tissues, and to excretory tissues. Preliminary

Table 3-2: The concentrations of thyroid hormone in various tissues of lake sturgeon.

	T4 (ng / gram tissue)		T3 (ng / gram tissue)			
Tissue	X	SEM	X	SEM	T3 / T4	n
Brain	0.62	0.06	5.88	3.12	11.32	6
Thyroid	2.41	0.97	21.31	2.02	10.55	5
L. I.	*0.09[4]	0.01	0.54	0.1	7.16	4
Gill	*0.08[1]	0.04	0.21	0.16	3.13	4
U. I.	*0.03[3]	0.22	0.77	0.26	3.06	4
Gonad	*0.05[2]	0.04	*0.11[1]	0.1	2.63	4
Muscle	*0.03[1]	0.01	*0.05[2]	0.05	1.99	4
Liver	*0.06[4]	0.03	*0.07[2]	0.04	1.39	4
Skin	0.10	0.03	*0.08[1]	0.07	0.95	3
Kidney	0.10	0.03	0.07	0.05	0.84	3
Gall Bladder**	6.02	2.88	3.57	1.06	0.71	4

All values were corrected for recovery and the weights expressed in ng TH / wet tissue weight.

* Calculated with some values at or below the detection limit of assay. Average detection limits of extracts: T4 = 0.12 ng / ml (0.02 -0.19), T3 = 0.02 ng / ml (0.006 - 0.035).

** ng per whole Gall Bladder.

Super script numbers indicate number of samples in each mean that were below detection lim

Table 3-3: The concentrations of TH in the whole body of lake sturgeon

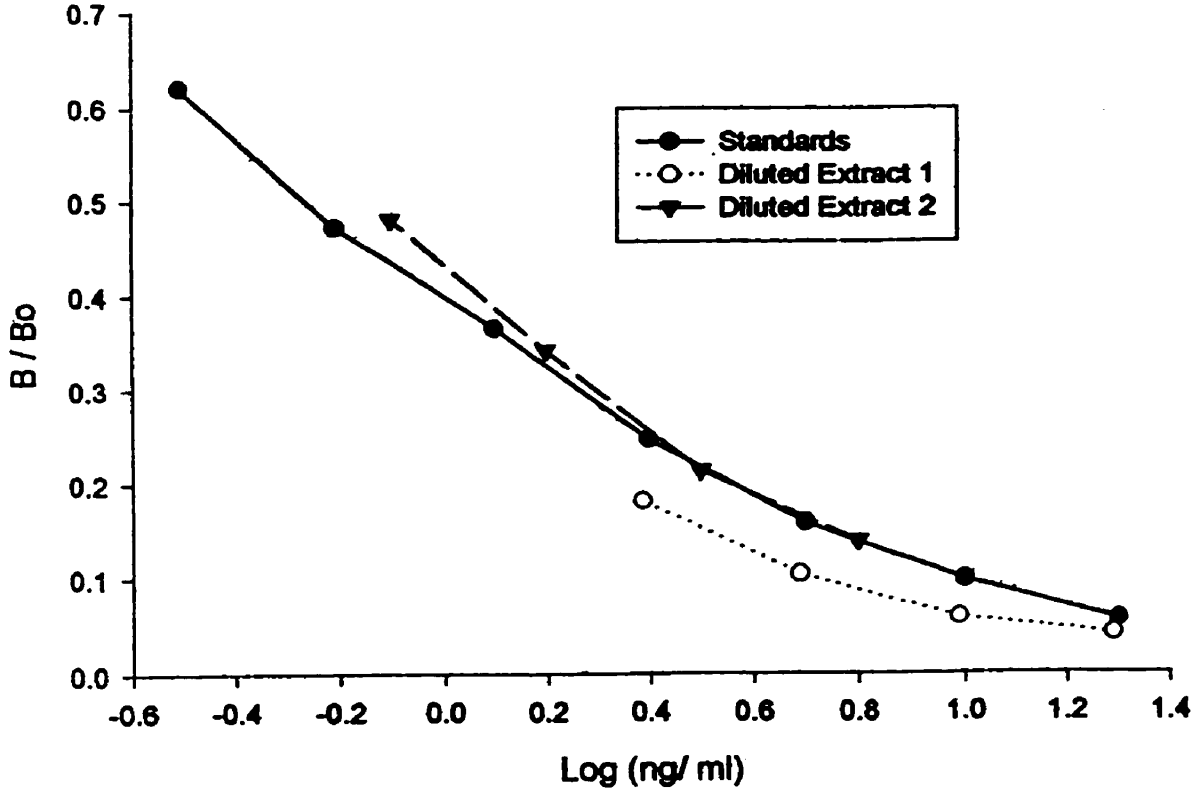
	T4 (ng / gram wet tissue)		T3 (ng / gram of wet tissue)		Molar Ratio	
Fish	X	SEM	X	SEM	T3 / T4	SEM
1	1.03	0.24	8.14	2.36	9.43	11.74
2	0.53[2]	0.11	5.84	1.00	13.15	10.85
3	2.06[5]	1.62	13.54	4.43	7.84	3.26
4	0.52[1]	0.31	19.37	7.68	44.50	29.57
5	0.52	0.12	15.29	1.73	35.09	17.21
Mean	**0.93**	**0.48**	**12.44**	**3.44**	**22.00**	**14.53**

Basibranchial thyroid region removed before body macerated.

Each value is the Mean ± SEM of 6 measurements.

Super script number indicates number of values included in each mean that were at the detection limit.

Figure 3-4: T3-concentration profile from 2 sturgeon whole body extracts diluted by 50 % at each successive dilution compared to a radioimmunoassay standard curve.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B / Bo
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Log (ng/ ml)
Standards
Diluted Extract 1
Diluted Extract 2

analysis of lake sturgeon plasma found low to negligible levels of TH (S. B. Brown, unpublished data). These results questioned the importance of TH in the lake sturgeon.

Plasma analysis of laboratory-raised sturgeon by RIA confirmed low hormone levels for both T4 (0.29 ± 0.05 ng / ml) and T3 (0.19 ± 0.01 ng / ml) in all cases. The T4 values are lower than those in wild sturgeon (T4 = 0.83 ± 0.85 ng / ml), however, the T3 levels did not differ between the two groups (T3 = 1.31 ± 1.64 ng / ml). The difference in plasma T4 levels may relate to the size and age differences between the laboratory and wild sturgeon as the wild sturgeon were much older and larger than those raised in the lab. These values are at the lower range of values reported in wild white sturgeon (T4 = 0 - 3.6 ng / ml, T3 = 0 – 6.9 ng / ml) (McEnroe and Cech, 1994). Compared to other fish, reptiles and mammals, the plasma TH levels of lake sturgeon are among the lowest (Table 2-1).

A low degree of binding of TH by lake sturgeon plasma proteins may limit the blood's ability to carry the TH and explain the low plasma TH levels. The percentage of plasma TH bound by protein (98.9 % for T4, and 99.60 % for T3) is lower than that in other species. Arctic charr plasma proteins normally bind 99.72 - 99.85 % T4 and 99.83 - 99.91 % T3 (Eales and Shostak, 1985b). Values as low as those in the lake sturgeon have not been reported for any other species. rT3 was bound by plasma proteins to the lowest extent (96.93 %). This value is much lower than that in rainbow trout (99.05 %) (Eales et al, 1983). Poor binding of rT3 may indicate its apparent lack of value as an inactive TH metabolite. This idea is supported by the very high hepatic deiodinase activity in breaking down rT3 (See Chapter 4).

The lake sturgeon FT4 (0.033 ng / ml) and FT3 (0.004 ng / ml) are very similar to the values found in arctic charr (FT4 = 0.043 ng / ml, FT3 = 0.002 ng / ml) (Eales and Shostak, 1985b). These values are also simialr to those of the mammal (FT4 = 0.172 ng / ml, FT3 = 0.003 ng / ml). This suggests that even though the TH is carried in low quantities in the plasma of the lake sturgeon, the quantity of the plasma TH that is available for exchange with the tissues is similar to that in the charr and mammal.

Low TH concentrations in the plasma of the sturgeon suggest that TH is not carried in great quantities in that blood compartment. However, RBCs may store a significant amount of the TH transported in the blood and release it as it is needed for transport from the blood to tissues. At steady state, the sturgeon RBCs contained 19.5 $\pm$ 0.5 % of T4, 6.1 $\pm$ 0.3 % of T3, and 6.9 $\pm$ 0.7 % of rT3 in whole blood (Fig. 3-3). In rainbow trout, 5-11% T4, 14-23% T3 and 23-24% of whole blood rT3 is carried in the RBCs (McLeese and Eales, 1998). A greater percentage of T3 than T4 in the RBCs is also found in other fish studied like the eel, *Anguilla anguilla*, 19% T3, 7% T4; carp, *Cyprinus carpio*, 41% T3, 14% T4; and Atlantic salmon, *Salmo salar*, 17% of T3, 15% of T4; Leloup and Fontaine, 1960). The RBCs of mammals also contain a significant percentage of blood TH. The rat RBCs contain approximately 23% of total blood T3 (Françon et al, 1990). The percentage of T4 is much greater, and T3 and rT3 much lower in sturgeon RBCs compared to other fish species. In contrast to other fish, the percentage of whole blood T4 in RBCs is greater than T3.

Like mammals, sturgeon may have cytosolic or membrane receptors in their RBCs. However, sturgeon RBC proteins appear to have greater affinity for T4 than T3 as the RBCs contain a greater percentage of blood T4 than T3 in equilibrium with the

plasma. Unlike mammals, mature fish RBCs are nucleated and rainbow trout RBC nuclei contain high-affinity TH receptors, but these are present in very small numbers (Sullivan et al, 1987; Bres and Eales, 1988). Nuclear receptors similar to those in the trout may also be present in sturgeon RBCs, but are unlikely to have a major influence on the RBC TH content.

The RBCs of lake sturgeon carry TH with whole blood percentages of T4 >> rT3 > T3 which contrasts with other fish that carry higher proportions of whole blood T3 than T4 in RBCs. Since the lake sturgeon RBCs carry a low percentage of blood THs, and the sturgeon's hematocrit is lower than in other fish species, the RBCs are not carrying a large amount of TH. Therefore, sturgeon blood as a whole is low in TH content.

Tissue Thyroid Hormones

Since TH is not transported in significant quantities in the blood between tissues, it might be predicted that the extrathyroidal tissues would not contain much TH. However, this depends on the kinetics of exchange of TH between the blood and tissues. Therefore, the T4 and T3 content of the tissues were examined to determine if any tissues have a significant capacity and affinity for TH.

All extrathyroidal tissues examined contained low TH levels except for the brain which had 5.9 ng T3 / gm tissue. High T3 concentrations occur in the brain (≅10-40 ng/gm tissue) of coho salmon undergoing parr-smolt transformation in fresh and saltwater (Specker et al, 1992). The molar activity ratio (T3/T4) in the sturgeon brain was also high (11.3) like that in the coho salmon (≅5.2) indicating that large quantities of T3 may be required in the brain of young sturgeon and metamorphosing salmon (Specker

et al, 1992). T3 is essential for development in growing fish brain (Timiras and Nzekwe, 1989).

High brain T3 levels could be due to the presence of high affinity TH-binding proteins and / or significant deiodination activity of T4 to T3. A TH-binding protein called transthyretin occurs in the brain of mammals, birds, and salmon. It is produced by the choroid plexus and released into the cerebrospinal fluid resulting in high levels of T4 in brain tissue (Dickson et al, 1985; Schreiber et al, 1990). The presence of transthyretin in the brain concentrates the hormone to significant quantities such that the brain is one of the most concentrated sites of TH outside of the thyroid follicles (Dickson et al, 1985). Bird transthyretin has a much greater affinity for T3 than T4 leading to high levels of T3 but not T4 in the bird brain. In masu salmon, transthyretin in blood serum has a higher affinity for T3 than T4 (Yamauchi et al, 1999). A transthyretin similar to that in salmon and birds may also occur in particularly high concentrations in the sturgeon brain and to lesser degrees in the other sturgeon tissues. This would result in a high concentration of T3 throughout the sturgeon tissues.

Alternately, similar to mammals, brain transthyretin in the sturgeon may have a higher affinity for T4, attracting high concentrations of T4 into the brain. Significant deiodination if present in the brain, could then convert a majority of the T4 to T3.

A final possible explanation for the high TH levels in the brain may involve contamination of the brain tissue by hemopoeitic tissue. This red-blood-cell-producing tissue is located in close proximity to the brain tissue and contains a large number of cells in early development. Immature RBC nuclei contain a much greater concentration of thyroid hormone receptors than mature cells in trout (Sullivan et al, 1987). If this is also

the case in sturgeon then this would result in a concentration of hormones in the hemopoietic tissue. However, the T3 concentrations found in the brain extracts are higher than what is likely bound to nuclear receptors in the immature red blood cells. Rainbow trout proRBCs contain, on average, only 1781 sites per nucleus (Sullivan et al, 1987). Therefore, the brain tissue likely contained the majority of the hormone extracted.

In rainbow trout, the liver (3.5 ng /gm), kidney (2.8 ng / gm), and intestine (2.5 ng / gm) contain the greatest concentrations of hormone outside of the thyroid (Fok et al, 1990). The upper (0.8 ± 0.3 ng / gm) and lower (0.5 ± 0.1) intestines of sturgeon were among the tissues containing the greatest concentration of T3, but it was still much less than that in the trout intestine (2.5 ng / gm) (Fok et al, 1990). Dietary sources of TH may explain the levels of TH in the upper intestine. Biliary excretion of TH into the lower intestine could contribute to the TH levels in this tissue. The gall bladder secretes bile containing THs into the lower intestine (T4 = 6.0 ± 2.9 ng / ml bile, T3 = 3.6 ± 1.1 ng / ml bile) of the sturgeon.

Greater concentrations of T3 than T4 were found in the thyroid gland with a molar activity ratio of 10.6 (T3/T4). High thyroidal T3 concentrations suggest that T3 rather T4 is the primary form of the TH released by the thyroid gland. If this is the case, the role of peripheral tissue T4-ORD activity in regulating the production of T3 may not be as important as it is in teleosts. Instead, it is predicted that T3-IRD activity may control the quantity of T3 in the body through inactivation by deiodination of T3 to 3,3'-T2.

High concentrations of T3 in the whole body extracts (less thyroid) could indicate that either some of the thyroid in the gill region was not removed or there is thyroidal

tissue at some other unidentified site in the sturgeon. For example, heterotopic thyroid tissue occurs in the kidney of goldfish (Hoar and Eales, 1963; Peter, 1970). Additionally, there may be some other tissue not studied that contains high concentrations of hormone.

Conclusions

The blood plasma or serum of both wild and laboratory-raised lake sturgeon contains very low quantities of both T4 and T3. Low binding by plasma TH proteins explains the inability of sturgeon plasma to hold significant quantities of TH. However, the proportion of plasma thyroid hormone available for exchange with the tissues is similar to that in other fish and mammals.

The red blood cells do not contain an unusually high proportion of the total blood TH when compared to other species. Therefore, lake sturgeon blood as a whole is low in thyroid hormone content.

All the tissues contain measurable quantities of TH (both T4 and T3), with greater amounts of T3 than T4 in most tissues and in the whole body extracts. The brain and thyroid contain the greatest amount of T3. High concentrations of T3 in the thyroid gland suggest that T3 may be the predominant form of the hormone released by the sturgeon thyroid. High brain T3 levels could be due to the presence of a high affinity T3-binding protein and / or significant T4-ORD activity.

Chapter 4

Sturgeon Deiodination Activity and Characteristics

Introduction

Thyroid hormone is taken up into the tissues from the blood by both active and passive transport systems (Riley and Eales, 1993a). Once inside the tissues the hormone can be converted into other forms by deiodinase enzymes (Eales and Brown, 1993).

There are four main deiodination pathways in teleosts: T4-ORD, T4-IRD, T3-IRD, and rT3-ORD illustrated in Figure 1-1. T4-ORD converts weakly biologically active T4 (presumed prohormone) into T3 (active form) by removing an outer-ring iodine. T3 binds with greatest affinity to receptors in fish cells (Bres and Eales, 1988). It is the form of the hormone responsible for the effects on fish growth, maturation, and development (Brown and Eales, 1993). T3-IRD inactivates T3 by removal of an inner-ring iodine to form 3,3'-T2. T4 can also be converted into an inactive (rT3) form by T4-IRD. This may serve to control the amount of T4 available for conversion to T3. rT3 can in turn be further broken down by rT3-ORD into 3,3'-T2 to salvage another iodide for recycling back to the thyroid. In mammals, there are three main deiodinase enzymes found in the tissues (types I, II, and III). These isozymes differ in their substrate preferences, mechanism of reaction with DTT cofactor, and inhibitor sensitivities (Table 4-1). In fish, several deiodinases have been described which resemble their mammalian counterparts in several but not all respects (Finnson et al, 1999).

In this chapter, lake sturgeon deiodination pathways were characterized and compared to other fish and mammal deiodinases reported in the literature. Study of lake

Table 4-1: Characteristics of the type I, II, and III mammalian deiodinase enzymes.

	Deiodinase Type		
Characterisitic	I	II	III
Preferred Substrate	sulfate conjugated rT3	sulfate conjugated T4	sulfate conjugated T3
DTT Kinetics	ping-pong	sequential	sequential
Response to T3 challenge	Increases	Unaffected	Not Studied
Inhibitor Sensitivity	Sensitive	Relatively Insensitive	Insensitive

sturgeon deiodination will determine the role deiodinase enzymes play in regulating the quantity of the various forms of TH present in the body.

Materials and Methods

Fish Maintenance

Eighteen laboratory-raised lake sturgeon weighing 307.9 ± 18.9 g and measuring 43.5 ± 0.8 cm total length were used in these experiments. They were held in fiberglass tanks in running dechlorinated Winnipeg City water at 12-15 °C and fed twice daily (10:00 and 16:00) with Martin's trout pellets to satiation. The fish were anesthetized with tricaine methane sulfonate (MS222, 0.083 g/l) (Syndel Laboratories LTD.) and then killed by concussion to the head. Immediately upon removal, the tissues were wrapped individually in aluminum foil, frozen in liquid nitrogen, and then stored at -76 °C until microsome preparation. The microsomal fraction of tissue homogenates contains the TH deiodinase activity.

Microsome Preparation

The microsomes were prepared according to the procedure of Shields and Eales (1986) with some modifications. After partial thawing, each tissue was individually homogenized in 2.5 ml. of buffer (pH=7.2, 0.03 M $Na_2HPO_4 \cdot 7H2O$, 0.01 M KH_2PO_4, 10 mM dithiothreitol (DTT), 0.01 M EDTA, 0.25 M sucrose) using a Polytron (Brinkman Instruments, Palo Alto, Calif.) at a setting of "five" for 5-10 sec, followed by two strokes with a motorized pestle (Tri-R Instruments Inc., New York). The homogenate was then filtered through cheese cloth and then ultra-centrifuged using a 50.2 Ti rotor for 20 min at 730g and then for 20 min at 25,200g. The supernatant was decanted off, and the pellet discarded after each spin sequence. The supernatant was then centrifuged one last time

for 67 min at 110,000g to produce the microsome pellet, which was resuspended in 2.5 ml of buffer. This was separated into 2 x 1-ml aliquots and frozen in cryovials at -76 °C. An additional 0.5 ml was frozen for the initial protein assay.

Protein Assay

The protein content of the microsome samples was determined using the Bio-Rad protein method (Bradford 1976). The absorbances of the samples were compared to those of known protein concentrations fitted to a standard curve, obtained using known amounts (0-1mg/ml) of bovine serum albumin (BSA). Five milliliters of Bio-Rad protein dye was added to 100 µl of the diluted microsomes or BSA standards, and allowed to stand for at least 15 min before the absorbance of each tube was read using a Spectronic 607 spectrophotometer (Milton Roy Co.) at a wavelength of 595 nm.

Deiodinase Assay

The deiodinase assay followed that of Shields and Eales (1986). The radioactively labeled T4 (*T4), *T3 or *rT3. (New England Nuclear; specific activity = 1070 - 3300 µCi / µgm.) used in the assays were radiolabelled with ^{125}I on one of the outer-ring iodines. The *T4 and *rT3 substrates were purified under a fumehood by removal of the free $^{125}I^-$ produced by their decomposition. Purification of *T3 was not necessary. The *T4 and *rT3 were purified by pipetting the unpurified hormone onto Sephadex LH-20 minicolumns (5-ml Quick-Sep column, Isolab Inc., OH) containing 0.25 g LH-20 Sephadex. The hormone binds to the gel but the free radioactive iodide remains unbound. Three millilitres of ddH20 was then eluted through the column to carry away the unbound $^{125}I^-$ which was discarded.. Then 2.5 ml of 0.1N ethanolic ammonia (NH_4OH / 100% ethanol 1:1) was eluted through the column to liberate the hormone

which was collected in a small test tube. The purified *T4 or *rT3 was then desiccated by placing the small test tube in a water-filled beaker heated on a hot plate to 58 °C. Air was gently blown on the solution through a hypodermic needle to increase the rate of evaporation. After the hormone was dry, it was reconstituted in 100 µl of 0.1N NaOH. An appropriate quantity of stock *T3 was also constituted in 0.1 N NaOH for T3 deiodinase assays. Five microliters of the *T4, *rT3 and *T3 solutions were placed in small test tubes and the radioactivity counted in a Beckman 8000 well-type gamma detector. The appropriate amount of these solutions was diluted with 0.1 N NaOH to give a final hormone substrate activity of 50,000 cpm / 10 µl. An equal volume of unlabelled hormone of a desired concentration in 0.1N NaOH was then added to the radiolabelled hormone to produce the final substrate to be used in the assay (~ 600 µl). Only a small percentage of the substrate need be radiolabelled hormone as this is sufficient to determine the percentage of the entire substrate deiodinated. The unlabelled hormone was used as it is not as expensive as the radiolabelled hormone.

To begin the assay, the microsomes were thawed and diluted to a protein concentration of 0.3 mg/ml with buffer (pH=7.2, 0.03 M $Na_2HPO_4 \cdot 7H_2O$, 0.01 M KH_2PO_4, 20 mM dithiothreitol (DTT), 0.01 M EDTA, 0.25 M sucrose). However, the pH and DTT concentrations used in the buffer were varied in those assays done to determine the optimum pH and DTT concentrations for T4-ORD, T3-IRD, and rT3-ORD. Three types of incubation tubes were included in each assay (blanks, quality controls, and experimentals). Blank tubes contained no microsomes. These were used to determine the amount of free $^{125}I^-$ that was not removed from the substrate during the purification as well as any $^{125}I^-$ that was removed from the hormone non-enzymatically. Quality control

tubes contained a trout hepatic microsome sample of known activity used to check reproducibility between assays. If the deiodination of the quality control was similar in all assays then they were considered to have been performed correctly and the results where regarded as being comparable. Experimental tubes contained the microsome samples to be tested.

Each experimental sample tube and quality control tube received 0.5 ml of the appropriate diluted microsomes, and 0.5 ml of buffer was put in the blank tubes. All tubes were then preincubated for 30 min at 12 °C in a temperature-controlled water bath to equilibrate them to the assay temperature. Ten microliters of substrate was then added at 30-sec intervals to each tube which was then vortexed before incubating at 12 °C for 1-2 hours depending on the assay and deiodination pathway studied. Following incubation, 10 µl of 2 mM potassium iodide (KI) was added to each tube in the same order as the substrate to stop the enzyme activity. Three total count reference (TCR) tubes containing an equal amount of *T4/T4 as that in the incubate tubes (10 µl), were counted in the gamma detector. The TCR tubes were used to estimate the total amount of radioactivity contained in the substrate added to each incubate tube. The quantity of hormone constituting the radiolabelled portion of the substrate was determined using the TCR value taking the radioactive decay of $^{125}I^-$ and the specific activity into account.

High-performance liquid-chromatography (HPLC) analysis was used to analyze the products of deiodination. 500 µl of methanol and 12 µl of methyl mercaptoimidazole was added to the 500 µl of the incubate before analysis. The mixture was transferred to 1.5-ml Eppendorf microcentrifuge tubes and centrifuged for 5 min at 13,000 g at room temperature to separate the precipitated proteins to the bottom of the tube. Four hundred

and twenty-five microlitres of each supernatant was pipetted into individual brown 700-µl autosampler vials for HPLC analysis.

The HPLC analysis was conducted according to the procedure of Sweeting and Eales (1992a). This method was used to measure the deiodination activity of T4-ORD, T4-IRD, T3-IRD, and rT3-ORD. Two solvents (0.1 % TFA in H_2O (solv. A) and 0.1% TFA in acetonitrile (solv. B)) were combined and pumped through an Alltech Econosphere C18; 5 µ column at a ratio that was changed slightly between runs according to the conditions of the column, but was typically 58 % A. A 200-µl aliquot of sample was injected into the column along with the solvent A and B mixture. As the sample passed through the column, the $^{125}I^-$, ^{125}I-labelled iodothyronine derivatives and original substrate were separated, and eluted from the column at different times. Each product has a different affinity for the column which determines the retention time on the column. The $^{125}I^-$ elutes from the column first, followed by 3,3'-T2, T3, rT3, and finally T4 in order of decreasing hydrophilicity. After exiting the column the solution passed through a UV monitor, a Ramona-90 gamma radiation spectrometer, and then to waste. The UV monitor was used to measure the UV absorbance of excess amounts of unlabelled authentic iodothyronines that were added to the incubate before it was eluted through the column. This allowed for identification of the labelled iodothyronines in the incubate as they had identical elution times to the unlabelled authentics. The data from the Ramona-90 gamma detector were fed into an IBM computer for storage, integration, and graphical display of the results using the Gilson 714 software package. The graphical display showed a series of peaks that represented the relative amounts of the

different products eluted from the column and detected by the radiation spectrometer or the UV monitor.

The deiodination activity was calculated using the proportion of original substrate converted to product as determined from the HPLC output. The proportions of products present in the incubates that did not contain enzyme, (blank controls) were subtracted from the products present in the experimental incubates. This corrected the samples for the products already present in the added substrate and those products produced from spontaneous dissociation of iodide from the hormone during the assay. T4-ORD activity was determined from the proportion of T3 present as determined from the HPLC tracings multiplied by 2. Both outer-ring iodines have an equal probability of being removed from the T4 by the T4-ORD but only one is radiolabelled. Therefore, on average only half of the T3 produced would be detected by the HPLC as $^{125}I^-$ removal from the other half would render it undetectable by the gamma detector in the HPLC apparatus.

The rT3-ORD activity was determined in a similar fashion to the T4-ORD activity except that the proportion of 3,3'-T2 X 2 was used in the calculation. The T4-IRD and T3-IRD activities were calculated using the proportions of rT3 and 3,3'-T2 respectively produced as end products. For each deiodination reaction, the activity was calculated by multiplying the proportion of substrate converted to product multiplied by the total original substrate divided by the incubation time in hours and the microsomal protein concentration of the incubate as determined from the protein assay. The results were expressed in pmoles of substrated deiodinated / hr / mg protein. The calculation for T4-ORD activity is shown below:

$$\text{T4 deiodination (pmol. hr.}^{-1}\text{ mg protein}^{-1}) = \frac{\text{T4 substrate (nM) x Fraction of *T4 deiodinated x 2}}{\text{Incubation time (hr) x Microsomal protein (mg)}}$$

The substrate preferences of the T4-ORD, rT3-ORD, T4-IRD, and T3-IRD enzymes were determined according to the procedure followed by Frith and Eales (1996). Upper intestine microsomes were incubated with a fixed amount of radiolabelled hormone (assumed preferred substrate) to which was added 1, 10, or 100 nM of the appropriate analog being tested (3,5,3'-triiodothyroacetic acid (TRIAC), tetraiodothyroacetic acid (TETRAC), 3,5-T2, rT3, T3, or T4). The enzyme activities were expressed as the percentage of the activity of the control occurring in the presence of the preferred substrate alone. The test with the analogs indicates the order of substrate preference of deiodinases.

The effects of three selenocysteine inhibitors (10, 100, 100 nM) where tested to determine the presence or absence of this group at the active site of the T4-ORD, rT3-ORD, T4-IRD, and T3-IRD enzymes extracted from the liver. This was done according to the procedure of Frith and Eales (1996). The inhibitors tested were 6-n-propyl-2-thiouracil (PTU), iodoacetate (IAC), and aurothioglucose (ATG). The activity was expressed as a percentage of the activity occurring in the absence of any inhibitor.

The chemicals used in the deiodinase assay, HPLC analysis, and protein assay where supplied by Fisher Scientific, J. T. Baker Chemical Co., Sigma Chemical Co., BDH Inc.

A one-way analysis of variance test (ANOVA) was done to determine if there were significant differences in deiodinase activity when pH, DTT, analog, and inhibitor concentrations were varied. If it were concluded from the ANOVA that differences existed, a Tukey HSD test was employed to determine where statistical differences occurred between means ($p < 0.05$).

Results

TH deiodination in lake sturgeon tissues was studied to determine their distribution and characteristics. Several lake sturgeon tissues (liver, lower intestine, upper intestine, head kidney, brain, notochord, muscle, and immature gonad) were assayed initially to determine their deiodination activity. From these preliminary analyses it was determined that the liver and intestine possessed the greatest deiodination activity. Therefore, these tissues as well as the thyroid were used to characterize the deiodination reactions.

Microsomes prepared from the liver, lower and upper intestines, and the thyroidal region of a group of laboratory-raised sturgeon where assayed to compare the T4-ORD, T4-IRD, and T3-IRD activities (Table 4-2). The liver contained more than twice the T4-ORD activity found in the lower intestine and thyroid. The greatest T3-IRD activity was also in the liver followed closely by the upper intestine. The upper intestine contained the greatest T4-IRD activity, which was 5X the activity in the liver, lower intestine and thyroid. The T4-ORD activity was greater than the T3-IRD activity in all tissues except for the upper intestine. In the liver, the activity of the T4-ORD which produces T3 was approximately 3 X greater than the activity of the T3-IRD which breaks down the T3.

Overall, the liver showed the greatest deiodination activity. rT3-ORD was the most active deiodination in the liver with more than 8 X the activity of the T4-ORD pathway (Table 4-3). The T3-IRD pathway was only a third as active as T4-ORD. The liver microsomes were used to characterize the T4-ORD, rT3-ORD, and T3-IRD reactions to determine their similarity with the enzymes found in teleost fish.

Table 4-2: The activity of three thyroid hormone deiodination pathways present in microsomal fractions of tissues obtained from 5 lake sturgeon

	T4-ORD		T4-IRD		T3-IRD	
	X	SEM	X	SEM	X	SEM
Liver	0.122	0.056	0.002	0.002	0.048	0.018
Lower Intestine	0.058	0.022	0.002	0.002	0.002	0.002
Upper Intestine	0.012	0.004	0.012	0.006	0.040	0.018
Thyroid	0.044	0.020	0.004	0.002	0.002	0.002

Values in pmoles TH deiodinated / hr / mg protein

Substrate Concentrations in Assays were 0.6 nM for T4 and 0.8 nM for T3

Table 4-3: Hepatic deiodinase activity in a group of 12 lake sturgeon

	X	SEM
T4-ORD	0.120	0.152
rT3-ORD	0.990	0.292
T3-IRD	0.046	0.028

All values are in pmoles TH deiodinated / hr / mg protein

Substrate concentrations in the assays were 0.6 nM for T4 and 0.8 nM for rT3 and T3

T4-ORD

There was significant hepatic T4-ORD activity as determined by the quantity of T3 produced using T4 as substrate (Fig. 4-1a). This pathway worked at an optimum pH of 6.0 – 7.5 (Fig. 4-2a), and 10 mM DTT in the incubate increased activity, with higher concentrations not increasing activity significantly (Fig. 4-3a).

Enzyme kinetic analysis using a Lineweaver-Burke double-reciprocal plot revealed an apparent K_m for T4-ORD of 6.7 ± 2.0 nM and a V_{max} of 26.1 ± 12.8 pmoles T4 deiodinated / mg protein / hr (n = 2) (Fig. 4-4).

T4-ORD had a substrate preference of T4 > TETRAC > rT3 > TRIAC > T3 = 3,5-T2 as judged by the degree of inhibition of deiodination of labeled substrate by the addition of various thyroid hormone analogs at three concentrations (Fig. 4-5).

Propylthiouracil did not inhibit T4-ORD activity, iodoacetate mildly inhibited, and ATG almost completely inhibited activity (Fig. 4-6).

T4-IRD

Insufficient activity was present in the liver microsomes to characterize the T4-IRD pathway.

T3-IRD

Hepatic T3-IRD activity was detected as judged from the quantity of 3,3'-T2 produced using T3 as substrate (Fig 4-1b). Activity was optimal at a pH of 6.7 – 7.8 (Fig. 4-2b), and at a DTT concentration of 15 mM (Fig. 4-3b).

Enzyme kinetic analysis using a Lineweaver-Burke double-reciprocal plot revealed an apparent K_m for T3-IRD of 3.3 ± 1.2 nM and a V_{max} of 1.3 ± 0.2 pmoles T3 deiodinated / mg protein / hr (n = 2) (Fig. 4-7).

Figure 4-1: Representative HPLC (High-Pressure Liquid Chromatography) profiles showing the substrate and products resulting from a) T4-deiodination, b) T3-deiodination, and c) rT3-deiodination. The percentage of acetonitrile in the eluent solvent used in the separation is shown by the dotted line. The solid line is the radioactivity eluted from the column at various retention times. The radioactive peaks corresponding to $^{125}I^-$, 3,3'-T2, T3, rT3, and T4 are labelled on the tracings.

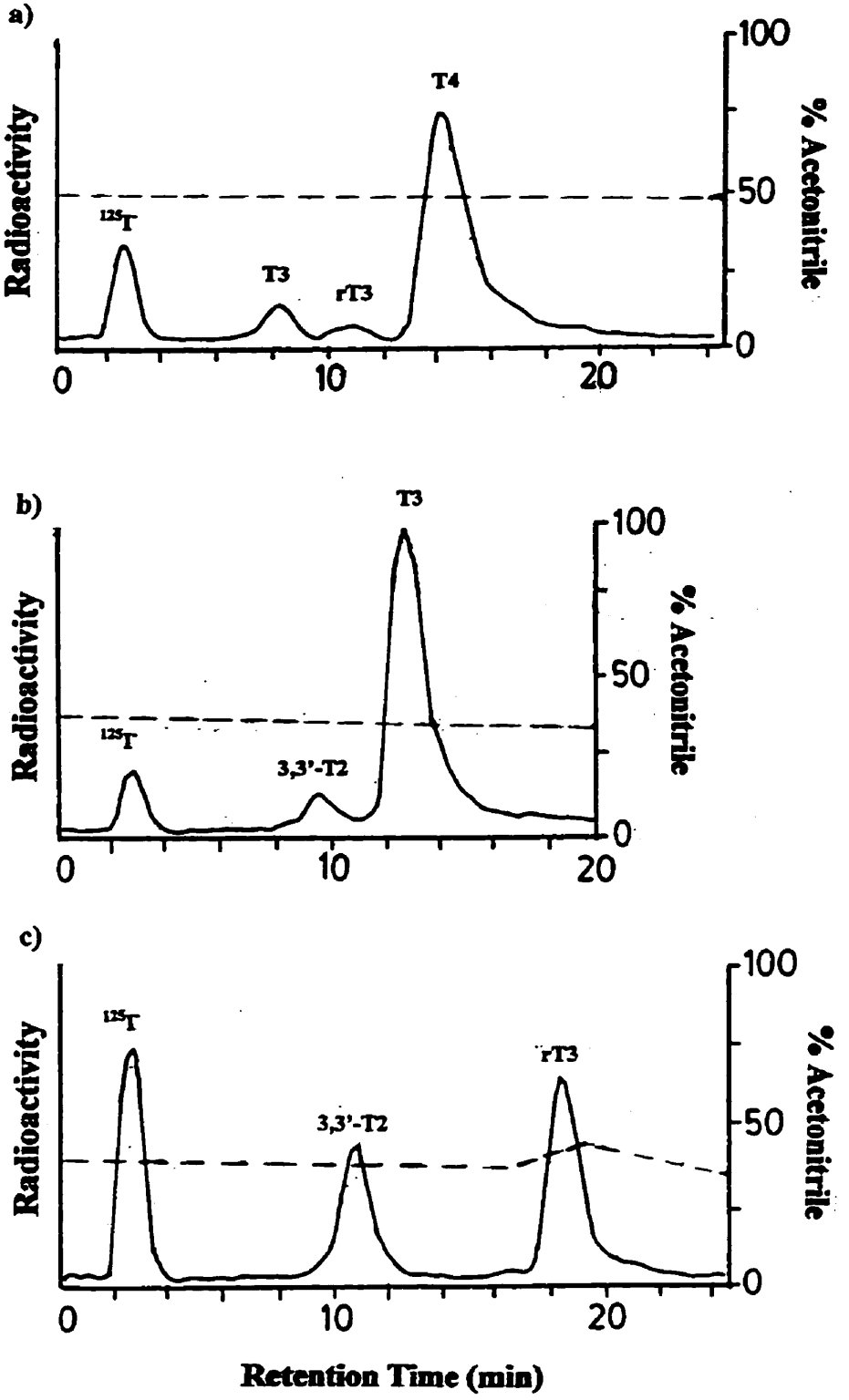

a)
Radioactivity
% Acetonitrile
100
50
0
^{125}T
T3
rT3
T4
0
10
20
b)
Radioactivity
% Acetonitrile
100
50
0
^{125}T
3,3'-T2
T3
0
10
20
c)
Radioactivity
% Acetonitrile
100
50
0
^{125}T
3,3'-T2
rT3
0
10
20
Retention Time (min)

Figure 4-2: The pH profiles of a) T4-ORD, b) T3-IRD c) rT3-ORD activities in sturgeon liver microsomes. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool. Results are expressed as the proportion of activity of the point with the greatest mean activity. The T4 assay was done with 0.8 nM substrate, and rT3 and T3 assays with 0.6 nM substrate. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p < 0.05$). Differences are indicated by lack of a similar letter over data points.

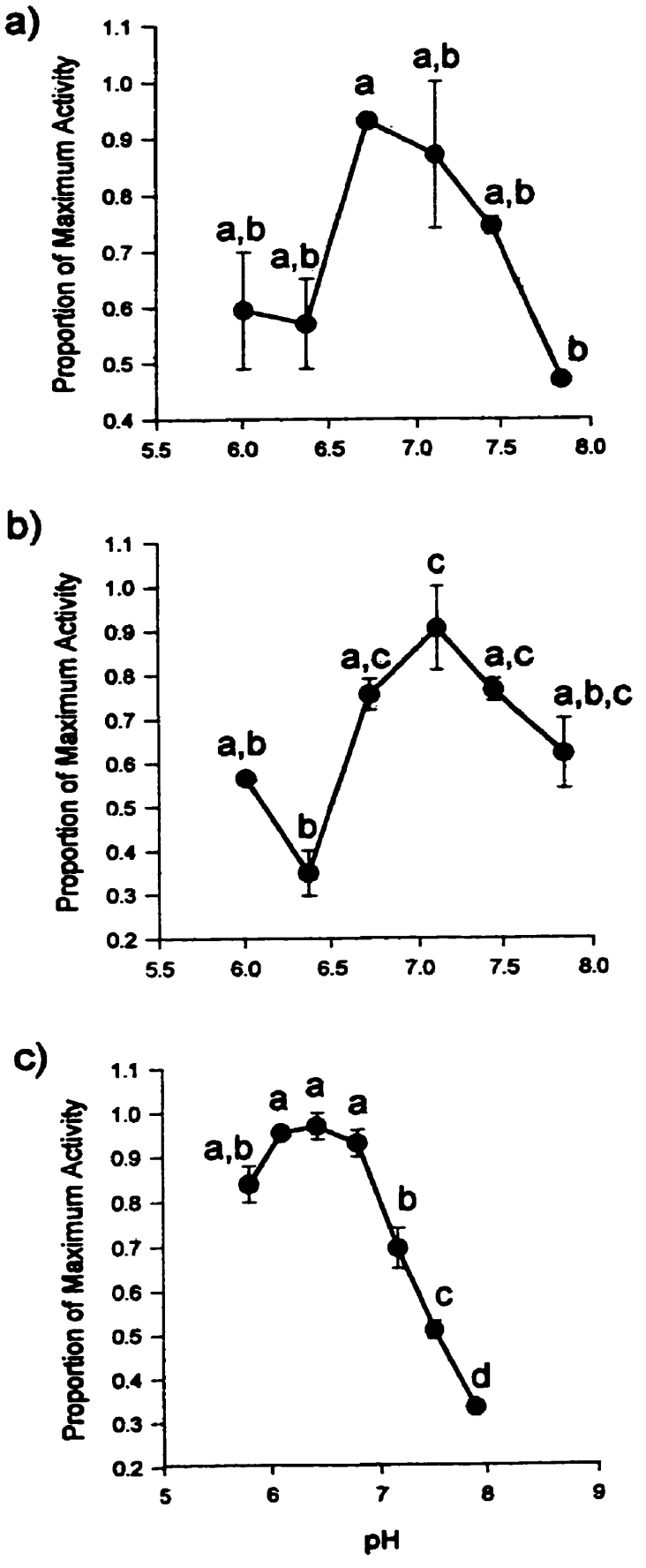

a)
Proportion of Maximum Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
5.5
6.0
6.5
7.0
7.5
8.0
a,b
a,b
a
a,b
a,b
b
b)
Proportion of Maximum Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
5.5
6.0
6.5
7.0
7.5
8.0
a,b
b
a,c
c
a,c
a,b,c
c)
Proportion of Maximum Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
5
6
7
8
9
pH
a,b
a
a
a
b
c
d

Figure 4-3: The effects of varying the concentration of DTT (dithiothreitol) on a) T4-ORD, b) T3-IRD, and c) rT3-ORD activities in liver microsomes. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool. Results are expressed as the proportion of activity of the point with the greatest mean activity. The T4 assay was done with 0.8 nM substrate, rT3 and T3 assays with 0.6 nM substrate. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p < 0.05$). Differences are indicated by lack of a similar letter over data points.

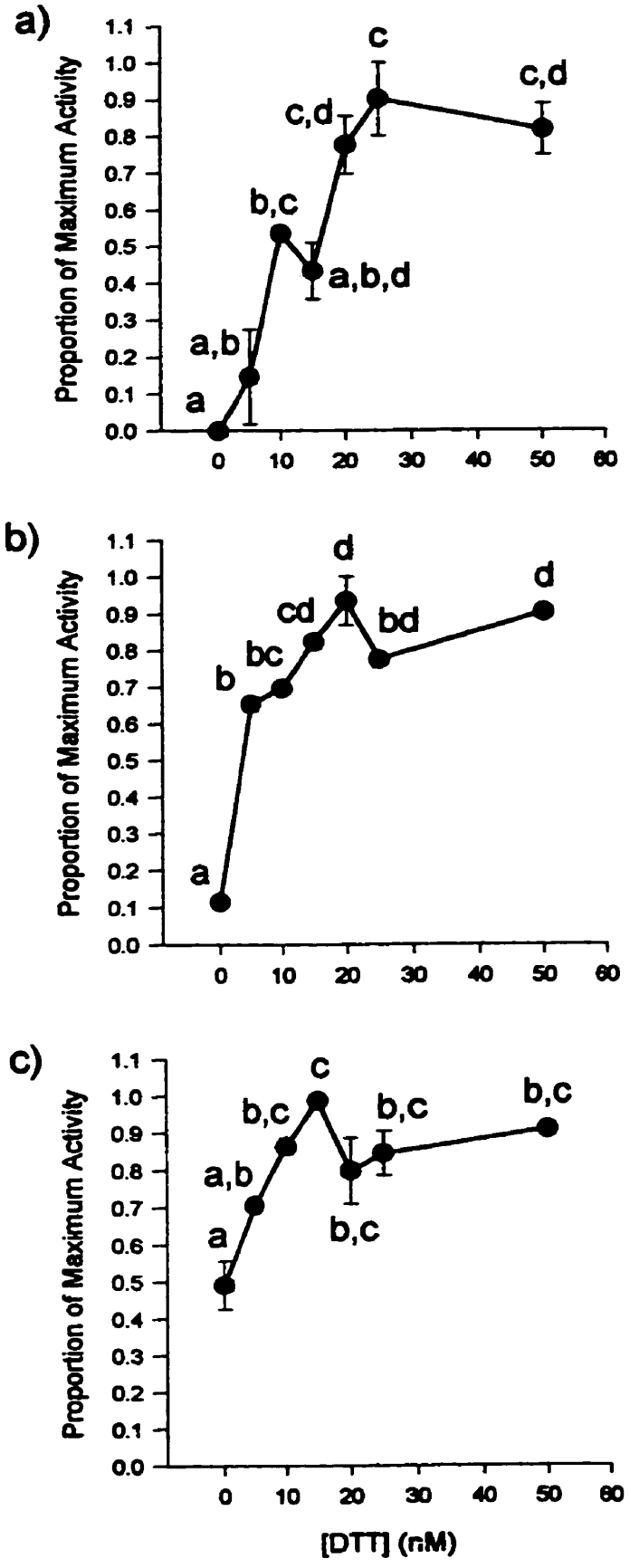

a)
Proportion of Maximum Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
a
a,b
b,c
c,d
c
a,b,d
c,d
b)
Proportion of Maximum Activity
a
b
bc
cd
d
bd
d
c)
Proportion of Maximum Activity
a
a,b
b,c
c
b,c
b,c
b,c
[DTT] (nM)

Figure 4-4: Lineweaver-Burke double reciprocal plot of T4-ORD activity. V = activity in pmoles T4 deiodinated / hr / mg protein; S = T4 substrate in nM. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool.

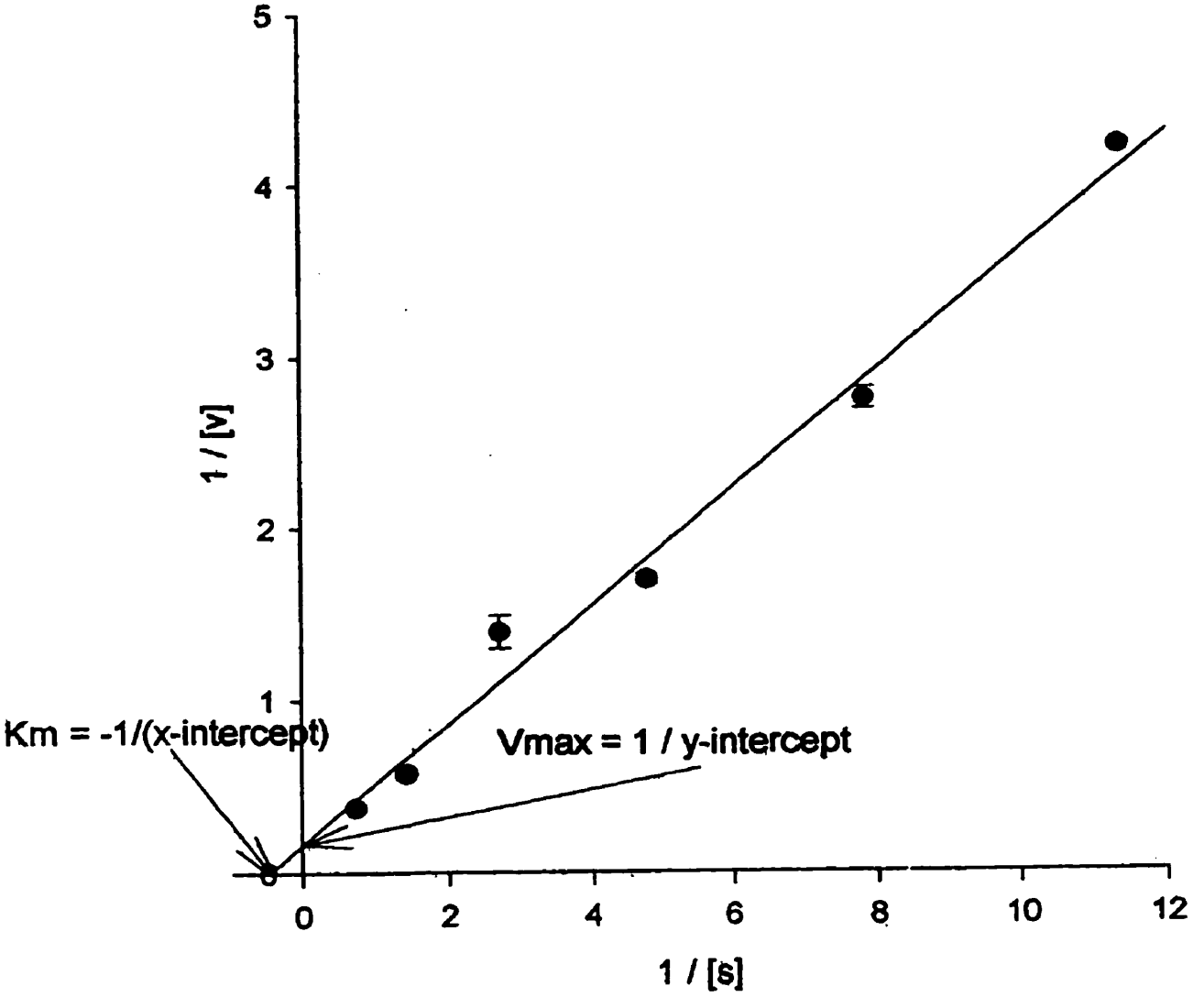
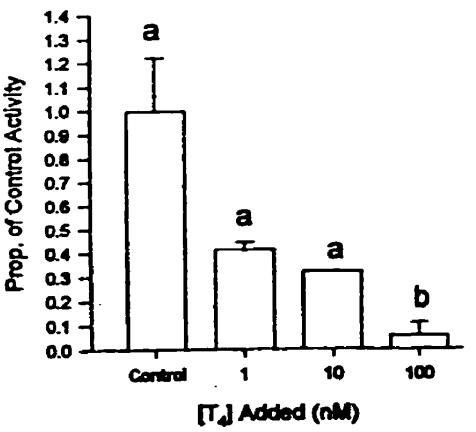
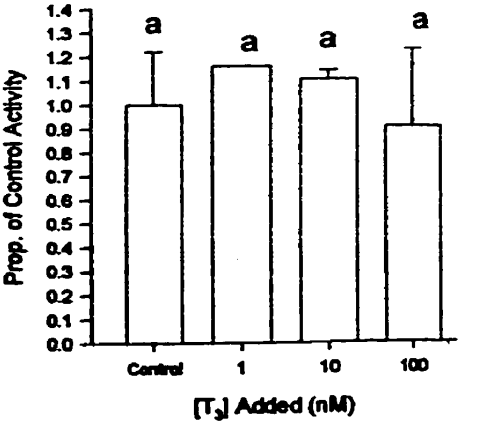
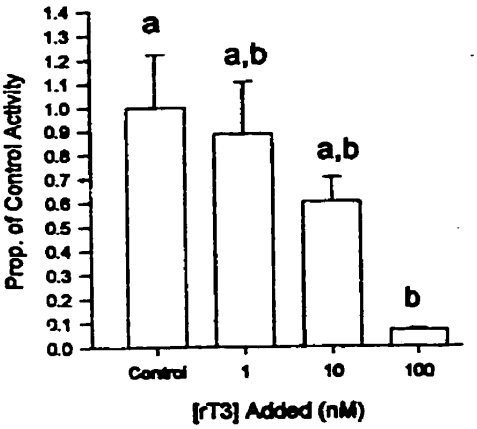
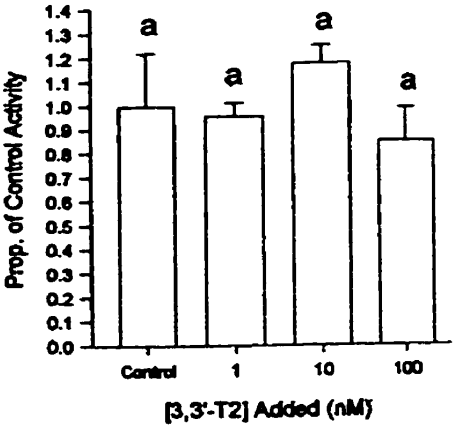

5
4
3
2
1
0
1 / [v]
Km = -1/(x-intercept)
Vmax = 1 / y-intercept
0
2
4
6
8
10
12
1 / [s]

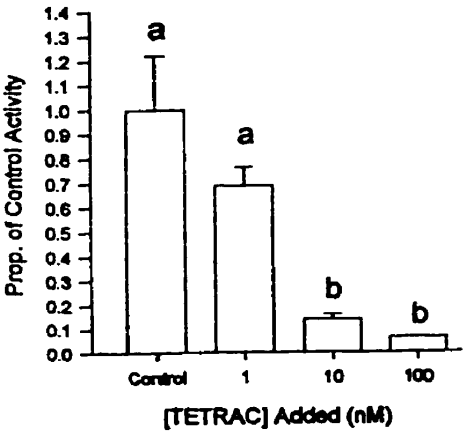
Figure 4-5: The effect of the addition of T4 and T4 analogs to microsomal incubates on the proportion of T4-substrate (0.8 nM) deiodinated by T4-ORD. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool. Results are expressed as the proportion of activity of the mean control activity which constitutes a microsomal incubate containing no added analog. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p \leq 0.05$). Differences are indicated by lack of a similar letter over a bar.


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
b
Control
1
10
100
[T4] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[T3] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a,b
a,b
b
Control
1
10
100
[rT3] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[3,3'-T2] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
b
b
Control
1
10
100
[TETRAC] Added (nM)

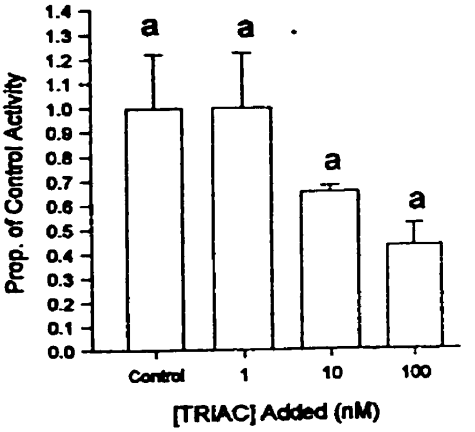
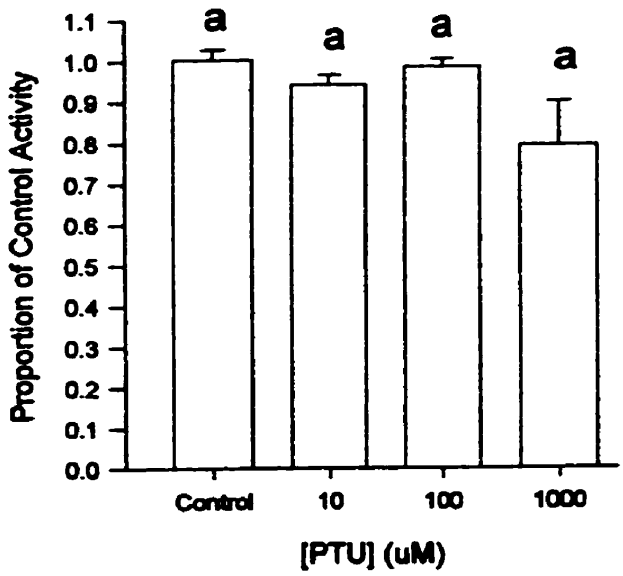
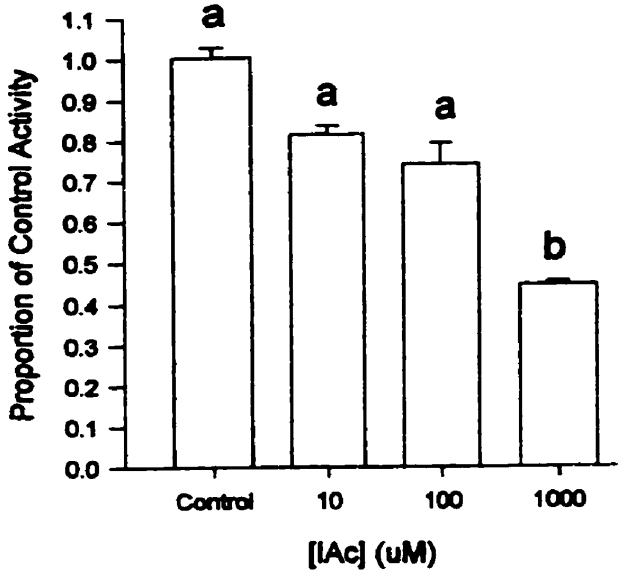

Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[TRIAC] Added (nM)

Figure 4-6: The effect of the addition of deiodination inhibitors to microsomal incubates on the proportion of T4-substrate (0.8 nM) deiodinated by T4-ORD. Each point represents the mean ± SEM of triplicate measurements from a common microsomal pool. Results are expressed as the proportion of the mean control activity which constitutes a microsomal incubate containing no added inhibitor. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p < 0.05$). Differences are indicated by lack of a similar letter over a bar.


Proportion of Control Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
10
100
1000
[PTU] (uM)


Proportion of Control Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
b
Control
10
100
1000
[IAc] (uM)

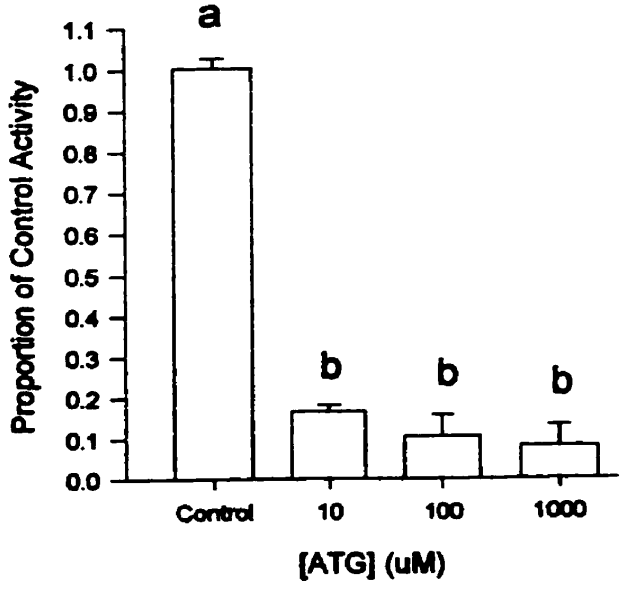
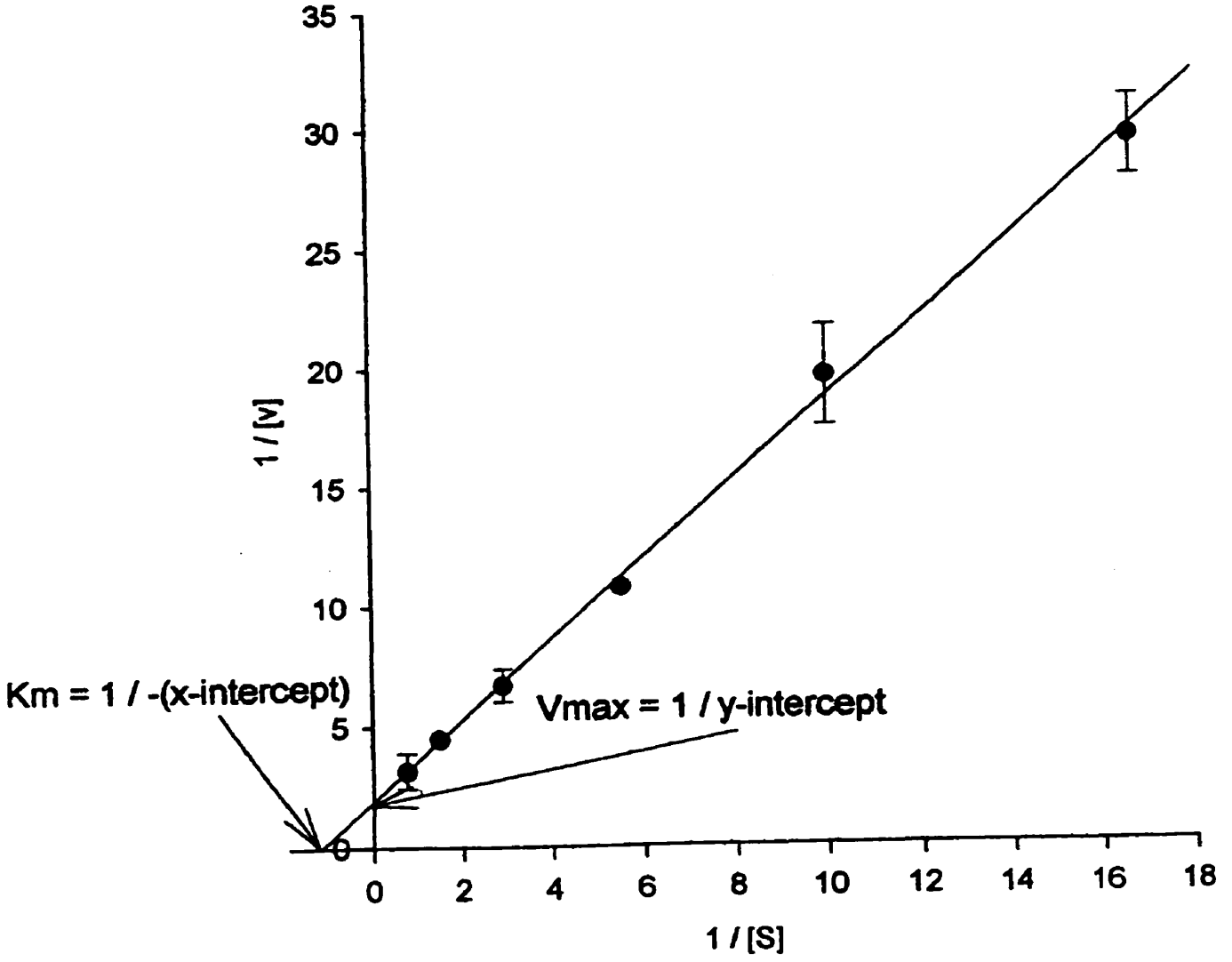

Proportion of Control Activity
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
b
Control
10
100
1000
[ATG] (uM)

Figure 4-7: Lineweaver-Burke double reciprocal plot of T3-IRD activity. V = activity in pmoles T3 deiodinated / hr / mg protein; S = T3 substrate in nM. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool.


35
30
25
20
15
10
5
0
1 / [V]
0
2
4
6
8
10
12
14
16
18
1 / [S]
Km = 1 / -(x-intercept)
Vmax = 1 / y-intercept

T3-IRD activity had a substrate preference of T3 > TRIAC > TETRAC > rT3 > T4 > 3,5-T2 as judged by the degree of inhibition of deiodination of labeled substrate by the addition of various thyroid hormone analogs at three concentrations (Fig. 4-8).

Propylthiouracil did not inhibit T3-IRD activity, iodoacetate mildly inhibited, and ATG almost completely inhibited (the enzyme's) activity (Fig. 4-9).

rT3-ORD

The rT3-ORD activity was the most active of the three deiodinations present in the liver as determined by the quantity of 3,3'-T2 produced using rT3 as a substrate (Fig. 4-1c).

Activity was maximal at a pH of 5.8 – 6.8 (Fig. 4-2c), and was stimulated maximally by 10 mM DTT (Fig. 4-3c).

Due to the extremely high saturation point of rT3-ORD, it was not possible to accurately characterize the kinetics of this deiodination pathway.

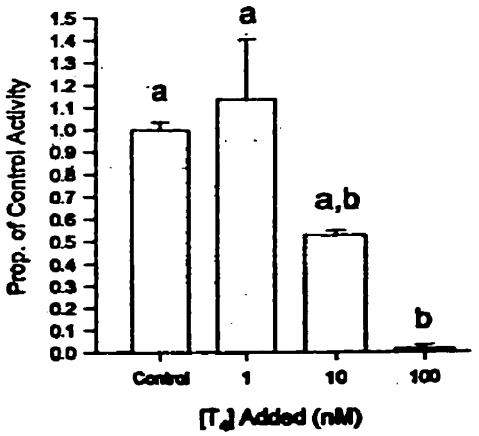
It was also not possible to determine the order of substrate preference for rT3-ORD as none of the analogs, including rT3 at the highest concentration tested (100 nM), reduced the proportion of labelled rT3 deiodinated to 3,3'-T2 (Fig. 4-10).

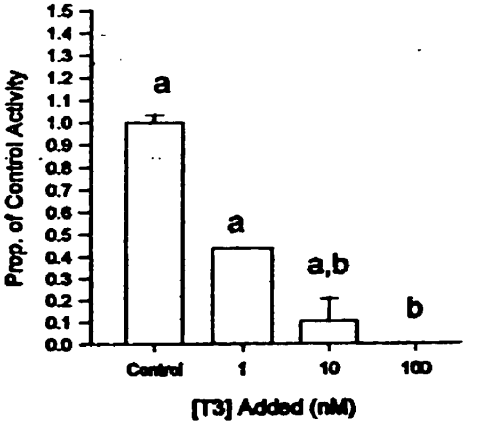
The selenocysteine inhibitor IAC affected rT3-ORD activity moderately, PTU had no effect and ATG almost completely eliminated activity at all concentrations (Fig. 4-11).

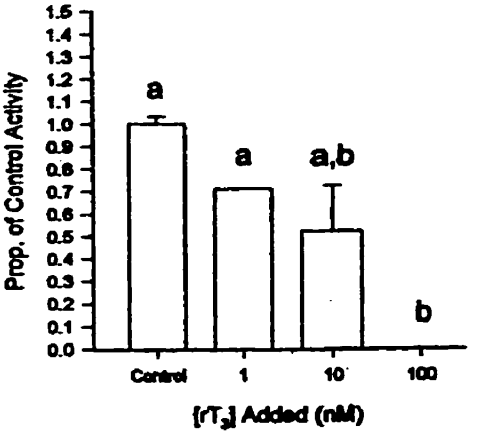
Temperature Affects on Deiodination

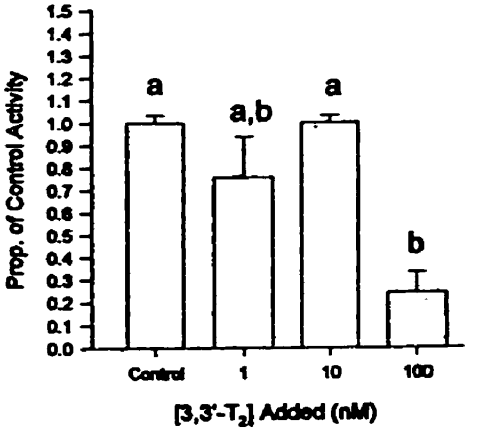
Enzyme activity for all deiodinases increased with incubation temperature to 32°C (Fig 4-12). At 39 °C T4-ORD, T4-IRD, rT3-ORD and T3-IRD activities decreased sharply. rT3-ORD activity did not change significantly at 39 °C compared to its activity recorded at 32 °C.

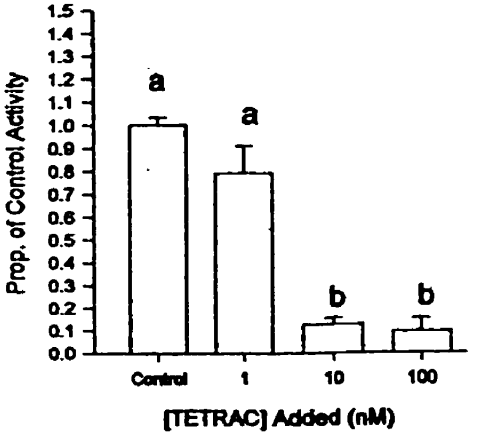
Figure 4-8: The effect of the addition of T3 and T3 analogs to microsomal incubates on the proportion of T3-substrate (0.6 nM) deiodinated by T3-IRD. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool. Results are expressed as the proportion of activity of the mean control activity which constitutes a microsomal incubate containing no added analog. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p < 0.05$). Differences are indicated by lack of a similar letter over a bar.


Prop. of Control Activity
a
a
a,b
b
Control
1
10
100
[T4] Added (nM)


Prop. of Control Activity
a
a
a,b
b
Control
1
10
100
[T3] Added (nM)


Prop. of Control Activity
a
a
a,b
b
Control
1
10
100
[rT3] Added (nM)


Prop. of Control Activity
a
a,b
a
b
Control
1
10
100
[3,3'-T2] Added (nM)


Prop. of Control Activity
a
a
b
b
Control
1
10
100
[TETRAC] Added (nM)

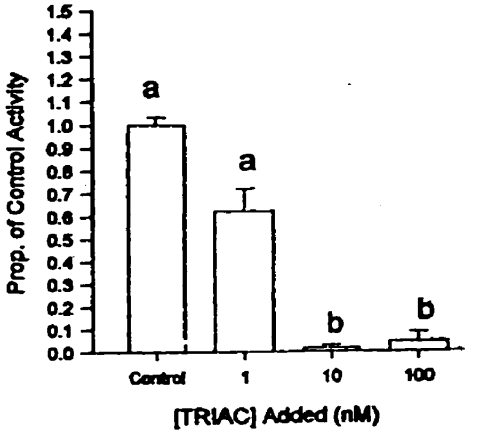
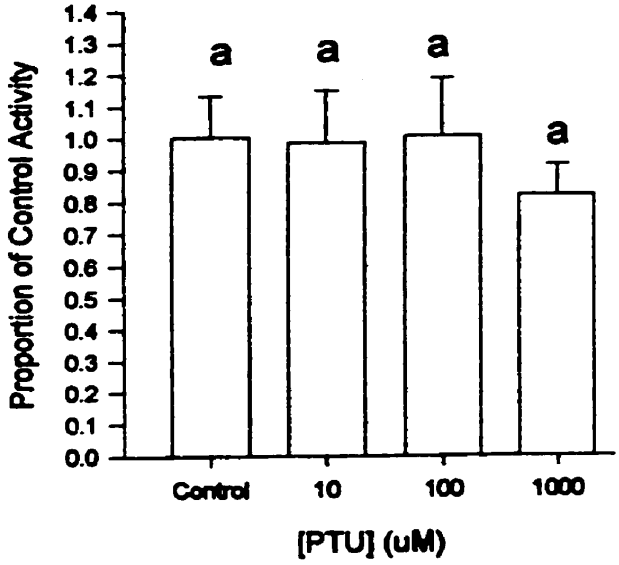
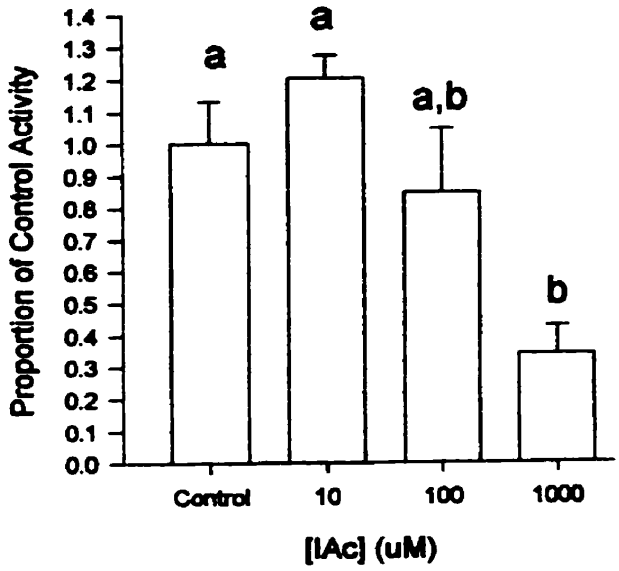

Prop. of Control Activity
a
a
b
b
Control
1
10
100
[TRIAC] Added (nM)

Figure 4-9: The effect of the addition of deiodination inhibitors to microsomal incubates on the proportion of T3-substrate (0.6 nM) deiodinated by T3-IRD. Each point represents the mean ± SEM of triplicate measurements from a common microsomal pool. Results are expressed as the proportion of the mean control activity which constitutes a microsomal incubate containing no added inhibitor. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p < 0.05$). Differences are indicated by lack of a similar letter over a bar.


Proportion of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
10
100
1000
[PTU] (uM)


Proportion of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a,b
b
Control
10
100
1000
[IAc] (uM)

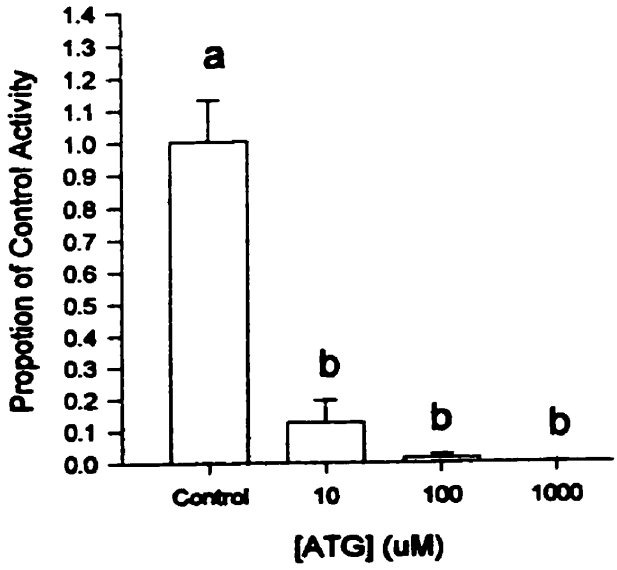
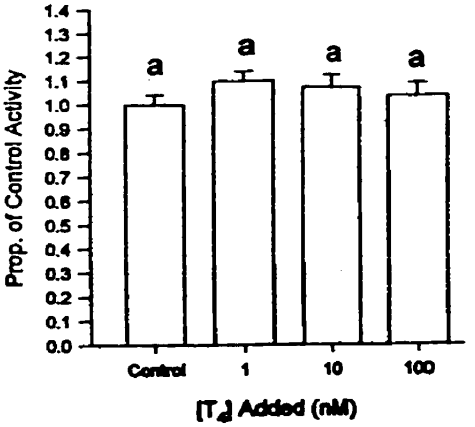
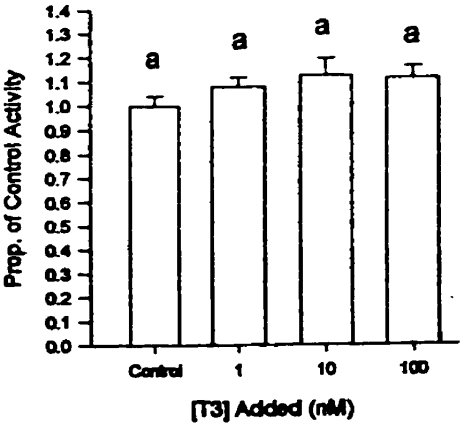
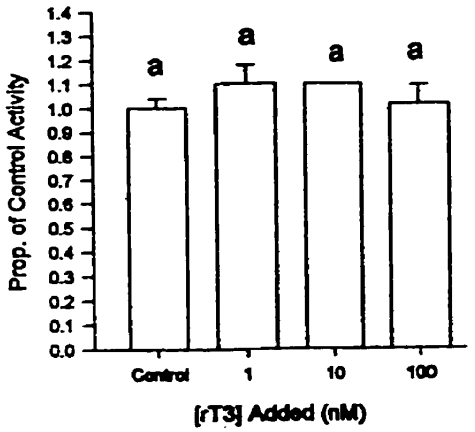
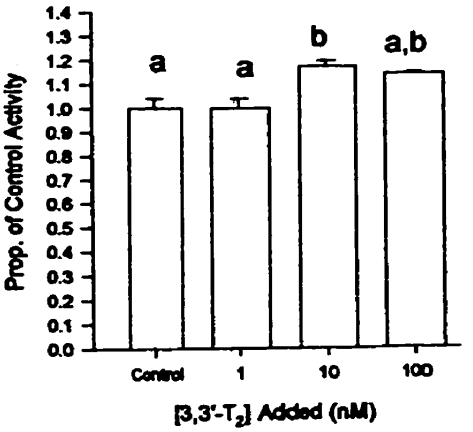

Propotion of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
b
Control
10
100
1000
[ATG] (uM)

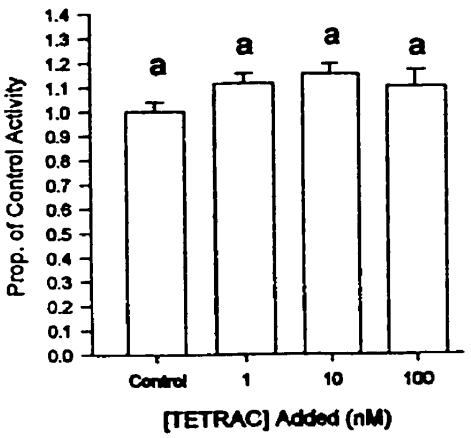
Figure 4-10: The effect of the addition of rT3 and rT3 analogs to microsomal incubates on the proportion of rT3-substrate (0.6 nM) deiodinated by rT3-ORD. Each point represents the mean ± SEM of duplicate measurements from a common microsomal pool. Results are expressed as the proportion of activity of the mean control activity which constitutes a microsomal incubate containing no added analog. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p \leq 0.05$). Differences are indicated by lack of a similar letter over a bar.


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[T4] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[T3] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[rT3] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
b
a,b
Control
1
10
100
[3,3'-T2] Added (nM)


Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[TETRAC] Added (nM)

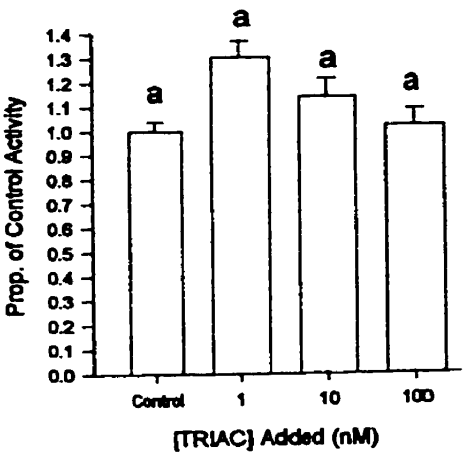
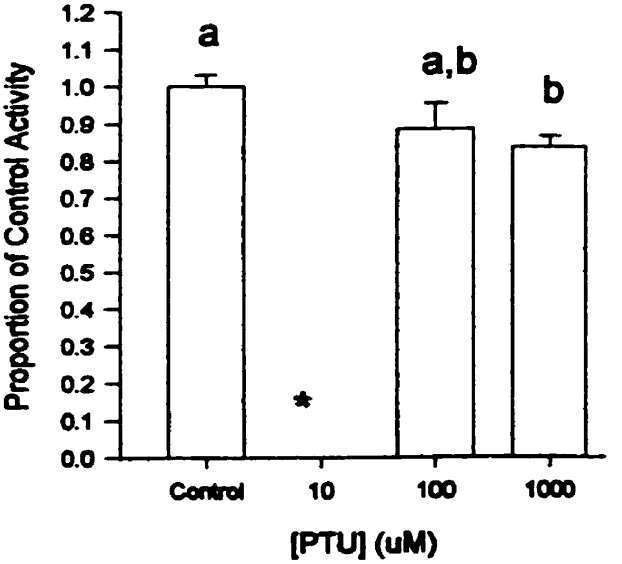
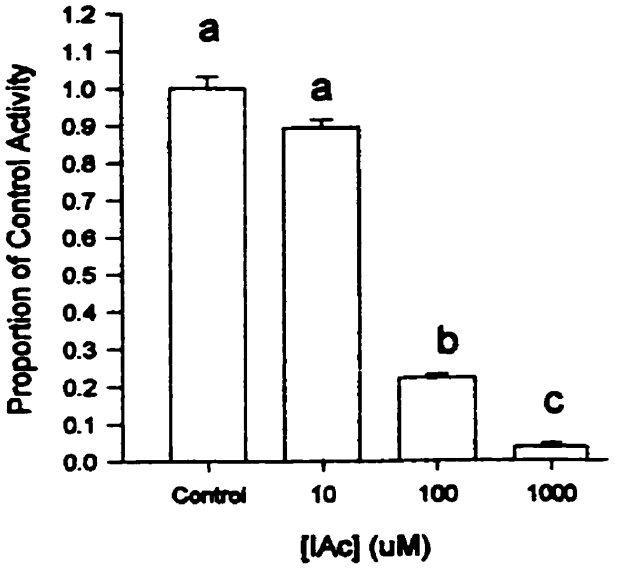

Prop. of Control Activity
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
a
a
Control
1
10
100
[TRIAC] Added (nM)

Figure 4-11: The effect of the addition of deiodination inhibitors to microsomal incubates on the proportion of rT3-substrate (0.6 nM) deiodinated by rT3-ORD. Each point represents the mean ± SEM of triplicate measurements from a common microsomal pool. Results are expressed as the proportion of the mean control activity which constitutes a microsomal incubate containing no added inhibitor. Differences between means were determined by one-way ANOVA followed by Tukey HSD tests to determine where differences exist within a set ($p < 0.05$). Differences are indicated by lack of a similar letter over a bar. * Value not available.


Proportion of Control Activity
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a,b
b
*
Control
10
100
1000
[PTU] (uM)


Proportion of Control Activity
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
b
c
Control
10
100
1000
[IAc] (uM)

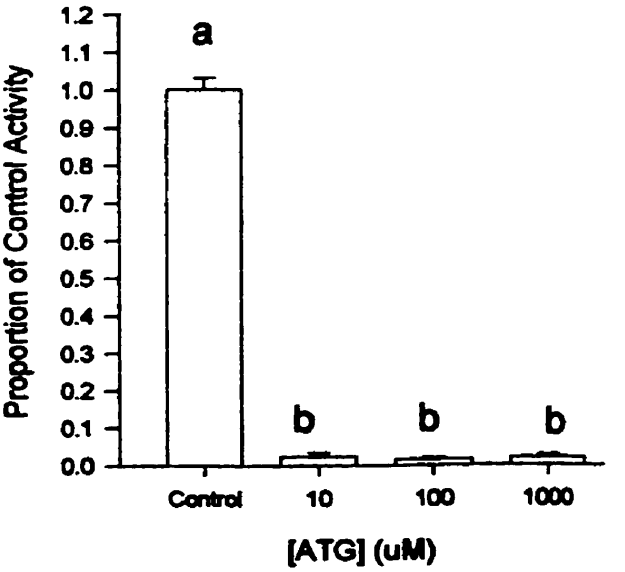
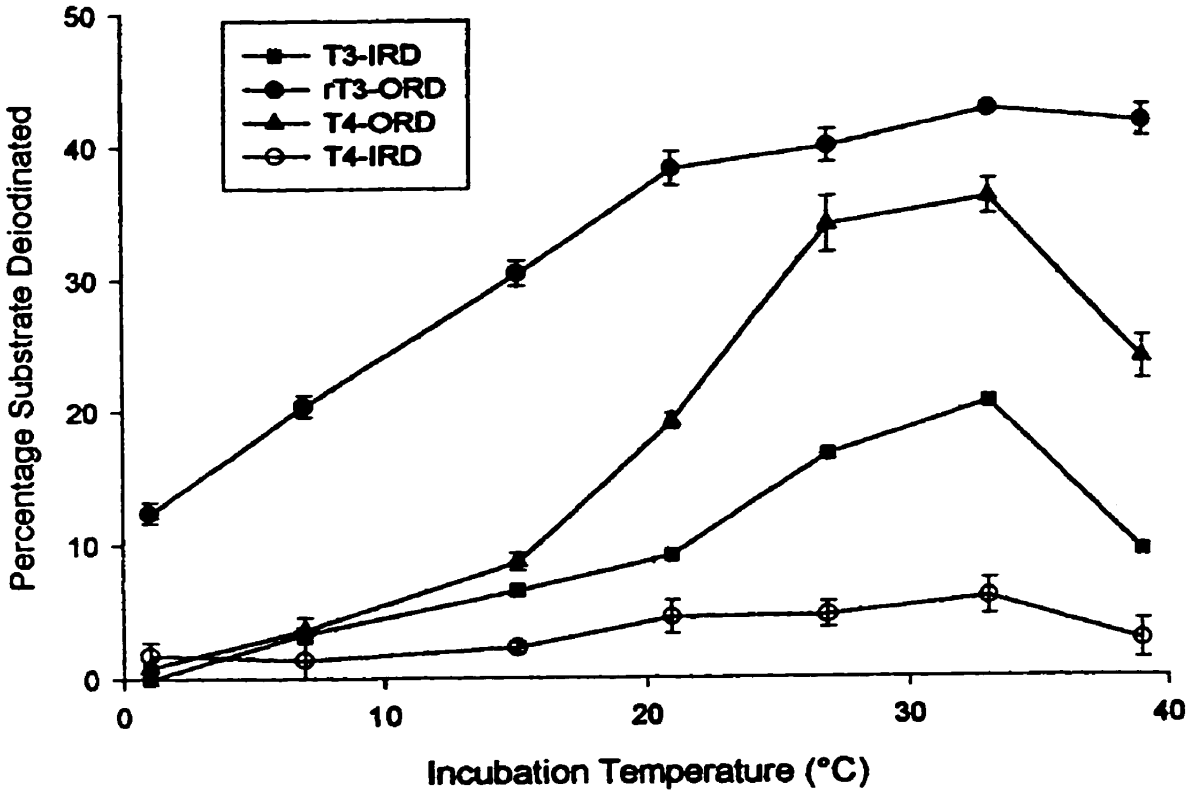

Proportion of Control Activity
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
b
b
Control
10
100
1000
[ATG] (uM)

Figure 4-12: The percentage of TH-deiodinated by liver microsomal T4-ORD, T4-IRD, rT3-ORD, and T3-IRD over a 38 °C-temperature range. Each point represents the mean ± SEM of triplicate measurements from a common microsomal pool. The T4 assay was done with 0.8 nM substrate, and the T3 and rT3 assays with 0.6 nM substrate.


T3-IRD
rT3-ORD
T4-ORD
T4-IRD
Percentage Substrate Deiodinated
Incubation Temperature (°C)
0
10
20
30
40
50

The temperature of the preincubation (30 min) preceding T4 and rT3 deiodination assays at 12 °C was varied to determine if there were differences in thermal sensitivity between the T4-ORD and rT3-ORD pathways. A difference would indicate that different enzymes catalyze the two outer-ring deiodination pathways. The profiles of the activities for the two pathways matched closely except at 45 °C where the T4-ORD activity was near zero and the rT3-ORD activity was at about 35 % of maximum activity (Fig. 4-13).

Sturgeon that were killed to study the deiodination properties were found to exhibit T4-ORD activities that changed with sampling time during the interval 9:13 to 16:09 (Fig. 4-14a). The fish sampled around 10:00 or earlier showed higher deiodination activity than those sampled later. This pattern matched that in another group of sturgeon sampled previously over a similar period (Fig. 4-14b) (My B.Sc. Hons. Thesis; Figure 19). Using this knowledge, the fish used in further experiments were sampled during a much shorter time interval, 09:00 to 10:30.

Discussion

Thyroid hormone deiodination pathways in lake sturgeon liver were characterized. This determined if similarities exist between sturgeon and other animal deiodinations.

I have previously (B.Sc. Hons. Thesis) examined deiodination activity in several lake sturgeon tissues (liver, lower intestine, upper intestine, head kidney, brain, notochord, muscle, and immature gonad). Of these, the liver contained the greatest deiodination activity followed by the lower and upper intestines. These tissues were

Figure 4-13: **T4-ORD and rT3-ORD activity of microsomal incubates preincubated at varying temperatures for 30 minutes before addition of substrate and incubation of all samples at 12 °C. Each point represents the mean ± SEM of duplicate measurements. A substrate concentration of 0.8 nM was used in the T4 assay and 0.6 nM used in the rT3 assay.**

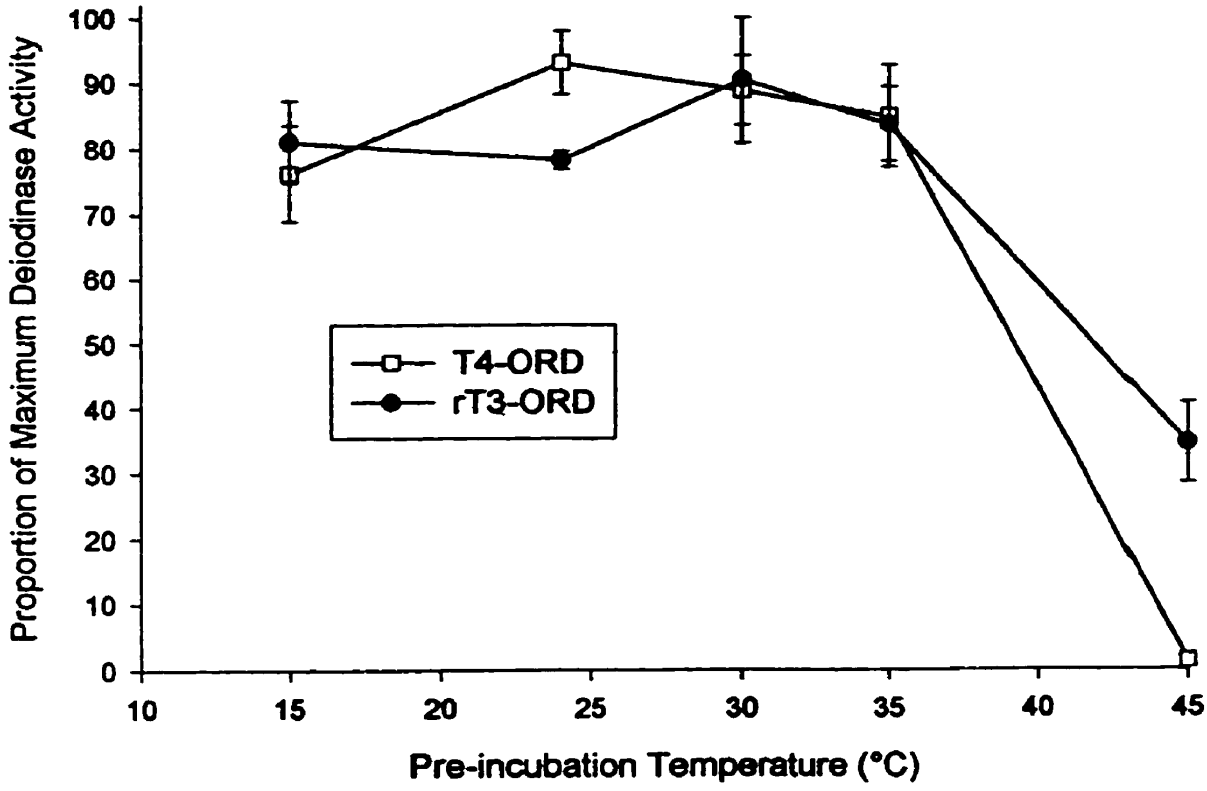
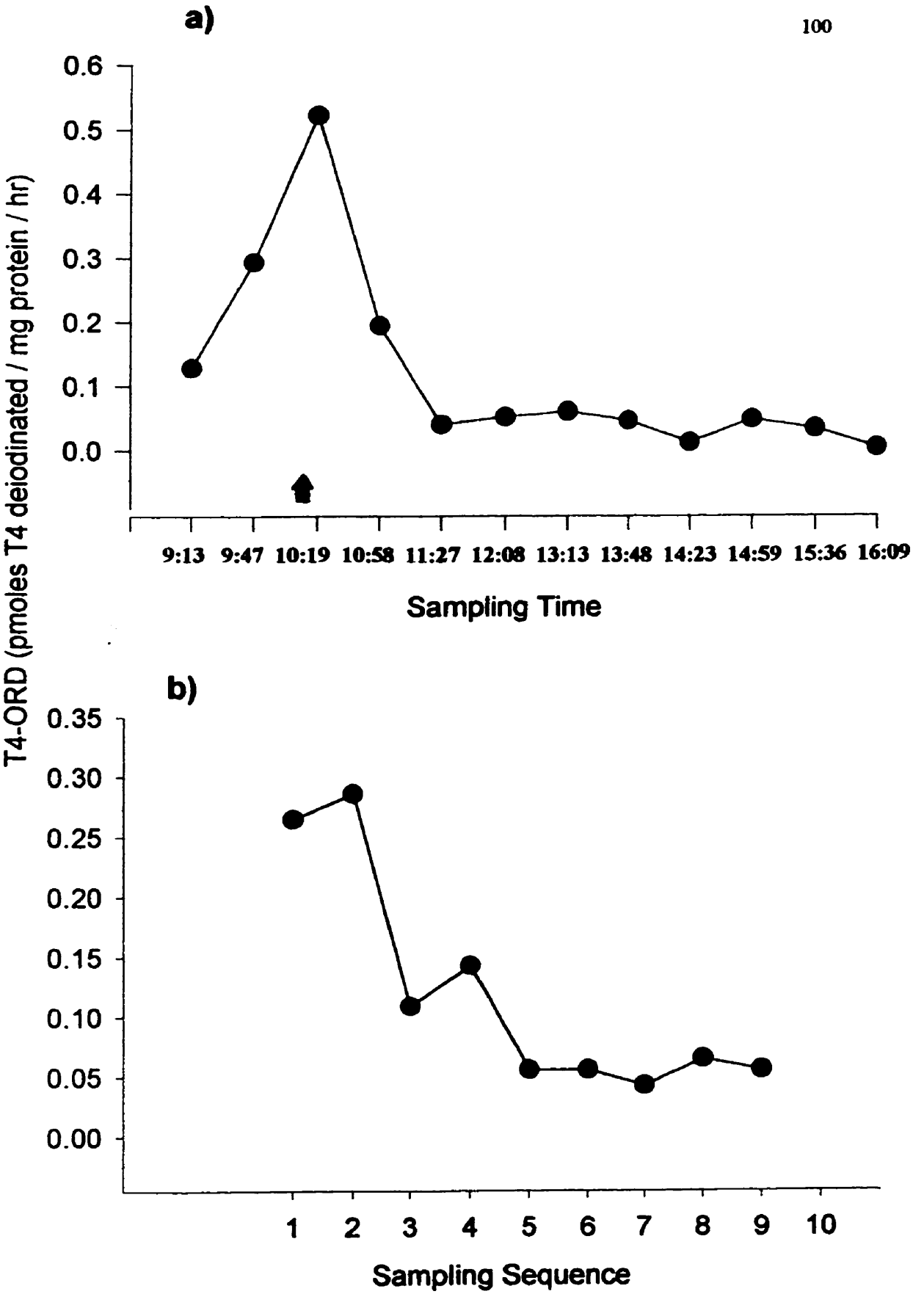

Proportion of Maximum Deiodinase Activity
100
90
80
70
60
50
40
30
20
10
0
T4-ORD
rT3-ORD
10
15
20
25
30
35
40
45
Pre-incubation Temperature (°C)

Figure 4-14: a) Time course of T4-ORD activity in liver microsomal incubates from laboratory-raised lake sturgeon killed at different times over a period of seven hours on May 7, 1997. Each point represents the hepatic deiodination activity in duplicate of a single fish killed at the time indicated (⬆ - arrow indicates time of feeding).
b) Time course of T4-ORD activity in liver microsomal incubates from a group of laboratory-raised lake sturgeon killed over a similar time period as in a) on September 2, 1996 (These data are from my Honors thesis). Each point represents the hepatic deiodination activity in duplicate of single fish. A substrate level of 0.8 nM was used in the incubations.


a)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
T4-ORD (pmoles T4 deiodinated / mg protein / hr)
9:13 9:47 10:19 10:58 11:27 12:08 13:13 13:48 14:23 14:59 15:36 16:09
Sampling Time
b)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1 2 3 4 5 6 7 8 9 10
Sampling Sequence

therefore selected for further study along with the thyroid, which was chosen due to its potential importance as the centre for production of thyroid hormone.

T4-ORD

The liver contained approximately 2 X greater T4-ORD activity than the lower intestine, followed by the thyroid and finally the upper intestine. The T4-ORD deiodinase pathway is found in several other fish species. It occurs in the liver and brain of rainbow trout (MacLatchy and Eales, 1992; Frith and Eales, 1996), Atlantic salmon liver, heart, gill, brain and skeletal muscle (Morin et al, 1993), larval sea lamprey liver and intestine (Eales et al, 1997), as well as in tilapia (Mol et al, 1997), and Atlantic cod liver (Cyr et al, 1998). It is most similar to type II mammalian deiodination (Leonard and Visser, 1986).

Significant deiodination in the liver of the sturgeon as in other fish highlights the importance of the liver as a metabolic centre for control of thyroid hormone metabolism. Blood passes through the liver via the hepatic portal system before going to other parts of the body. This allows the hepatic deiodinases to regulate the quantity of hormone that passes to the rest of the body tissues. The liver was used to characterize the T4-ORD, as well as the other tissue deiodination pathways in the lake sturgeon.

The T4-ORD activity functioned at an optimum pH of 6.0 – 7.5. This value is similar to the optimum pH of the deiodination pathway in rainbow trout brain (7.3) (Frith and Eales, 1996) and liver (7.0) (Shields and Eales, 1986; MacLatchy and Eales, 1992), larval sea lamprey liver and intestine (7.3) (Eales et al, 1997), and tilapia liver (6.5-7.5) (Mol et al, 1997). Optimal activity in this pH range shows that the T4-ORD and other deiodination pathways function best in the likely physiological pH range of the sturgeon.

Maximal T4-ORD activity occurred at a DTT concentration of 10 mM in the lake sturgeon. This is similar to the concentration required to produce maximum deiodination in the rainbow trout brain (10 mM); (Frith and Eales, 1996); and liver (10- 11 mM); (MacLatchy and Eales, 1992; Shields and Eales, 1986), larval sea lamprey liver (5 mM); (Eales et al, 1997), and tilapia liver (25-30 mM); (Mol et al, 1997). The ability of DTT to increase deiodination activity shows its importance as a cosubstrate in the deiodination reaction mechanism. At the optimum DTT concentration, the deiodinase enzymes would have the required amount of DTT for reduction of the enzymes back to their active form at the maximum rate.

Enzyme kinetic analysis using a Lineweaver-Burke double-reciprocal plot revealed a K_m of 6.7 nM and a V_{max} of 26.1 pmoles T4 deiodinated / mg protein / hr. The sturgeon K_m was higher but still close to the values obtained for this deiodination pathway in rainbow trout brain (1.2-2.5 nM) (Frith and Eales, 1996), liver (0.098 nM) (MacLatchy and Eales, 1992), Atlantic salmon liver (0.42 nM) (Morin et al, 1993), upstream migrant sea lamprey intestine (1.3 nM) (Eales et al, 1997), and tilapia liver (0.75 - 1.3 nM) (Mol et al, 1993; Mol et al, 1997). The V_{max} was also higher than the values obtained for this deiodination pathway in rainbow trout brain (0.10- 0.14 pmol) (Frith and Eales, 1993) and liver (3.74 pmol) (MacLatchy and Eales, 1992), Atlantic salmon liver (1.2 pmol) (Morin et al, 1993), upstream migrant sea lamprey intestine (0.35pmol), and tilapia liver (0.13 – 0.21pmol) (Mol et al, 1993; Mol et al, 1997). The much higher Vmax in the sturgeon liver shows that there is a much greater quantity of the enzyme present in the sturgeon liver than in other species.

Lake sturgeon T4-ORD had a substrate preference of T4 > TETRAC > rT3 > TRIAC > T3 = 3,5-T2. Those THs with two iodines on their outer-ring are preferred as substrates. This resembles the substrate preference of the T4-ORD pathway in rainbow trout liver and brain (Frith and Eales, 1993), larval sea lamprey intestine and liver (Eales et al, 1997), and tilapia liver (Mol et al, 1997), and Atlantic cod liver (Cyr et al, 1998).

When potential deiodination inhibitors were added to the incubates, they had varied effects on T4-ORD activity. Propylthiouracil did not inhibit T4-ORD activity, iodoacetate mildly inhibited, and ATG almost completely inhibited activity. This agreed with the results obtained for rainbow trout brain and liver (Frith and Eales, 1993), upstream migrant sea lamprey intestine (Eales et al, 1997), tilapia liver (Mol et al, 1993), and Atlantic cod liver (Cyr et al, 1998). The much stronger inhibition by aurothioglucose than propylthiouracil suggests that the T4-ORD may work through a sequential reaction mechanism with DTT rather than a ping-pong mechanism. PTU inhibits activity by binding to the selenolate iodide form of the enzyme which would occur in a ping-pong reaction mechanism after the enzyme removes an iodine from the TH. However, in a sequential reaction mechanism the deiodinase, thiol cofactor, and TH substrate all bind together before deiodination takes place meaning there would not be a free selenolate iodide form of the enzyme to which PTU could bind.

Higher T4-ORD activity occurred in the liver of sturgeon sampled earlier in the day (before 10:30) compared to those sampled later. The increased activity may involve anticipation of feeding as the fish were fed at around 10:15 every day. Increased T4-ORD around the time of feeding would increase the quantity of T3 in the tissues during a time of nutrient intake.

Daily melatonin cycles associated with the photocycle may also be involved in producing the cycle in T4-ORD activity. This possibility would require further study and has yet to be reported for any species.

A daily cycle in deiodination activity has not previously been reported for any fish species. However, diel variations in plasma T3 levels have been reported in rainbow trout in response to feeding (Eales et al, 1981). A detectable change in plasma THs did not accompany the cycle in liver deiodination in the sturgeon.

T4-IRD

The upper intestine contained the greatest T4-IRD activity with more than 3 X the activity found in the thyroid, lower intestine and liver. This deiodination pathway was not characterized, as the liver microsomes did not contain a sufficient amount of activity. The upper intestine could have been used but significant T4-IRD activity in this tissue was not known at the time the deiodination pathways were characterized.

T3-IRD

The liver contained the greatest T3-IRD activity, followed closely by the upper intestine. The thyroid and lower intestine contained almost undetectable levels of T3-IRD activity. This deiodination pathway is found in several other fish species. It occurs in rainbow trout brain (Frith and Eales, 1996), Atlantic salmon liver (Morin et al, 1993), liver of coho salmon (Sweeting et al, 1994), and resembles type III mammalian deiodinase (Leonard and Visser, 1986). The liver was used to characterize this deiodination pathway in the lake sturgeon.

Optimum T3-IRD activity occurred at a pH of 6.7 - 7.8. This matches closely the optimum for T3-IRD in rainbow trout (7.0) (Frith and Eales, 1996), and tilapia brain (6-7) (Mol et al, 1997).

Lake sturgeon T3-IRD increased with DTT concentration to a maximum at 15 mM DTT. This is a little higher than that required by the T3-IRD pathway in rainbow trout brain (10 mM) (Frith and Eales, 1996), and a little lower then that for tilapia brain (20mM) (Mol et al, 1997).

T3-IRD activity had a substrate preference of T3 > TRIAC > TETRAC > rT3 > T4 > 3,5-T2. This is similar to the results obtained for T3-IRD in the brain of rainbow trout (Frith and Eales, 1996), as well as in tilapia brain and gill (Mol et al, 1997). Those substrates with two iodines in their inner-ring interfere with the pathway to the greatest extent.

When potential inhibitors of deiodination were added to the T3-incubates they had varied effects. Propylthiouracil did not inhibit T3-IRD activity, iodoacetate mildly inhibited, and ATG almost completely inhibited the enzymes activity. Inhibition by aurothioglucose but not propylthiouracil suggests that T3-IRD may operate through a sequential reaction mechanism for the same reasons discussed for the T4-ORD pathway.

Kinetic analysis using a Lineweaver Burke double-reciprocal plot revealed a K_m of 3.3 nM and a V_{max} of 1.3 pmoles T3 deiodinated / hr / mg protein. The Km is a little lower than those reported in rainbow trout brain but the Vmax falls within the range previous reported for the trout brain (K_m = 5.2-5.4 nM; V_{max} = 1.06-1.97 pmoles T3 deiodinated / hr / mg protein) (Frith and Eales, 1996).

rT3-ORD

The liver was used to characterize the rT3-ORD deiodination as significant rT3-ORD activity was found to exist in this tissue. The rT3-ORD activity in the liver, was the most active of the three deiodination pathways characterized. This deiodination pathway also occurs in the rainbow trout liver (Finnson et al, 1999) and tilapia kidney (Mol et al, 1997) and is similar in certain respects to type I mammalian deiodination (Leonard and Visser, 1986)

Optimum rT3-ORD activity occurred at a pH of 5.8 – 6.8. This is similar to the optimum for rT3-ORD in the tilapia kidney (6-7) (Mol et al, 1997), and rainbow trout liver (7.0) (Finnson et al, 1999).

Lake sturgeon rT3-ORD activity occurred optimally at a DTT concentration of 10 mM. This is the same as the optimal DTT concentration of tilapia kidney rT3-ORD (10 mM) (Mol et al, 1993; Mol et al, 1997), and is a little higher than the optimum rainbow trout liver rT3-ORD (2.5 mM) (Finnson et al, 1999). The results show that DTT is required in the reaction mechanism as a cosubstrate as with other fish deiodinases.

It was not possible to determine the order of substrate preference for rT3-ORD as none of the analogs, or rT3, at the highest concentration tested (100 nM), reduced the proportion of labelled rT3 deiodinated to 3,3'-T2. Mol et al (1997) determined a substrate preference of rT3 > T4 > T3 for tilapia rT3-ORD activity when using analog concentrations of at least 1 micromolar. In a natural physiological situation, the rT3-ORD deiodinase would be never exposed to concentrations of thyroid hormones as high as those required to saturate the enzyme.

Adding deiodinase inhibitors to the incubations of rT3 substrate and liver microsomes had similar effects on rT3 deiodination as it did for T4 and T3 deiodination. The selenocysteine inhibitor IAC, affected rT3-ORD activity moderately, PTU had no effect and ATG almost completely eliminated activity at all concentrations. Similar results were obtained for kidney rT3-ORD activity in tilapia (Mol et al, 1997). As with the T4-ORD and T3-IRD pathways, the results suggest a possible sequential reaction mechanism.

The extremely high saturation level of this enzyme made determination of the enzyme kinetics of rT3-ORD in the sturgeon liver impossible. Substrate concentrations as high as 5 μM were used with no significant change in proportion of substrate deiodinated than when using only 0.00015 micromolar rT3. Therefore, a rT3-ORD with an extremely high saturation level exists in the liver. High-K_m rT3-ORD activity occurs in tilapia kidney (K_m = 0.33 - 1.25 μM; V_{max} = 31 – 96 pmol) (Mol et al, 1993; Mol et al, 1997).

Tissue Distribution of Deiodination

The lake sturgeon intestine showed significant deiodination activity. Intestinal deiodination is negligible in salmonids and mammals (Eales and Brown, 1993). Of the fish examined to date, only phylogenetically primitive fish like the lamprey and hagfish have significant intestinal deiodination activity (Eales et al, 1997). Intestinal deiodination is also significant in frogs during their metamorphosis from tadpole to adult as TH is important in the development of the gut at this stage (Shi, 1995).

Evolving vertebrates may have developed a use of the TH in their bodies before they developed the capacity to produce it themselves. Most organisms may have first

been exposed to TH through consumption of early TH producing organisms. This first TH source may have been marine algae which contain THs and are believed to produce TH using solar energy (Chino et al, 1994).

In this early environment, deiodination activity in the intestine would have been advantageous to regulate the amount and form of TH entering the blood stream. As they developed the capacity to produce thyroid hormone themselves, organisms may then have begun to confer deiodination activity to the more centralized and metabolically active liver tissue which is the main deiodination centre in more recently evolved vertebrates like teleosts and mammals.

Lake sturgeon consume a lot of benthic invertebrates and also scavenge on dead fish which may be a source of THs. Their entrance into the body across the intestinal wall could be regulated by the intestinal deiodination. Since blood from the intestine passes through the liver before entering the systemic circulation, it would also be advantageous to have high deiodination activity in the liver, which is also the case in the sturgeon.

The presence of significant thyroidal T4-ORD activity is understandable given the significantly higher concentrations of T3 than T4 in the sturgeon tissue. This deiodination pathway would convert the T4 produced in the thyroid to T3 before release of the hormone into the blood.

Temperature Effects on Deiodination

Varying the incubation temperature of deiodination led to a sharp increase in T4-ORD, T4-IRD, T3-IRD and rT3-ORD activity with increasing temperature until greater

than 32 °C. These temperatures exceed those to which the sturgeon would be exposed in a natural situation (≤ 15 °C) and also exceed their upper lethal temperature (~25 °C).

Blue tilapia (*Oreochromis aureus*) deiodinase enzymes also function optimally at temperatures greater than the lethal temperature (Mol et al, 1997). Its T4-ORD is optimal at 37 °C even though its upper lethal temperature is 30 °C. The tilapia's optimum T4-ORD temperature is equivalent to the normal body temperature of an endotherm, which is the maximal temperature for the mammalian deiodinase. Temperature optima of deiodinases have not been studied in most other fish. However, in Atlantic cod (*Gadus morhua*), the T4-ORD activity increased significantly through the temperature range from 1 to 12 °C (Cyr et al, 1998). Rainbow trout outer-ring and inner-ring deiodinase activities also increased steadily through a temperature range from 4 to 18 °C (Johnston and Eales, 1995). These temperature-dependant increases in deiodination were due to an increasing enzyme substrate affinity (decreasing K_m) of the deiodinase enzymes.

The thermal sensitivities of the two outer-ring deiodination pathways studied were compared to determine if the same enzyme catalyzes both of these deiodinations. Both T4-ORD and rT3-ORD activity varied approximately the same as pre-incubation temperature was varied except at 45 °C where the T4-ORD activity was almost completely eliminated but the rT3-ORD activity remained at about 35 % of its maximum activity. This shows that the rT3-ORD pathway is less sensitive to temperature induced denaturation at higher temperatures (~ 45 °C) than the T4-ORD pathway and suggests that the two deiodination pathways may be catalyzed by different deiodinase enzymes.

Conclusions

The characteristics of lake sturgeon deiodination were comparable in several respects to those in other fish and mammals. The activities of lake sturgeon hepatic T4-ORD, T3-IRD, and rT3-ORD are stimulated by DTT, inhibited by ATG, and IAC but not PTU inhibitors, and have pH optima, temperature sensitivies, substrate preferences and enzyme kinetics similar to deiodination in other fish and mammals.

From analysis of the various deiodination activities in lake sturgeon tissue microsomes, it was determined that significant T4-ORD activity exists in the liver and less so in the lower intestine and thyroid. Clearly the low TH levels in the blood of the lake sturgeon are not related to a lack of capacity to convert T4 to T3 in the peripheral tissues. Significant inner-ring deiodination of T4 occurs in the upper intestine and less so in the thyroid, lower intestine, and liver. Inner-ring deiodination of T3 is most active in the liver and upper intestine. This activity may reduce the quantity of T3 present in these tissues by deiodinating any T3 produced. Significant T4-ORD producing T3 occurs in the thyroid tissue with very low T3-IRD breaking down T3. This suggests that T4 produced in the thyroid gland may be converted to T3 before release into the blood stream. Significantly higher concentrations of T3 than T4 in the thyroid gland extracts support this theory.

High deiodination activity in the intestine of the sturgeon suggests that this tissue is an important centre for control of thyroid hormone levels in the body. Intestinal deiodinase activity is not significant in salmonids (Eales and Brown, 1993). The intestine is an important deiodinating centre in more primitive animals like the lamprey (Eales et al, 1997). In these animals, the intestine contains the greatest deiodinase activity in the

body with little activity in the liver. This seems to suggest that in an evolutionary context, the intestine was originally the site of thyroid hormone deiodinating control but through time more advanced organisms like teleosts and mammals shifted control of this process to the liver, which is a more metabolically active centralized organ.

In early evolutionary history, animals may have utilized thyroid hormone that they obtained through their diet before developing the capacity to produce the hormone. Any hormone entering the system would have to pass through the intestinal wall before reaching the other tissues. Therefore, the intestine would be strategically located to convert the hormone into the active T3 form or inactivate it to rT3 or T2 before releasing it into the system. This would allow the intestine to function in the control of the hormone content of the body. In the sturgeon, this function is retained and the low content of TH in the body of this fish may suggest that dietary sources of TH are still an important part of the whole-body hormone supply.

Chapter 5

DIETARY EFFECTS ON THYROID STATUS

Introduction

The thyroid system of salmonid fish is regulated by the quantity and quality of the diet. Reduction in the quantity of food reduces stimulation of the thyroid system. Starvation of brook trout significantly reduced *in vivo* conversion of *T4 to *T3 suggesting reduced T4-ORD activity (Higgs and Eales, 1977, 1978). Starvation also reduces the number of thyroid hormone receptors in the liver of rainbow trout (Van der Kraak and Eales, 1980; Bres et al, 1990) and coho salmon (Darling et al, 1982). Both of these processes reduce thyroid hormone action.

The quality of the diet produces chronic effects on thyroid hormone metabolism. Rainbow trout fed a low protein diet (0.32 %) for 38 days had a suppressed thyroid system with decreased T4-ORD and decreased plasma T3 levels compared to fish fed diets containing greater percentages of protein (47%) (Eales et al, 1992). In the long term, protein is the most important dietary component for stimulation of increased body T3 levels. Of the amino acids contained in protein, glycine has the greatest stimulatory effect (Riley et al, 1993b). It increases hepatic T4-ORD activity accompanied by increased plasma T3 levels in rainbow trout.

In teleost fish, THs play an important part in reproduction, development and growth (Eales and Brown, 1993). The active thyroid hormone (T3), stimulates growth through indirect mechanisms involving the stimulation of cellular processes. Lower diet rations that suppress the production of T3 also decrease the growth rate of Arctic charr

(Eales and Shostak, 1985a). A correlation between the thyroid status and growth of lake sturgeon may also exist.

Two groups of lake sturgeon fed on two diets of different quality showed significantly different growth rates. The function of the thyroid system was compared between these groups to determine if as in salmonids, there is a relationship between growth rate, diet quality, and thyroid status in lake sturgeon.

Materials and Methods

One of two groups of eight two-year-old sturgeon previously fed on #4 Martin's trout pellets (MTP) was switched to a diet of ocean plankton (zooplankton) (OP) of underdetermined nutritional composition, while the other group remained on the MTP diet (Prot. = 42%, Crude Fat = 16%, Fibre = 3.0%, Sodium = 0.35%, Calcium = 1.0%, Phosphorus = 0.75%, Vit. A = 7500 IU/kg, Vit. C = 180 IU/kg, Vit. D3 = 2500 IU/kg, Vit. E = 95 IU/kg). All fish were PIT tagged under the skin on there dorsal side at the start of the experiment for identification of individual fish. Both groups were fed twice daily (10:00 and 16:00) for 36 days (May 13, 1997 to June 18, 1997) a 1.0 % body weight ration. The fish in both groups were of similar average weights at the beginning of the experiment (OP-fed = 252.21 g; MTP-fed = 238.39 g) ($P > 0.05$).

At completion of this period, the sturgeon were killed and the liver and lower intestine were removed and immediately frozen in liquid nitrogen. The microsomal fractions from the tissues were assayed to compare the deiodination activities between the two groups. This procedure is described in chapter 4. The assays were done with an incubation period of 2 hours for the T4 and T3 assays and 1 hour for the rT3 assay, pH = 7.2, DTT = 10mM DTT, substrate level of 0.2 nM, approximate microsomal protein

concentration of 0.3 mg / ml, and incubation temperature = 12 °C. Blood plasma samples were obtained for determination of T4 and T3 content using the RIA procedure described in Chapter 3. The blood plasma proteins were analyzed by the G-25 Sephadex column procedure outlined in Chapter 3, to determine if any differences in plasma TH concentrations could be due to the binding properties of the plasma proteins.

The TH content of both diets was determined by extraction of 1-gram samples using the methods for TH extraction from tissues (See Chapter 3).

The sturgeon were weighed, and the fork and total lengths were measured at the beginning of the feeding period, one week into the period, and then every two weeks after that for the duration of the feeding period. The specific growth rate (SGR) of each sturgeon was calculated using the weights obtained on May 20, 1997 and June 18, 1997. It was calculated as follows: $SGR = Ln (W2 / W1)^{x} 100 / days$, where W2 is the weight of the sturgeon on June 18, 1997 and W1 is the weight on May 20, 1997 and "days" is the 29 days between these two dates. The SGR was also calculated similarly using the total-length changes over the 29-day period: $SGR = Ln (TL2 / TL1)^{x} 100 / days$.

Correlation coefficients were calculated between SGR (weight), SGR (total length), plasma T3 levels and plasma T4, T3 and all deiodination activities. Correlations were considered significant if $P < 0.05$.

Statistical analysis involved comparison of means with t-tests if the data fitted a normal distribution and there was homogeneity of variance. Otherwise, the Mann-Whitney test was used. Treatments were considered different if $P < 0.05$.

Results

The OP diet decreased the growth rate by weight of the sturgeon (SGR = 0.61 ± 0.08) compared to the MTP-fed fish (SGR = 1.29 ± 0.07) ($P < 0.05$). Growth rate by total length was also lower for the sturgeon fed OP (0.23 ± 0.02) compared to MTP (0.33 ± 0.02) ($P < 0.05$).

There was no significant difference in the plasma T4 level due to diet. However, the plasma T3 concentration was significantly greater in the fish fed MTP diet than in the fish fed the OP diet (Fig. 5-1) ($P < 0.05$).

An index of the degree of binding of T4 and T3 by the plasma thyroid-hormone binding proteins was determined using a 15% dilution of the plasma. There was no significant difference due to diet in either T3 or T4 binding to plasma proteins. The OP-fed fish had 73 ± 6 % of the T3 bound while the fish fed MTP had 69 ± 5 % T3 bound (Fig. 5-2). The OP-fed fish had 18 ± 5 % of the T4 bound while the fish fed the dry feed had 15 ± 4 % T4 bound.

Deiodination assays were performed on liver (Fig. 5-3) and lower intestine (Fig. 5-4) microsomal fractions of both diet groups. There was no difference in T4-ORD activity between diet groups in the liver ($P > 0.05$) or intestine ($P > 0.05$). But T3-IRD activity was significantly greater in the OP-fed than in the MTP-fed fish in both the liver ($P < 0.05$) and intestine ($P < 0.05$). In contrast, rT3-ORD was significantly greater in the liver but not the intestine of the OP-fed fish ($P > 0.05$). No significant differences in T4-IRD existed between groups for either liver or intestine.

Samples of the two diets were also analyzed for their TH contents (Fig. 5-5). There were no significant differences in T3 content ($P > 0.05$). However, the OP diet

Figure 5-1: Concentration of T4 and T3 in the blood plasma of lake sturgeon fed on a diet of Martin's trout pellets and on ocean plankton (n = 12) determined by radioimmunoassay of blood plasma samples. Each bar shows the mean ± SEM of 12 different samples measured in duplicate. Significant differences between diets are indicated by an asterick (*).

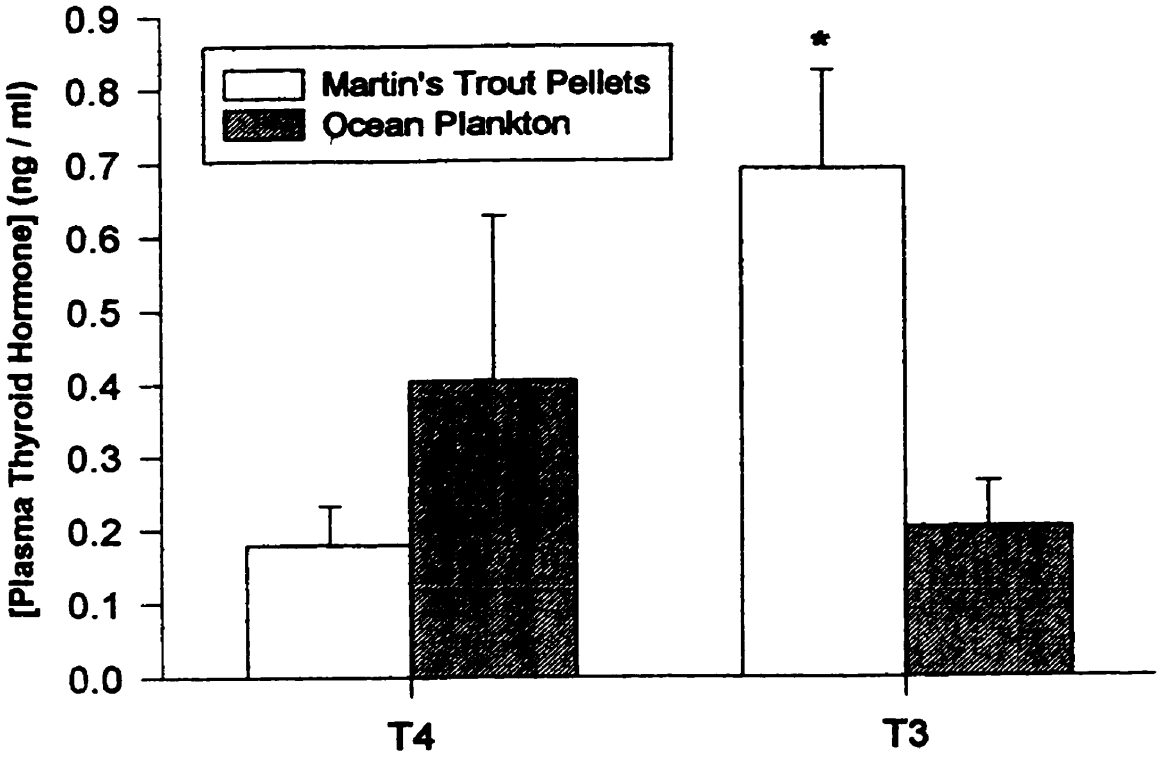

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
[Plasma Thyroid Hormone] (ng / ml)
Martin's Trout Pellets
Ocean Plankton
*
T4
T3

Figure 5-2: **Percentages of T4 and T3 bound by plasma proteins in the blood plasma of lake sturgeon fed on diets of Martin's trout pellets and ocean plankton as determined by the G-25 column method. Each bar shows the mean ± SEM of 12 samples measured in** duplicate.

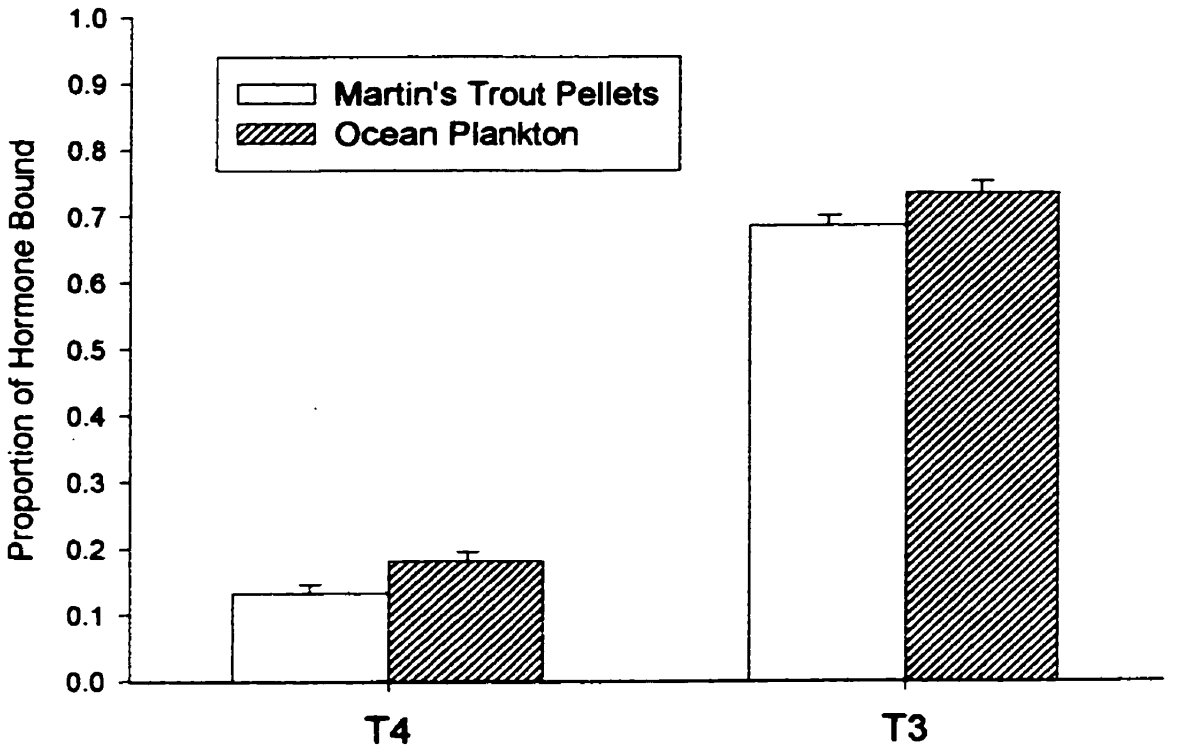

Martin's Trout Pellets
Ocean Plankton
Proportion of Hormone Bound
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
T4
T3

Figure 5-3: The hepatic microsomal activity of four deiodination pathways in two groups of lake sturgeon fed on diets of Martin's trout pellets and ocean plankton. The substrate level in the assays was 0.8 nM for T4 and 0.6 nM for rT3 and T3-deiodination assays. Each bar shows the mean ± SEM of 12 different samples incubated in duplicate. Significant differences between diets are indicated by an asterick (*).

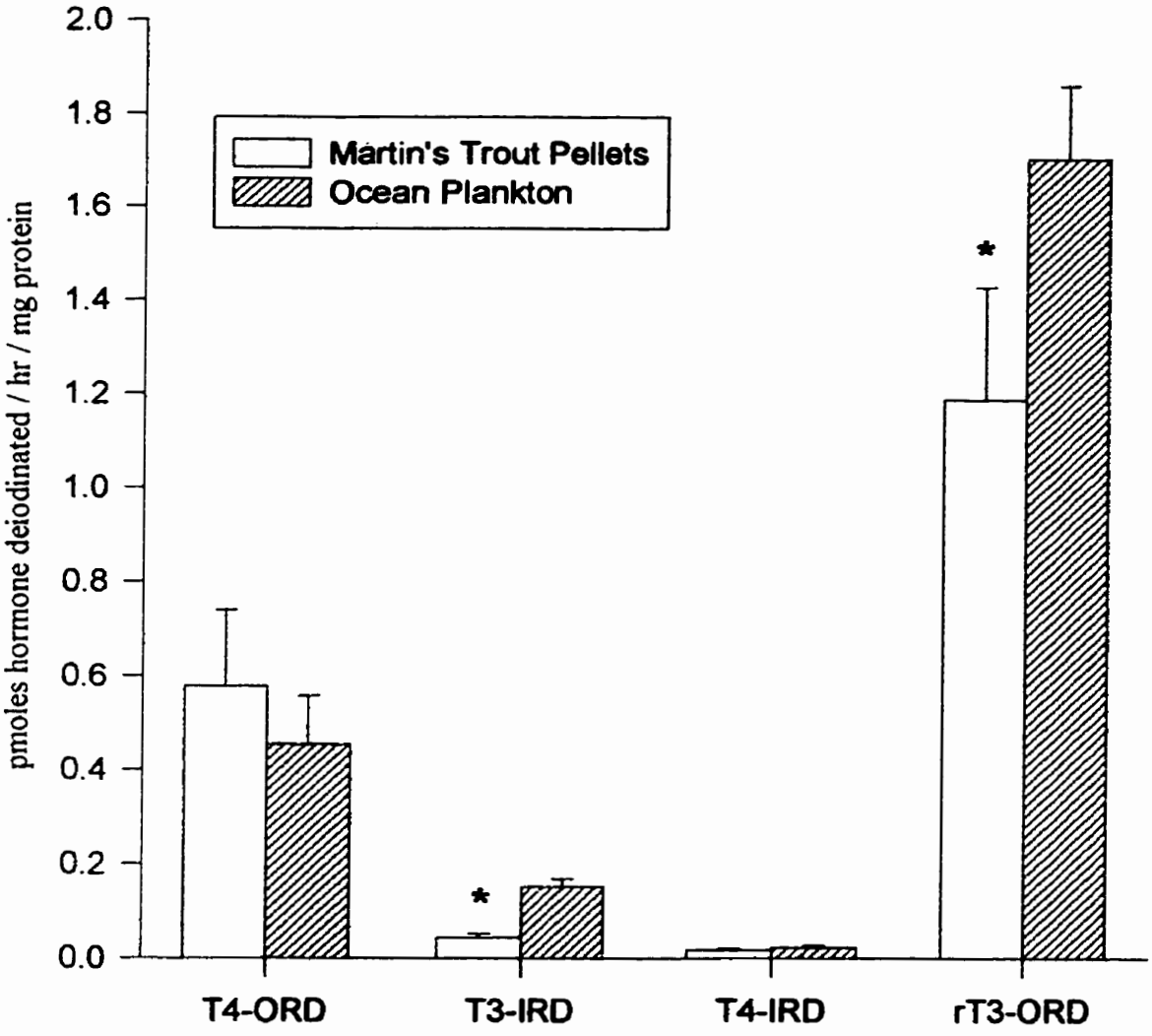

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pmoles hormone deiodinated / hr / mg protein
Martin's Trout Pellets
Ocean Plankton
*
*
T4-ORD
T3-IRD
T4-IRD
rT3-ORD

Figure 5-4: The lower intestine microsomal activity of four deiodination pathways in two groups of lake sturgeon fed on diets of Martin's trout pellets and ocean plankton (n = 12). measurements. The substrate level in the assays was 0.8 nM for T4, and 0.6 nM for rT3 and T3-deiodination assays. Each bar shows the mean ± SEM of 12 different samples incubated in duplicate. Significant differences between diets are indicated by an asterick (*).

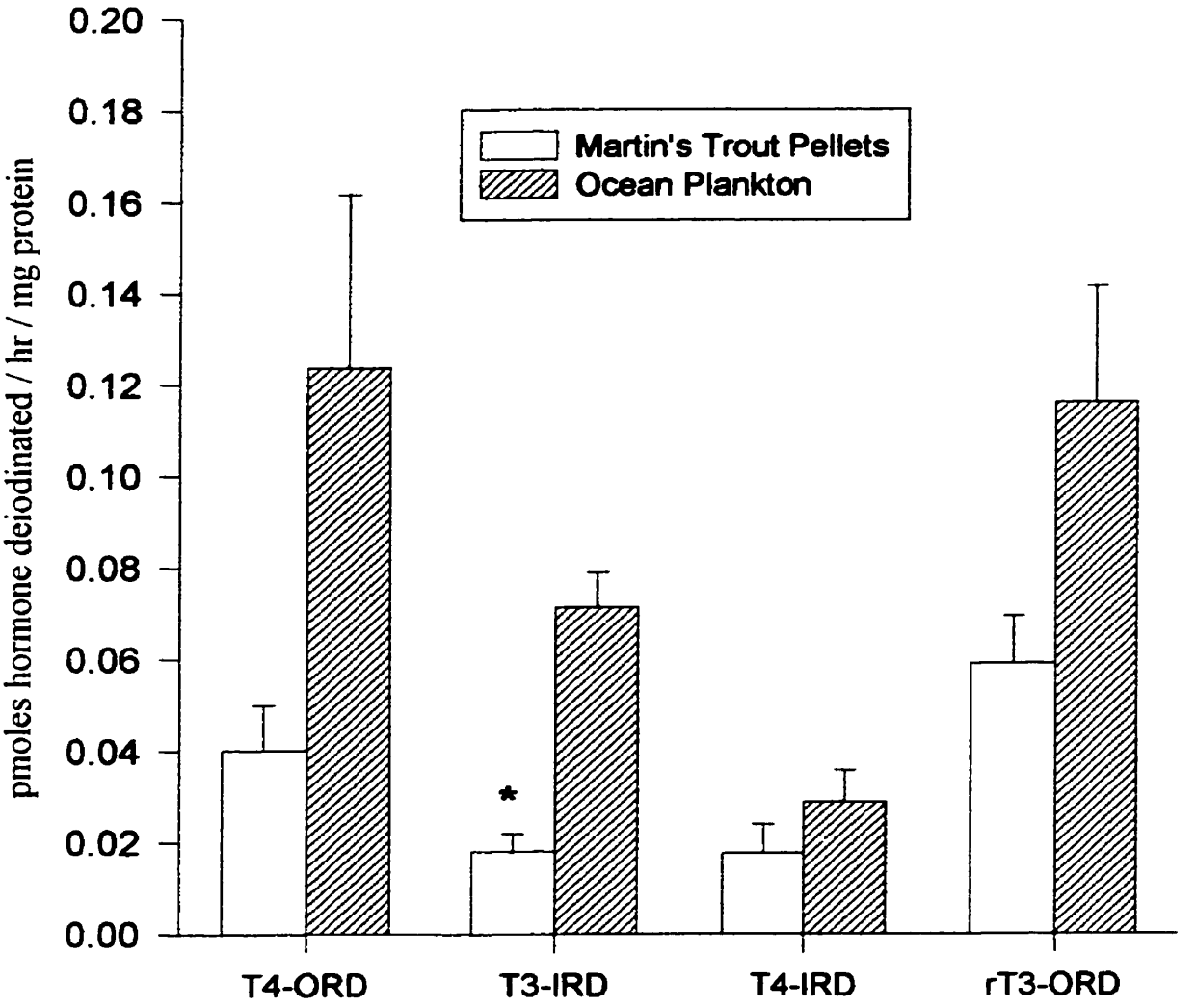
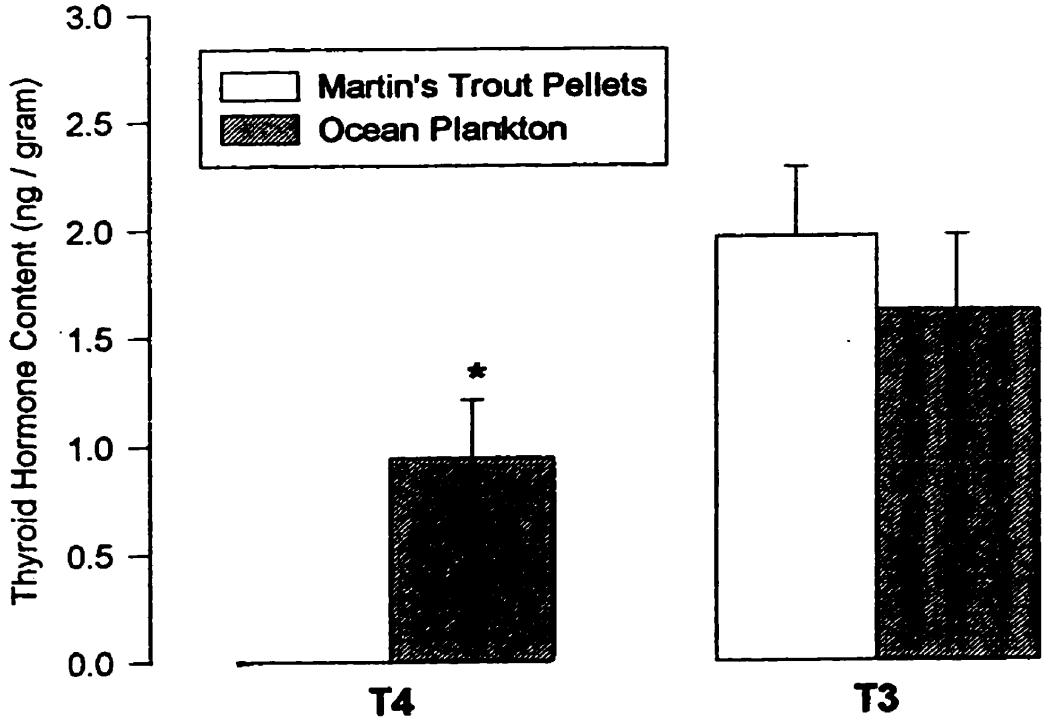

0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
pmoles hormone deiodinated / hr / mg protein
Martin's Trout Pellets
Ocean Plankton
*
T4-ORD
T3-IRD
T4-IRD
rT3-ORD

Figure 5-5: Concentrations of T4 and T3 in samples of Martin's trout pellets and ocean plankton used for feeding in the two diet groups of lake sturgeon. Each bar shows the mean ± SEM of 12 different samples incubated in duplicate. Significant differences between diets are indicated by an asterick (*).


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Thyroid Hormone Content (ng / gram)
Martin's Trout Pellets
Ocean Plankton
*
T4
T3

contained significantly more T4 than the MTP diet, which contained undetectable levels of the hormone ($P < 0.05$).

When data from both diet conditions were pooled, significant negative correlations existed between the liver ($r = -0.563$) and intestine ($r = -0.587$) T3-IRD activity and SGR (weight) ($P < 0.01$). There were also significant negative correlations between the liver ($r = -0.477$) and intestine ($r = -0.451$) T3-IRD and SGR (total length) as well as between the liver ($r = -0.453$) and intestine ($r = -0.491$) T3-IRD and plasma T3 levels ($P < 0.05$). Plasma T3 levels were also significantly correlated with SGR (weight) ($r = 0.576$, $P < 0.01$), but not with SGR (total length) ($r = 0.364$, $P > 0.05$) (Table 5-1).

Discussion

Two groups of lake sturgeon were previously fed on the same MTP diet before one group was switched to an OP diet. This was done to determine how lake sturgeon modify their thyroid system when faced with a change in diet that modifies growth rate.

Sturgeon fed the OP diet grew slower in both length and weight than those fed the MTP. Reduced growth rate of the OP fed sturgeon was accompanied by several changes in the thyroid system.

The plasma T4 levels did not differ between the two diet groups. This is not suprising considering both the hepatic and intestinal T4-ORD and T4-IRD pathways that deiodinate T4 did not differ significantly between diet groups. Elevations in T4 occur temporarily in rainbow trout refed a single meal after 3 days starvation (Himick and Eales, 1990). A difference in plasma T4 levels in sturgeon may have been found if sampling had been done at a different time of day. Flood and Eales (1983) reported that fed rainbow trout compared to starved trout did not differ in plasma T4 concentrations

Table 5-1: Correlation matrix displaying correlation coefficients (r) for comparisons between lake sturgeon thyroidal parameters with data from both diet groups pooled.

		SGR (Weight)	SGR (Total Length)	Plasma T3
	Plasma T3	0.576*	0.364	X
	Plasma T4	0.192	0.042	-0.120
T4-ORD	Liver	-0.120	-0.216	-0.017
	Intestine	-0.342	-0.383	-0.358
T4-IRD	Liver	-0.307	-0.314	0.091
	Intestine	-0.302	-0.303	-0.083
T3-IRD	Liver	-0.563*	-0.477*	-0.453*
	Intestine	-0.587*	-0.451*	-0.491*
rT3-ORD	Liver	-0.490*	-0.457*	-0.357
	Intestine	-0.441*	-0.241	-0.365

Correlations are considered significant if $r > 0.404$ ($p < 0.05$, $df = 22$) without considering the sign of r.

* indicates statistically significant correlations ($P < 0.05$).

SGR = Specific growth rate

when sampled at 11:00 but did differ significantly when sampled at 16:00. This difference was due to a diurnal cycle in plasma TH levels in fed but not starved trout, which lead to a rise in plasma T4 above that of the starved trout in the early morning hours. In chapter 3, I observed a diurnal hepatic deiodination cycle in two groups of sturgeon fed twice daily with Martin's trout pellets and sampled from 9:00 to 18:00. However, significant changes in TH levels did not accompany the altered deiodination activities. A hormone cycle may exist in the tissues due to the cycle in deiodinase activity without effecting the plasma TH levels significantly.

The plasma T3 levels were decreased in the OP-fed sturgeon compared to the MTP-fed fish. Reduction of plasma T3 is a common response of salmonid fish exposed to poor quality diet or starvation (Higgs and Eales, 1977, 1978; Shields and Eales, 1986).

The degree of binding of T4 and T3 to plasma proteins did not differ between diet groups. Therefore, reduced plasma T3 in the fish fed the OP diet was not due to decreased binding capacity or affinity of the plasma proteins. However, it may have been due to a reduction in the secretion of T3 from the thyroid gland, which was not studied in this experiment.

Reduced plasma T3 levels in the OP fed fish may have been due to the increased hepatic and intestinal T3-IRD activity degrading T3. The inner-ring deiodination pathways of salmonids have not been examined with respect to dietary affects. However, in contrast to the sturgeon, reduced plasma T3 levels in starved brook trout and rainbow trout are accompanied by a decreased conversion of T4 to T3 (Higgs and Eales, 1977, 1978; Flood and Eales, 1983; Shields and Eales, 1986). In rainbow trout, increasing

ration (0% to 3.0 % body weight) increases hepatic T4-ORD activity along with increasing plasma T3 levels (Sweeting and Eales, 1993).

rT3-ORD activity, which is responsible for degrading rT3 to 3,3'-T2, was significantly greater in the intestine of the OP fed fish, but not in the liver. Increased rT3-ORD occurs in livers of fasted rainbow trout (Finnson and Eales, 1999), and in contrast, a reduction in hepatic rT3-ORD occurs in the rat (McNabb, 1992).

The differences in thyroid status between the two diet groups may have been stimulated directly by nutrients within the food, or indirectly through growth hormone stimulated effects of deiodination. Growth hormone stimulates increased hepatic T4-ORD leading to elevations in plasma T3 levels in rainbow trout (MacLatchy and Eales, 1990).

Differences in the level of T4 in the two diets may have affected the results. The OP diet contained significant amounts of T4 whereas the MTP diet contained no detectable T4. This may have subjected the OP-fed sturgeon to a T4 challenge. Rainbow trout fed a diet supplemented with 12 ppm T4 slightly decreased activity of a hepatic high-K_m T4-ORD compared to controls (MacLatchy and Eales, 1993). T4 is absorbed poorly from the intestinal lumen of salmonids (Sinclair and Eales, 1974; Collicut and Eales, 1974; Whitaker and Eales, 1993) and does not elevate plasma T4 levels significantly. The absorption of T4 from the gut of the lake sturgeon would have to be studied to determine if T4 could be getting into the system and affecting the thyroid status of the OP-fed fish.

The fish fed the MTP diet had a higher specific growth rate (by weight and length) and higher plasma T3 levels than those fed the OP diet. Significant correlations

existed between these SGR values, plasma T3, and T3-IRD activity which suggests a link between thyroidal status and growth of lake sturgeon. Correlation of increased specific growth rate and higher T3 plasma concentrations like that found in the sturgeon also occurs in Arctic charr (*Salvelinus alpinus*) (Eales and Shostak, 1985a). This observation suggests that the thyroid hormone may be involved in growth of lake sturgeon, as it is in salmonids.

Conclusions

Sturgeon fed MTPs compared to OP exhibit higher growth rates along with decreased T3-IRD activity in intestine and liver and increased plasma T3 levels. The results support the hypothesis that a correlation exists between growth rate, T3 levels, and diet quality, as in salmonids. However, the sturgeon in contrast to salmonids studied, does not alter the T4-ORD pathway in response to changes in diet quality. Instead, when faced with a diet with lower growth promoting quality it increases the breakdown of the larger T3 pool (as opposed to the smaller T4 pool) by increasing T3-IRD activity in the liver and intestine.

Chapter 6

T3-Challenge Feeding Experiment

Introduction

All animals studied to date, that produce and secrete thyroid hormone (TH) within their bodies, maintain T4 and T3 levels in blood plasma within set concentration ranges that are maintained through secretion of hormone from the thyroid, modifications in deiodination, and uptake and release of hormone between the peripheral tissues and the blood (Brown and Eales, 1993). The level of the set point in the system is determined by the physiological state of the animal which is effected by such factors as diet quality (MacKenzie et al, 1998), salinity (Parker and Specker, 1990), temperature (Eales et al, 1986), and stressors (Johnston et al, 1996).

In the previous chapter, the composition of the diet affected the quantity of T3 within the blood plasma as well as the growth rate. This suggested a link between T3 levels and growth rate, which may indicate that the TH is responsible for stimulating growth either indirectly or directly in the lake sturgeon. T3 promotes growth directly by occupying nuclear receptors increasing the rate of transcription-related events or indirectly by promoting transcription of genes for growth promoting hormones such as growth hormone in fish (Farchi-Pisanty et al, 1997; Luo and McKeown, 1991). If elevations in T3 levels do promote growth in lake sturgeon, then when more food resources are available, higher quantities of T3 in the system would be desirable as faster growth could be accommodated. However, unwanted elevations in hormone content in the body can have a deleterious effect on survival when insufficient resources are available to accommodate the increased stimulation for growth.

Elevated levels of TH in the system are dealt with through autoregulatory responses, which tend to favour return of TH levels back to a set concentration. For example, rainbow trout respond by decreasing production of T3 (T4-ORD) and increasing the breakdown of T3 (T3-IRD) and T4 (T4-IRD) in the liver (Eales et al, 1990).

In this chapter, lake sturgeon were fed T3-containing food to elevate their body T3 levels to determine if, as in trout, an autoregulatory response to elevated T3 concentrations exists.

Materials and Methods

A group of eight lake sturgeon was fed a diet of Martin's trout pellets supplemented with 12 ppm T3 sprayed onto the food while dissolved in methanol) to observe the effect of a dietary T3 challenge on the sturgeon's thyroid system. Eight additional control sturgeon were fed the same diet without the added T3. The fish were weighed before and after the experiment to determine any effects of the dietary T3 on growth. The fish in both groups were fed 1.3% body weight once a day at about 10:00. After two weeks on this feeding regime the fish were killed and liver, lower intestine, brain, and blood samples were obtained for analysis.

Blood plasma samples were analyzed by RIA to determine T4 and T3 concentrations. The microsomal fractions from liver, lower intestine, and brain were analyzed in deiodination assays to determine T4-ORD, T4-IRD, T3-IRD, and rT3-ORD activities. The assays were done with an incubation period of 2 hr for the T4 and T3 assays and 1 hr for the rT3 assays. The phosphate-buffered incubates had a pH of 7.2, DTT concentration of 10 mM, substrate level of 0.2 nM for liver and intestine assays,

0.04 nM for brain assays, and an approximate microsomal protein concentration of 0.3 mg / ml.

Statistical analysis involved comparison of means by t-tests or when the data did not conform to normality a Mann-Whitney test was used to determine significant differences. Treatments were considered different if $P < 0.05$.

Results

At the start of the experiment, the control fish weighed 875 ± 43 grams and the T3-fed group weighed 878 ± 46 grams. At completion of the experimental feeding period the control fish weighed 1069 ± 19 grams and the T3-fed group weighed 1028 ± 78 grams. The control fish did not weigh significantly different from the T3-treated fish at the start ($P > 0.05$) or end ($P > 0.05$) of the feeding period. The plasma T4 levels did not differ significantly between groups ($P > 0.05$). However, the concentration of T3 in the plasma of the experimental fish was significantly greater than that of the controls ($P < 0.05$) (Fig. 6-1).

Hepatic T4-ORD activity in the liver of lake sturgeon decreased in response to elevated plasma T3 levels induced by the dietary T3 challenge (Fig. 6-2). No other significant differences in T4-ORD, rT3-ORD, T4-IRD or T3-IRD were recorded between groups in any of the tissues studied (liver, lower intestine or brain) (Fig. 6-2 to 6-4).

Discussion

Lake sturgeon, when faced with a dietary T3-challenge, elevate plasma T3 concentrations and decrease hepatic T4-ORD activity.

Elevations in plasma T3 levels upon a dietary induced T3-challenge also occurred in all studies on salmonid teleosts including coho salmon (Darling et al, 1982), rainbow

Figure 6-1: Concentration of T4 and T3 in the blood plasma of lake sturgeon fed on a diet of Martin's trout pellets supplemented with 12 ppm T3 compared to fish fed on the same diet lacking the added T3 determined by radioimmunoassay. Each bar shows the mean ± SEM of 12 different samples measured in duplicate. Significant differences between treatments are indicated by an asterick (*).

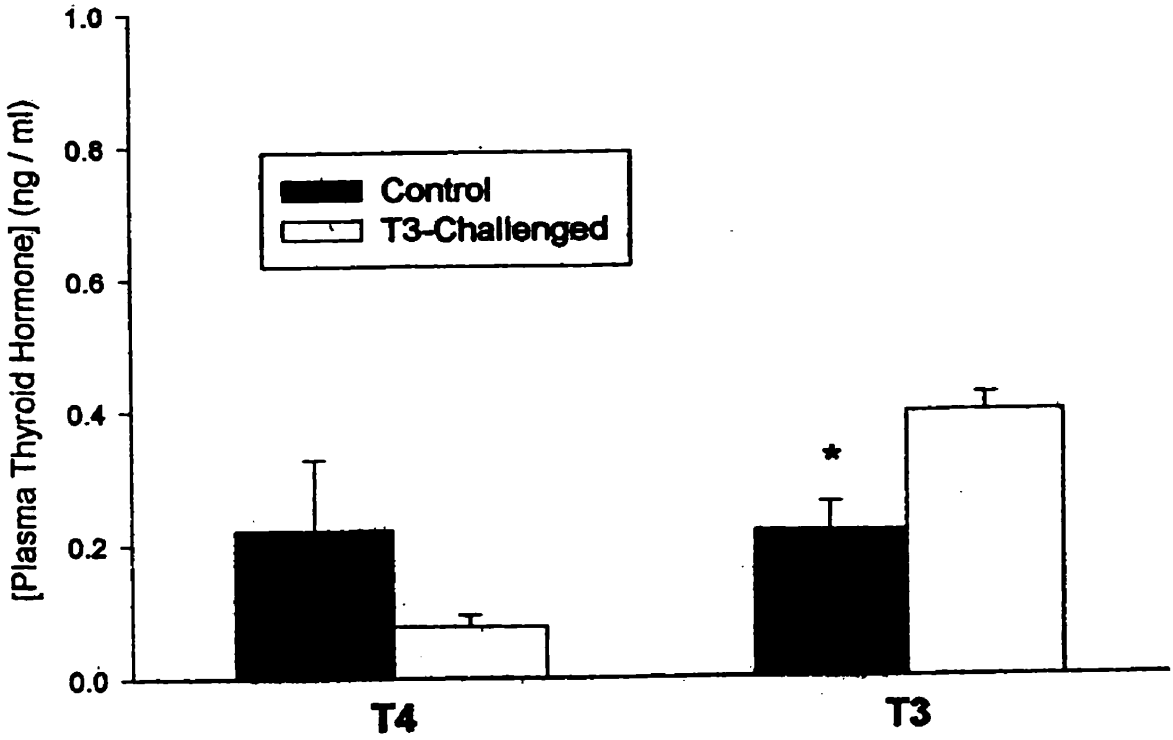

1.0
0.8
0.6
0.4
0.2
0.0
[Plasma Thyroid Hormone] (ng / ml)
Control
T3-Challenged
*
T4
T3

Figure 6-2: The hepatic microsomal activity of four deiodination pathways in lake sturgeon fed on T3-supplemented (12 ppm) Martin's trout pellets compared to controls fed on the same diet without added T3. The substrate level in all the assays was 0.4 nM. Each bar shows the mean ± SEM of duplicate measurements. Significant differences between treatments are indicated by an asterick (*).

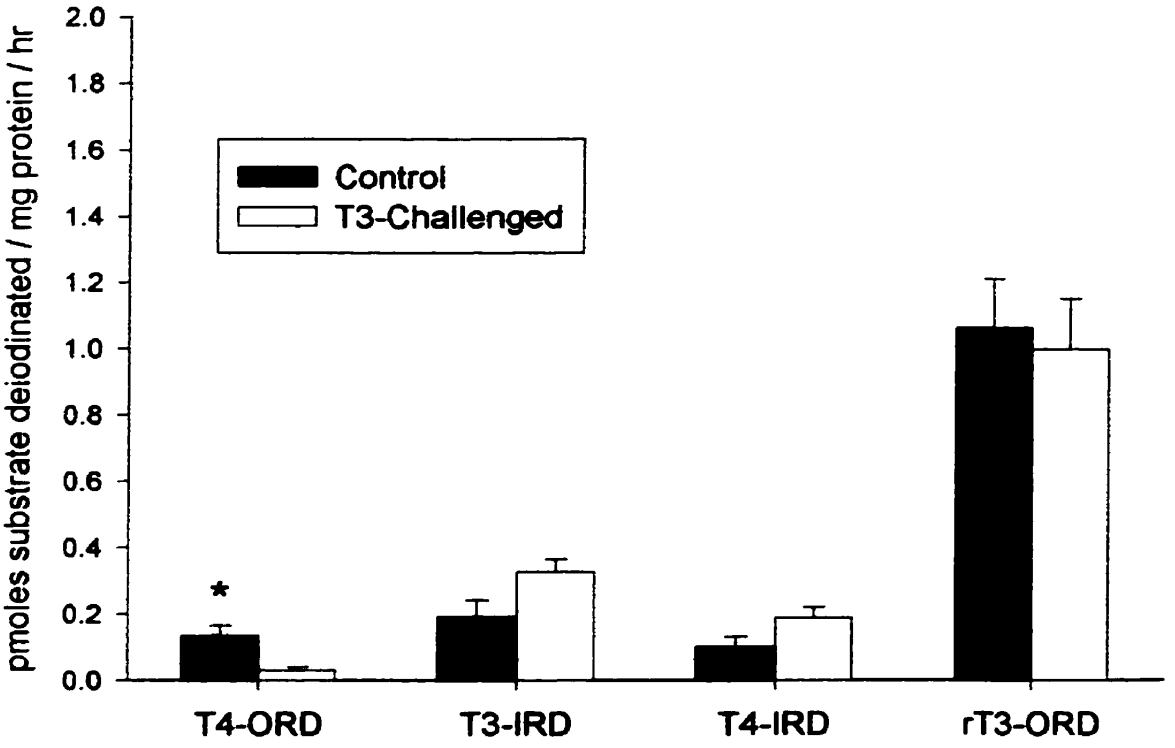

pmoles substrate deiodinated / mg protein / hr
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
T3-Challenged
*
T4-ORD
T3-IRD
T4-IRD
rT3-ORD

Figure 6-3: The lower intestine microsomal activity of four deiodination pathways in lake sturgeon fed on T3-supplemented (12 ppm) Martin's trout pellets compared to controls fed on the same diet without added T3. The substrate level in all the assays was 0.4 nM. Each bar shows the mean ± SEM of 12 different samples measured in duplicate.

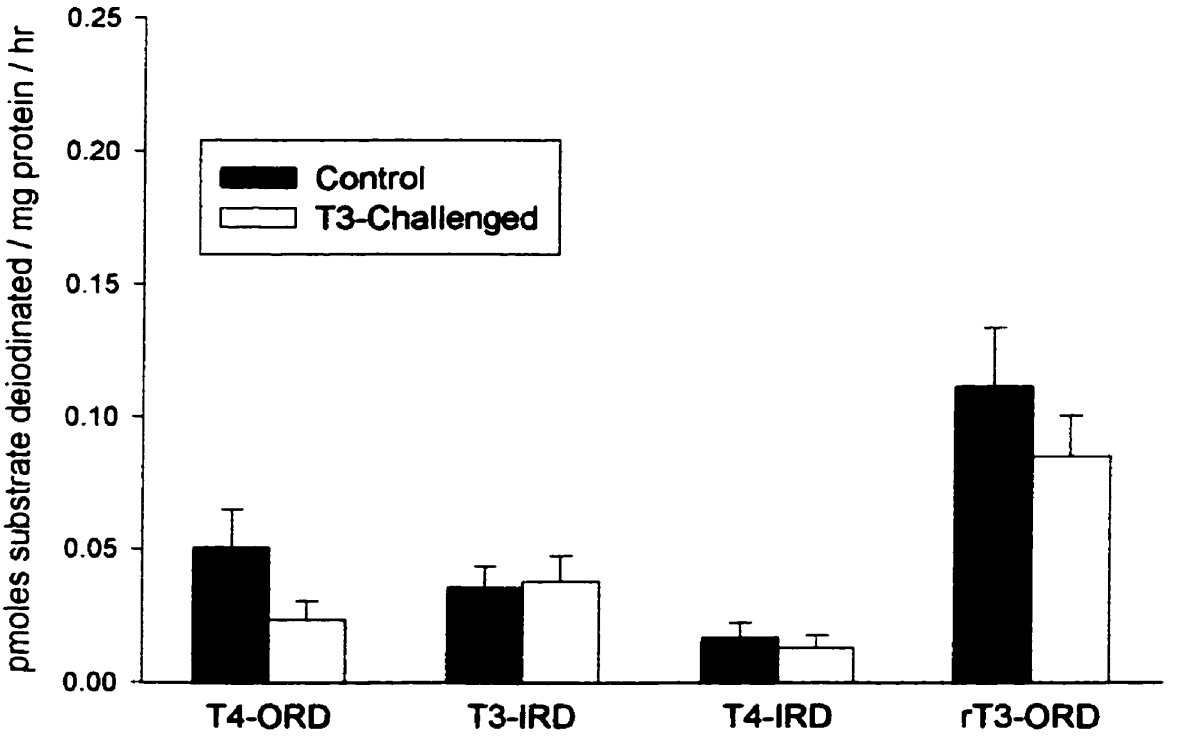
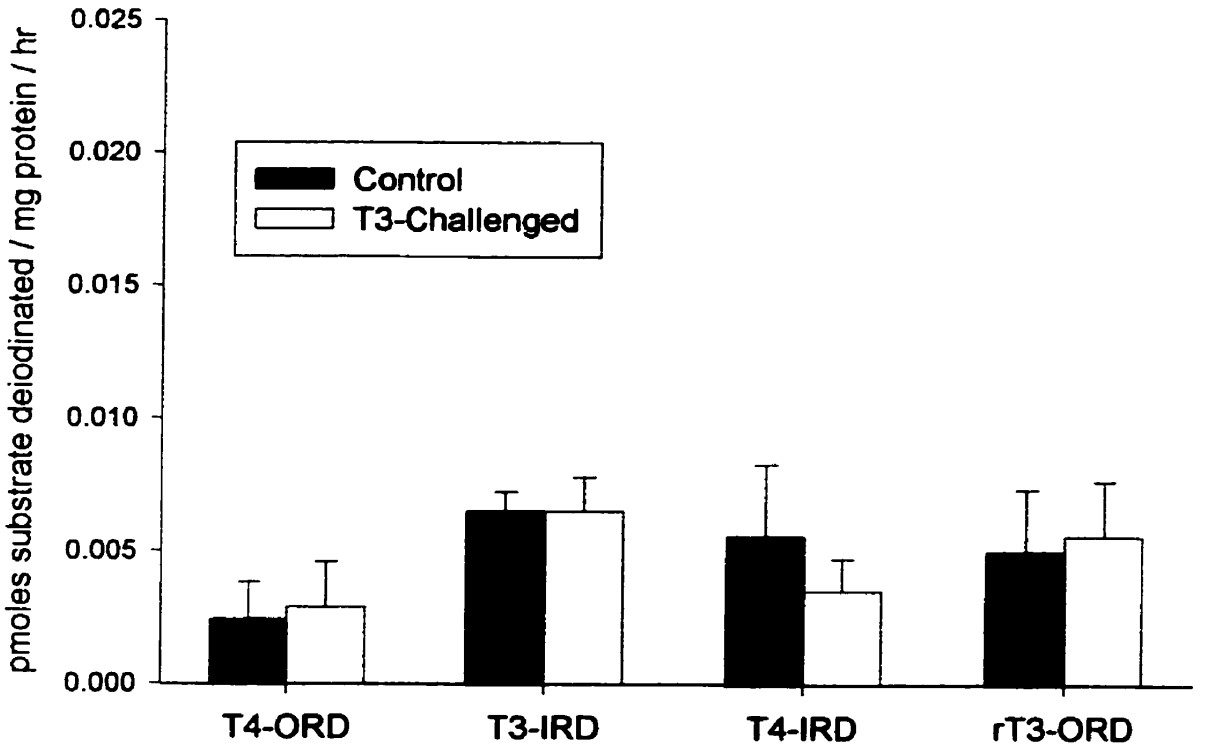

pmoles substrate deiodinated / mg protein / hr
0.25
0.20
0.15
0.10
0.05
0.00
Control
T3-Challenged
T4-ORD
T3-IRD
T4-IRD
rT3-ORD

Figure 6-4: The brain microsomal activity of four deiodination pathways in lake sturgeon fed on T3-supplemented (12 ppm) Martin's trout pellets compared to controls fed on the same diet without added T3. The substrate level in all the assays was 0.2 nM. Each bar shows the mean ± SEM of 12 different samples measured in duplicate.


0.025
0.020
0.015
0.010
0.005
0.000
pmoles substrate deiodinated / mg protein / hr
Control
T3-Challenged
T4-ORD
T3-IRD
T4-IRD
rT3-ORD

trout (Eales et al, 1990; Sweeting and Eales, 1992b; Eales and Finnson, 1991; Fines et al, 1999) and Atlantic salmon (Saunders et al, 1985).

Reduction in hepatic T4-ORD activity in response to T3 challenge is similar to what occurs in the rainbow trout, which show decreased hepatic T4-ORD activity 24 hours after receiving a T3 supplement (Sweeting and Eales, 1992b). Decreasing activity of this enzyme may serve as an attempt to maintain total body T3 concentrations at the required level through this autoregulatory response, which reduces the production of T3.

T4-IRD activity producing rT3 was not increased in sturgeon tissues by the T3 challenge. This is in contrast to rainbow trout liver (Sweeting and Eales, 1992b; Finnson and Eales, 1999). The average control activity was lower than the experimental values but the difference was not statistically significant. The lower plasma T4 concentration in the sturgeon compared to the rainbow trout might make it less important that the sturgeon diverts unneeded T4 to rT3 as not as much T4 is normally available for deiodination to T3.

T3-IRD activity producing 3,5-T2 was also not changed significantly in any of the tissues. This is also in contrast to what occurs in the liver of T3-challenged rainbow trout which increase hepatic T3-IRD activity (Fines et al, 1999; Sweeting and Eales, 1992b).

A possible explanation for the lack of effects of the T3-challenge on T4-IRD activity and T3-IRD as well as the T4-ORD activity in other tissues may involve the quantity of T3 that can be transported in the blood from the intestines to the other tissues. Low binding by plasma thyroid hormone binding proteins in the sturgeon would limit the quantity of T3 carried in the blood. The sturgeon used in this study were fed the same concentration of T3 in their food (12 ppm) as the rainbow trout used in previous studies

(Sweeting and Eales, 1992b; Fines et al, 1999; Finnson and Eales, 1999). The blood plasma levels also increased about 2 X in the sturgeon blood as in the rainbow trout studies. However, the quantity of thyroid hormone that can be carried in the sturgeon blood plasma is much less than the capacity of the rainbow trout plasma due to poor binding by the sturgeon plasma proteins. Therefore, the absolute amount of T3 carried to the tissues of the T3-challenged sturgeon would not have been as great as it was for the rainbow trout. Nor would the quantity of T3 taken up from the intestine. This would have resulted in a weaker stimulus for modifications in deiodination in the tissues.

A reduction in T4-ORD activity occurred in the liver but not in the intestine or brain. This suggests that the liver may be the main regulator of plasma T4 / T3 concentrations in response to a weak T3 challenge. Additionally, the much lower T4-ORD activity in the brain and intestine than in the liver might make it more difficult to detect a significant reduction in activity in these tissues as it is harder to accurately measure low deiodination activities.

Plasma T4 levels had not changed significantly after 7 days. It was expected that plasma T4 levels would not have changed. The effect of T3 challenge on plasma T4 levels is inconsistent and varies with the age and species of fish. Sometimes plasma T4 levels increase (Sweeting and Eales, 1992b), decrease (Rivas et al, 1982) or do not change (Eales et al, 1990). However, in all cases, plasma T4 is not greatly affected.

In studies with rainbow trout, plasma T4 was elevated for up to 4 weeks after the start of T3-supplementation (Sweeting and Eales, 1992b). After which time T4 levels were normal. The initial rise in plasma T4 was presumably due to a T3 stimulated increase in thyroidal T4 secretion. However, the sturgeon thyroid contains mostly T3 and

so might also secrete primarily T3. Therefore, T3 stimulation of thyroid hormone release would not increase plasma T4 levels in the sturgeon.

An unusual phenomenon in this study was that the activity of the T4-IRD and T3-IRD pathways in the liver were greater than the T4-ORD pathway in both the control and T3-treated fish. This is suprising as in all other analysis of sturgeon liver deiodinase, the T4-ORD pathway is considerably more active than both inner-ring pathways. The control liver T4-ORD activity is about the same as that recorded in other liver microsome assays but the T3 and T4-IRD pathways appear to be more active. The increased inner-ring deiodinase activity could be due to the change in feeding regime given to the sturgeon at the commencement of this experiment. Previously the sturgeon had been fed twice daily at 10:00 and 16:00 a 1.0 % body weight ration at each feeding. However, for the T3 challenge experiment, the fish were fed a 1.3 % body weight ration once daily at 10:00. This reduction in the quantity of food consumed by the sturgeon may have induced the inner-ring deiodinase pathways to reduce a T3 body pool that was higher than the sturgeon required at the new lower ration level.

Conclusions

The lake sturgeon responds to a T3-supplemented diet, which increases plasma T3 levels by decreasing the production of T3 from T4 without altering the rate of deiodination of T3. This does not exclude the possibility that T3 is removed from the system through increased conjugation followed by deiodination of the conjugate or by non-enzymatic means.

The T3 autoregulatory response of the thyroid system observed in the sturgeon may occur in its daily life. Invertebrates contain thyroid hormones from ingestion of

plankton that have produced hormone with the help of solar energy (Eales, 1997). Lake sturgeon consume a large number of invertebrates like insect larvae and crustaceans, as well as other fish (Scott and Crossman, 1998). These dietary sources expose them to TH supplements on a daily basis in nature.

Chapter 7

General Conclusions

The first objective of this thesis was to confirm the low plasma T3 and T4 levels in lake sturgeon by analysis of laboratory-raised and wild lake sturgeon. The sturgeon were found to have low plasma T3 levels that were consistently greater than the low plasma T4 concentrations.

The second objective was to analyze the blood properties of the sturgeon to determine if they could explain the low plasma TH levels. The affinity and capacity of the plasma TH-binding-proteins as well as the equilibrium TH content of the red blood cells were examined. The sturgeon TH-binding-proteins bound T4, T3 and rT3 to a much lower extent than in other fish studied but they bound the THs with similar relative affinities and capacities to those of teleosts (T3 > T4 > rT3). The absolute quantity of T4 and T3 free in the plasma was also similar to that in other fish and mammals. The RBCs were found to hold whole blood proportions of T4, T3 and rT3 within the range of other fish studied, however the sturgeon RBCs are unusual in holding a greater proportion of T4 than T3. It was concluded that low binding of TH by the plasma proteins likely determines the low concentrations of THs in the sturgeon blood plasma.

The third objective was to analyze sturgeon tissues for their T4 and T3 content to determine if the THs are sequestered in great quantities in any sturgeon tissues. It was concluded that almost all the sturgeon tissues contain greater concentrations of T3 than T4 and that the thyroid and brain contain the greatest concentrations of T3. High thyroidal T3 concentrations suggest that T3 rather than T4 is the primary TH released from the sturgeon thyroid gland.

The fourth objective was to assay sturgeon tissues for the presence of TH-deiodination activity. The liver was the most active deiodinating tissue containing the greatest T4-ORD and T3-IRD activity of the tissues assayed. The lower intestine also contained significant T4-ORD and the upper intestine contained significant T3-IRD activity. However, the brain and thyroid contained the greatest T4-IRD activity. Significant T4-ORD activity in the thyroid combined with presence of T4-IRD activity support the hypothesis that T3 and not T4 is the primary TH released from the thyroid gland. The liver was used to characterize the TH-deiodination pathways which resemble in many respects the deiodination pathways in other fish.

The final objective was to determine the responsiveness of the sturgeon thyroid system to dietary components. The sturgeon responded to a diet with lower growth promoting qualities by increasing hepatic and intestinal T3-IRD activity accompanied by a decrease in plasma T3 levels. The growth rate, plasma T3 levels, and T3-IRD activity were found to be significantly correlated suggesting a role of T3 in growth of lake sturgeon.

The response of the lake sturgeon thyroid system to a dietary induced T3 challenge was also examined. The plasma T3 levels were elevated, and the hepatic T4-ORD activity was reduced in the T3-challenged fish compared to controls.

Sturgeon thyroid function compared to teleosts

The lake sturgeon thyroid system appears to differ in several ways from the teleost system. One marked difference between the sturgeon and teleosts is in the T4 and T3 content of the thyroid. In sturgeon, T3 is the predominant TH in the thyroid tissue compared to greater concentrations of T4 than T3 in the teleost thyroid tissue. High T3

levels in the sturgeon thyroid suggest that T3 may be the predominant form of TH released from the thyroid gland. Significant T4-ORD activity in the thyroid gland indicates that T4 is the initial synthetic product which is then quickly deiodinated to T3 before release. This differs from rainbow trout thyroids which release predominantly T4 (Eales and Brown, 1993).

Assuming that T3 is the main form of TH released by the sturgeon thyroid, the quantity of active TH in the system would be determined mainly by the amount of T3 released from the thyroid as well as the T3-IRD activity in the peripheral tissues breaking down the T3. This contrasts with the teleosts in which the T4-ORD activity in the peripheral tissues is most important in controlling the quantity of T3 in the system. Much greater quantities of T3 than T4 in the sturgeon tissues may also be related to predominant T3 release by the thyroid. The sturgeon brain and thyroid gland contained the greatest concentrations of TH especially T3.

Another difference between sturgeon and teleosts is the lower binding of THs by sturgeon blood plasma proteins. This reduced the quantity of TH that can be carried by the blood compared to the teleosts which may serve to reduce the rate of TH loss through filtration in the kidneys in a freshwater environment that is low in iodide required for the production of more TH.

The properties of deiodination in the sturgeon tissues are similar to those in teleosts. However, the distribution of deiodination activity differs. Sturgeon have significant deiodination activity in their liver as in teleosts, however additionally, marked deiodination is also present in their intestines. This appears to be a primitive

characteristic described to date only in fish like the lamprey (Eales et al, 1997) and hagfish (McLeese et al, 2000).

The sturgeon also differs from teleosts in its response to changes in diet quality. The sturgeon responds a diet with lower growth promoting quality by increasing the activity of deiodination of T3 in the liver and intestine. Perhaps in an attempt to reduce the quantity of T3 in the tissue pool as less of the active thyroid hormone (T3) is required when feeding on a diet with less growth promoting quality. In contrast, the teleosts strategy is to reduce T3 production through reduction in hepatic T4 to T3 deiodination in addition to increasing T3-IRD activity.

The sturgeon and teleosts are alike in that thyroidal status is correlated with specific growth rate. However, the mechanisms for achieving this are different. In sturgeon, T3-IRD is highly negatively correlated and plasma T3 levels are positively correlated with the specific growth rate. In teleosts, hepatic T4-ORD and plasma T3 levels are correlated with specfic growth rate.

The strategy employed by the sturgeon to cope with a T3-challenge is similar to that in teleosts in reducing hepatic T4-ORD activity. However, induction of hepatic T3-IRD activity does not occur in the sturgeon as in teleosts. This may be related to the lower quantities of T3 that can be carried within the sturgeon blood compared to teleosts, leading to a lower T3 stimulation of thyroid hormone deiodination.

Sturgeon thyroid system compared to mammals

The overall strategy that the lake sturgeon adopts in its thyroid system control may relate to its environment. The lake sturgeon lives a mostly freshwater existence. This environment is poor in iodide content as opposed to iodide-rich saltwater. Iodide is

an essential component in the production of TH and is often the limiting ingredient. Deficiencies in iodide lead to lowered body TH levels and the development of goitres (enlargement of the thyroid gland caused by iodide insufficiency). Mammals and freshwater fish including the lake sturgeon, generally contain low levels of iodide in their blood plasma (Rats = 0.7 ug / 100 ml, humans = 0.5, lake sturgeon = 1.15) (Leloup, 1970). An exception to this can be found in brook trout raised on a commercially prepared diet. These fish have high plasma iodide levels (1800 µg / 100 ml) mainly due to efficient uptake of iodide from their intestine and the high iodide concentration of their diet (31-35 µg I / gram dry weight) (Gregory and Eales, 1975).

In contrast to the low plasma iodide levels in mammals and freshwater fish, fish that commonly live in salt water usually have much greater concentrations of iodide in their plasma (Rainbow trout = 7.3 to 78.3 ug / 100 ml, Atlantic salmon = 2 to 81.3) (Leloup, 1970) as this medium is rich in iodide.

The sturgeon thyroid system more closely matches the strategy employed by those animals that are low in iodide, like mammals, which adopt a strategy of thyroid hormone metabolism that better conserves iodide within their bodies. Mammals have a centralized control of T3 production whereas teleosts regulate T3 levels peripherally. Rats may obtain as much as 55 % of their T3 directly from the thyroid. Teleosts produce most of their T3 in the peripheral tissues by deiodination, which releases iodide into the plasma where it may be lost from the body. The sturgeon, had 10 X more T3 than T4 in the thyroid tissues suggesting that a significant quantity of the body T3 is obtained directly from thyroid as in mammals which would indicate a similar centralized control.

Future direction

An insight into the function of the lake sturgeon thyroid system has been established, however there are several important aspects that require further study before a full picture of the sturgeon thyroid system can be drawn.

The lake sturgeon is a member of the genus *Acipenser* with most of its members living a significant proportion of their lives in saltwater (Scott and Crossman, 1998). This suggests that the lake sturgeon may be a recently freshwater adapted fish of marine origin. Saltwater is rich in iodide so marine fish would not require highly efficient iodide pumps in their gills to bring sufficient iodide into their bodies. However, freshwater is poor in iodide content and therefore, an efficient iodide pump would be required. The lake sturgeon may therefore have a poor iodide transport system in the gills. Therefore, the rate of iodide uptake through the gills needs to be studied. Since iodide can also be brought into the body through the diet, the efficiency of the intestine in transporting iodide into the blood stream should also be examined.

Once inside the plasma, the iodide would likely have to been bound to plasma proteins like iodurophorine to allow significant quantities to be held in the blood plasma. Therefore, the quantity of iodide in the tissues and blood plasma as well as the affinity of any proteins binding the iodide needs to be studied. Examining the lake sturgeons iodide economy would determine if the low TH levels in the tissues and blood of lake sturgeon may be related to a deficiency in iodide required to produce the hormone.

Another aspect of the lake sturgeon thyroid system that requires further study is the identity of the predominant TH released from the thyroid gland. In this study it was established that the thyroidal region of the sturgeon contains much greater quantities of

T3 than T4. This suggested that T3 and not T4 is the primary form of TH released from the thyroid tissue. This theory could be more confidently supported if TSH was administered to lake sturgeon. TSH as in teleosts might increase thyroidal secretion of TH raising either the T4 and / or T3 levels in the blood indicating which TH form is predominantly released by the thyroid of lake sturgeon.

Finally, the rate of plasma clearance of the TH should also be examined as the rate at which the hormone enters as well as leaves the blood stream could help to explain the low plasma TH in the lake sturgeon.

Literature Cited

Bandyopadhyay, S. and Bhattacharya, S. 1993. Purification and properties of Indian major carp (*Cirrhimus mrigala*, Ham.) pituitary thyrotropin. Gen. Comp. Endocrinol. 90: 192-204.

Beckett, G. J., Beddows, S. E., Morrice, P. C., Nicol, F. and Arthur, J. R. 1987. Inhibition of hepatic deiodination of thyroxine is caused by selenium deficiency in rats. Biochem. J. 248: 443-447.

Beckett, G. J., MacDougall, D. A., Nicol, F. and Arthur, R. 1989. Inhibition of type I and type II iodothyronine deiodinase activity in rat liver, kidney and brain produced by selenium deficiency. Biochem. J. 259: 887-892.

Beech, S. G., Walker, S. W., Beckett, G. J. Arthur, J. R., Nicol, F. and Lee, D. 1995. Effect of selenium depletion on thyroidal type-I iodothyronine deiodinase activity in isolated human thyrocytes and rat thyroid and liver. Analyst 120: 827-831.

Bermano, G., Nicol, F., Dyer, J. A., Sunde, R. A., Beckett, G. J., Arthur, J. R. and Hesketh, J. E. 1995. Tissue-specific regulation of selenoenzyme gene expression during selenium deficiency in rats. Biochem. J. 311: 425-430.

Berry, M. J., Kieffer, J. D. and Larsen, P. R. 1991a. Evidence that cysteine, not selenocysteine, is in the catalytic site of type II iodothyronine deiodinase. Endocrinology 129: 550-552.

Berry, M. J., Kieffer, J. D., Harney, J. W. and Larsen, P. R. 1991b. Selenocysteine confers the biochemical properties characteristic of the type I iodothyronine deiodinase. J. Biol. Chem. 266: 14155-14158.

Berry, M. J., Banu, L. and Larsen, P. R. 1991c. Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349: 438-440.

Berry, M. J. and Larsen, P. R. 1992. The role of selenium in thyroid hormone action. Endocr. Rev. 13: 207-219.

Botta, J. A., Mendoza, D., Morero, R. D. and Farias, R. N. 1983. High affinity L-triiodothyronine binding sites on washed rat erythrocyte membranes. J. Biol. Chem. 258: 6690-6692.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brown, C. L., Doroshov, S. I., Cochran, M. D., and Bern, H. A. 1989. Enhanced survival of striped bass fingerlings after maternal triiodothyronine treatment. Fish Physiol. Biochem. 7: 295-299.

Brown S. B. and Eales, J. G. 1977. Measurement of L-thyroxine and 3,5,3'-triiodo-L-thyronine levels in fish plasma by radioimmunoassay. Can. J. Zool. 55: 293-299.

Bres, O. and Eales, J. G. 1988. High-affinity, limited capacity triiodothyronine-binding sites in nuclei from various tissues of the rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 69: 71-79.

Bres, O., Cyr, D. G. and Eales, J. G. 1990. Factors influencing the affinity and capacity of T3-binding sites in hepatic nuclei of rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 254: 63-71.

Buettner, C., Harney, J. W. and Larsen, P. R. 1998. The 3'-untranslated region of human type 2 iodothyronine deiodinase mRNA contains a functional selenocysteine insertion sequence element. J. Biol. Chem. 273: 33374-33378.

Byamungu, N., Corneillie, S., Mol, K., Darras, V. and Kuhn, E. R. 1990. Stimulation of thyroid function by several pituitary hormones results in an increase in plasma thyroxine and reverse triiodothyronine in tilapia (*Tilapia nilotica*). Gen. Comp. Endocrinol. 80: 33-40.

Chan H. H. and Eales, J. G. 1976. Influence of bovine TSH on plasma thyroxine levels and thyroid function in brook trout, *Salvelinus fontinalis* (Mitchill). Gen. Comp. Endocrinol. 28: 461-472.

Chanoine, J. P., Safran, M., Farwell, A. P., Dubord, S., Alex, S., Stone, S., Arthur, J. R., Braverman, L. E. and Leonard, J. L. 1992. Effects of selenium deficiency on thyroid hormone economy in rats. Endocrinology 131: 1787-1792.

Chino Y., Saito, M., Yamasu, K., Suyemitsu, T. and Ishihara, K. 1994. Formation of the adult rudiment of sea urchins is influenced by thyroxine. Dev. Biol. 161: 1-11.

Collicutt, J. M. and Eales, J. G. 1974. Excretion and enterohepatic cycling of ^{125}I-L-thyroxine in channel catfish, *Ictalurus punctatus*. Rafinesque.

Croteau, W., Whittemore, S. L., Schneider, M. J. and St. Germain, D. L. 1995. Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. J. Biol. Chem. 270: 16569-16575.

Cyr, D. G. and Eales, J. G. 1988a. *In vitro* effects of thyroid hormones on gonadotropin-induced estradiol-17ß secretion by ovarian follicles of rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 69: 80-87.

Cyr, D. G. and Eales, J. G. 1988b. The influence of thyroidal status on ovarian function in rainbow trout, *Salmo gairdneri*. J. Exp. Zool. 248: 81-87.

Cyr, D. G. and Eales, J. G. 1992. Effects of short-term 17 beta-estradiol treatment on the properties of T4-binding proteins in the plasma of immature rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 262: 414-419.

Cyr, D. G., Idler D. R., Audet, C., McLeese, J. M. and Eales, J. G. 1998. Effects of long-term temperature acclimation on thyroid hormone deiodinase function, plasma thyroid hormone levels, growth, and reproductive status of male Atlantic cod, *Gadus morhua*. Gen. Comp. Endocrinol. 109: 24-36.

Darling, D. S., Dickhoff, W. W. and Gorbman, A. 1982. Changes of thyroid hormone receptor and deiodinase activities in coho salmon. Amer. Zool. 22: 854. (Abstract).

Davey, J. C., Becker, K. B., Schneider, M. J., St. Germain, D. L. and Galton, V. A. 1995. Cloning of a cDNA for the type II iodothyronine deiodinase. J. Biol. Chem. 270: 26786-26789.

Davis, P. J. and Osawa, Y. 1983. Binding of thyroid hormone by heme proteins may signal protein age. J. Theor. Biol. 103: 133-136.

DeJesus, E. G., Inui, Y. and Hirano, T. 1990. Cortisol enhances the stimulating action of thyroid hormones on dorsal fin-ray resorption of flounder larvae *in vitro*. Gen. Comp. Endocrinol. 79: 167-173.

Detlaf, T. A. and Davydova, S. I. 1974. Effect of triiodothyronine on ripening of oocytes in stellate sturgeon after the action of low temperatures and reservation of females. Ontogenez. 5: 454-462.

Dickson, P. W., Aldred, A. R., Marley, P. D., Guo-Fen, T., Howlett, G. J. and Schreiber, G. 1985. High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. Biochem. Biophys. Res. Comm. 127: 890-895.

Eales, J. G. 1987. Kinetics of T4 and T3 binding to plasma sites in salmonid teleost fish. Gen. Comp. Endocrinol. 65: 288-299.

Eales, J. G. 1997. Iodine metabolism and thyroid-related functions in organisms lacking thyroid follicles: Are thyroid hormones also vitamins. Proc. Soc. Exper. Biol. Med. 214: 1-19.

Eales, J. G. and Brown, S. B. 1993. Measurement and regulation of thyroidal status in teleost fish. Rev. Fish Biol. Fisheries. 3: 299-347.

Eales, J. G., Brown, S. B., Cyr, D. G., Adams, B. A. and Finnson, K. W. 1999. *Thyroid hormone deiodination as an index of thyroidal status in fish*, Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment: Eighth Volume, ASTM STP 1364, D. S. Henshel, M. C. Black, and M. C. Harrass, Eds., American Society for Testing and Materials, West Conshohocken, PA.

Eales, J. G., Chang, J. P., Van Der Kraak, G., Omeljaniuk, R. J., and Uin, L. 1982. Effects of temperature on plasma thyroxine and iodide kinetics in rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 47: 295-307.

Eales, J. G. and Finnson K. R. 1991. Response of hepatic thyroxine 5'-deiodinase of rainbow trout, *Oncorhynchus mykiss*, to chronic ingestion of 3,5,3'-triiodo-L-thyronine. J. Exp. Zool. 257: 230-235.

Eales, J. G., Higgs, D. A., Uin, L. M., MacLatchy, D. L., Bres, O., McBride, J. R. and Dosanjh, B. S. 1990. Influence of dietary lipid and carbohydrate levels and chronic 3,5,3'-triiodothyronine treatment on thyroid function in immature rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 80: 146-154.

Eales, J. G. and Himick, B. A. 1988. The effects of TRH on plasma thyroid hormone levels of rainbow trout (*Salmo gairdneri*) and Arctic charr (*Salvelinus alpinus*). Gen. Comp. Endocrinol. 72: 333-339.

Eales, J. G., Holmes, J. A., McLeese J. M. and Youson, J. H. 1997. Thyroid hormone deiodination in various tissues of larval and upstream-migrant sea lampreys, *Petromyzon marinus*. Gen. Comp. Endocrinol. 106: 202-210.

Eales, J. G., Hughes, M. and Uin, L. 1981. Effect of food intake on diel variation in plasma thyroid hormone levels in rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 45: 167-174.

Eales, J. G., MacLatchy, D. L., Higgs, D. A. and Dosanjh, B. J. 1992. The influence of dietary protein and caloric content on thyroid function and hepatic thyroxine 5'-monodeiodinase activity in rainbow trout, *Oncorhynchus mykiss*. Can. J. Zool. 70: 1526-1535.

Eales, J. G., Morin, P. P., Tsang, P. and Hara, T. J. 1993. Thyroid hormone deiodination in brain, liver, gill, heart and muscle of Atlantic salmon (*Salmo salar*) during photoperiodically-induced parr-smolt transformation. II. Outer- and inner-ring 3,5,3'-triiodo-L-thyronine and 3,3',5'-triiodo-L-thyronine (reverse T3) deiodination. Gen. Comp. Endocrinol. 90: 157-167.

Eales, J. G., Omeljaniuk, R. J. and Shostak, S. 1983. Reverse T3 in rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 50: 395-406.

Eales, J. G. and Shostak, S. 1985a. Correlations between food ration, somatic growth parameters and thyroid function in arctic charr, *Salvelinus alpinus* L. Comp. Biochem. Physiol. 80A: 522-558.

Eales, J. G. and Shostak, S. 1985b Free T4 and T3 in relation to total hormone, free hormone indices, and protein in plasma of rainbow trout and Arctic charr. Gen. Comp. Endocrinol. 58: 291-302.

Eales, J. G., Van Der Kraak, G. J., Chang, J. P. and Omeljaniuk R. J. 1986. Effects of temperature on triiodothyronine plasma levels, kinetics, and hepatocyte nuclear binding in rainbow trout, *Salmo gairdneri*. Can. J. Zool. 64: 2658-2664.

Eisenstein, Z., Hagg, S., Vagenakis, A. G., Fang, S. L., Ransil, B., Burger, A., Balsam, A., Braverman, L. E. and Ingbar, S. H. 1978. Effects of starvation on the production and peripheral metabolism of 3,3',5'-triiodothyronine in euthyroid obese subjects. J. Clin. Endocrinol. Metab. 47: 889-893.

Falkner, N. W. and Eales, J. G. 1973. Investigation of iodothyronine binding to plasma proteins in brook trout, *Salvelinus fontinalis*, using precipitation, dialysis, and electrophoretic methods. J. Fish. Res. Board Can. 30: 1131-1140.

Fanjul, A. N. and Farias, R. N. 1991. Novel cold-sensitive cytosolic 3,5,3'-triiodo-L-thyronine-binding proteins in human red blood cell. Isolation and characterization. J. Biol. Chem. 266: 16415-16419.

Farchi-Pisanty, O., Sternberg, H. and Moav, B. 1997. Transcriptional regulation of fish growth hormone gene. Fish Physiol. Biochem. 17: 237-246.

Fines, G. A., Plohman, J. and Eales, J.G. 1999. Effect of experimental 3,5,3'-triiodothyronine hyperthyroidism on thyroid hormone deiodination in brain regions and liver of rainbow trout, *Oncorhynchus mykiss*. Can. J. Zool. 77: 1185-1191.

Finnson, K. W. and Eales, J. G. 1997. Glucuronidation of thyroxine and 3,5,3'-triiodothyronine by hepatic microsomes in rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol 117C: 193-199.

Finnson, K. W. and Eales, J. G. 1998. Sulfation of thyroid hormones by liver of rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. 120C: 415-420.

Finnson, K. W. and Eales, J. G. 1999. Effect of T3 treatment and food ration on hepatic deiodination and conjugation of thyroid hormones in rainbow trout, Oncorhynchus mykiss. Gen. Comp. Endocrinol. 115: 379-386.

Finnson, K. W., McLeese, J. M. and Eales, J. G. 1999. Deiodination and deconjugation of thyroid hormone conjugates and type I deiodination in liver of rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 115: 387-397.

Flood, C. G. and Eales, J. G. 1983. Effects of starvation and refeeding on plasma T4 and T3 levels and T4 deiodination in rainbow trout, *Salmo gairdneri*. Can. J. Zool. 61: 1949-1953.

Fok, P. and Eales, J. G. 1984. Regulation of plasma T3 levels in T4-challenged rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 53: 197-202.

Fok, P., Eales, J. G. and Brown, S. B. 1990. Determination of 3,5,3'-triiodo-L-thyronine (T3) levels in tissues of rainbow trout (*Salmo gairdneri*) and the effect of low ambient pH and aluminum. Fish Physiol. Biochem. 8: 281-290.

Francon, J., Osty, J., Chantoux, F. and Blondeau, J. P. 1990. Erythrocyte-associated triiodothyronine in the rat: a source of hormone for target cells. Acta Endocrinol. Copenh. 122: 341-348.

Frith, S. D. and Eales, J. G. 1996. Thyroid hormone deiodination pathways in brain and liver of rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 101: 323-332.

Gavin, L. A., McMahon, F. A. and Moeller, M. 1981. Carbohydrate in contrast to protein feeding increases the hepatic content of active thyroxine-5'-deiodinase in the rat. Endocrinology. 109: 530-537.

Glick, Z., Wu, S. Y., Lupien, J., Reggio, R., Bray, G. A. and Fisher, D. A. 1985. Meal-induced brown fat thermogenesis and thyroid hormone metabolism in rats. Amer. J. Physiol. 249: E519-E524.

Gregory, L. A. and Eales, J. G. 1975. Factors contributing to high levels of plasma iodide in brook trout, *Salvelinus fontinalis* (Mitchill). Can. J. Zool. 53: 267-277.

Higgs, D. A. and Eales, J. G. 1977. Influence of food deprivation on radioiodothyronine and radioiodide kinetics in yearling brook trout, *Salvelinus fontinalis* (Mitchill), with a consideration of the extent of L-thyroxine conversion to 3,5,3'-triiodo-L-thyronine. Gen. Comp. Endocrinol. 32: 29-40.

Higgs, D. A. and Eales, J. G. 1978. Radiothyroxine kinetics of yearling brook trout, *Salvelinus fontinalis* (Mitchill), on different levels of dietary intake. Can. J. Zool. 56: 80-85.

Higgs, D. A. and Eales, J. G. 1979. Influence of diet composition on radiothyroxine kinetics in brook trout, *Salvelinus fontinalis* (Mitchill). Can. J. Zool. 57: 396-402.

Higgs, D. A., Fagerlund, U. H. M. and McBride J. R. 1979. Influence of orally administered L-thyroxine or 3,5,3'-triiodo-L-thyronine on growth, food consumption, and food conversion of underyearling coho salmon (*Oncorhynchus kisutch*). Can. J. Zool. 56: 1974-1979.

Himick, B. A. and Eales, J. G. 1990. The acute effects of food and glucose challenge on plasma thyroxine and triiodothyronine levels in previously starved rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 78: 34-41.

Himick, B. A., Higgs, D. A. and Eales, J. G. 1991. The acute effects of alteration in the dietary concentrations of carbohydrate, protein, and lipid on plasma T4, T3, and glucose levels in rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 82: 451-458.

Hoar, W. S. and Eales, J. G. 1963. The thyroid gland and low-temperature resistance of goldfish. Can. J. Zool. 41: 654-669.

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

Hulbert, A. J. and Williams, C. A. 1988. Thyroid function in a lizard, a tortoise and a crocodile, compared with mammals. Comp. Biochem. Physiol. 90A: 41-48.

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

Inui, Y. and Miwa, S. 1985. Thyroid hormone induces metamorphosis of flounder larvae. Gen. Comp. Endocrinol. 60: 450-454.

Inui, Y., Tagawa, M., Miwa, S., and Hirano, T. 1989. Effects of bovine TSH on the tissue thyroxine level and metamorphosis in prometamorphic flounder larvae. Gen. Comp. Endocrinol. 74: 406-410.

Johnston, C. E. and Eales, J. G. 1995. Effects of acclimation and assay temperature on outer- and inner-ring thyroxine and 3,5,3'-triiodo-L-thyronine deiodination by liver microsomes of rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 272: 426-434.

Johnston, C. E. Gordillo, C., and Eales, J. G. 1996. Transition from a hatchery to a laboratory environment induces inner-ring monodeiodination pathways for thyroid hormones in liver of rainbow trout, *Oncorhynchus mykiss*. Can. J. Zool. 74: 2178-2183.

Kaplan, M. M. and Utiger, R. D. 1978. Iodothyronine metabolism in liver and kidney homogenates from hyperthyroid and hypothyroid rats. Endocrinology 103: 156-161.

Kaul, K. and Bhattacharya, S. 1988. Thyroid hormone stimulation of the perch (*Anabas testudineus* Bloch) ovarian mitochondrial steroidogenesis. Ind. J. Exp. Biol. 26: 413-417.

Keiffer, J. D., Mover, H., Federico, P. and Maloof, F. 1976. Pituitary-thyroid axis in neonatal and adult rats: Comparison of the sexes. Endocrinology 98: 295-304.

Larsen, P. R. and Berry, M. J. 1995. Nutritional and hormonal regulation of thyroid hormone deiodinases. Ann. Rev. Nutr. 15: 323-352.

Larsen, D. A., Swanson, P., Dickey, J. T., Rivier, J. and Dickhoff, W. W. 1998. *In vitro* thyrotropin-releasing activity of corticotropin-releasing hormone-family peptides in coho salmon, Oncorhynchus kisutch. Gen. Comp. Endocrinol. 109: 276-285.

Leatherland, J. F., Cho, Y. and Hilton, J. 1984. Effect of diet on serum thyroid hormone levels in rainbow trout (*Salmo gairdneri* Richardson). Comp. Biochem. Physiol. 78A: 601-605.

Leatherland, J. F. and Farbridge, K. J. 1992. Chronic fasting reduces the response of the thyroid to growth hormone and TSH, and alters the growth hormone-related changes in hepatic 5'-monodeiodinase activity in rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 87: 342-353.

Leatherland, J. F. and Flett, P. A. 1988. Effect of propanolol in combination with TSH and ovine prolactin on plasma thyroid hormone levels and in vitro hepatic monodeiodination of thyroxine in brook charr, *Salvelinus fontinalis* (Mitchill). Comp. Biochem. Physiol. A. 91: 371-376.

Leloup, J. 1970. Mémoires du muséum national d'histoire naturelle: les mécanismes de régulation de l'iodurémie et leur controle endocrinien chez les téléostéens en eau douce. Paris Éditions du museum.

Leloup, J. and Fontaine, M. 1960. Iodine metabolism in lower vertebrates. Ann. N. Y. Acad. Sci. 86 : 316-353.

Leonard, J. L., Siegrist-Kaiser, C. A. and Zuckerman, C. J. 1990. Regulation of type II iodothyronine 5'-deiodinase by thyroid hormone. J. Biol. Chem. 265: 940-946.

Leonard, J. L. and Visser, T. J. 1986. *Biochemistry of Deiodination*. In Thyroid Hormone Metabolism (G. Henneman, Ed.), pp.189-230. Dekker, New York / Basel.

Licht, P., Denver, R. J. and Stamper, D. L. 1990. Relation of plasma thyroxine binding to thyroidal activity and determination of thyroxine binding proteins in a turtle, *Pseudemys scripta*. Gen. Comp. Endocrinol. 80: 238-256.

Lindahl, K., Lundqvist, H. and Rydevik, M. 1983. Plasma thyroxine levels and thyroid gland histology in Baltic salmon (*Salmo salar* L.) during smoltification. Can. J. Zool. 61: 1954-1958.

Luo, D. and McKeown, B. A. 1991. The effect of thyroid hormone and glucocorticoids on carp growth hormone-releasing factor (GRF)-induced growth hormone (GH) release in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. 99A: 621-626.

MacKenzie, D. S., VanPutte, C. M. and Leiner, K. A. 1998. Nutrient regulation of endocrine function in fish. Aquaculture 161: 3-25.

MacLatchy, D. L. and Eales, J. G. 1990. Growth hormone stimulates hepatic thyroxine 5'-monodeiodinase activity and 3,5,3'-triiodothyronine levels in rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 78: 164-172.

MacLatchy, D. L. and Eales, J. G. 1992. Properties of T4 5'-deiodinating systems in various tissues of the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 86: 313-322.

MacLatchy, D. L. and Eales, J. G. 1993. Effect of T3 and T4 challenge on inner- and outer-ring deiodination of T3 and T4 in the liver, kidney, and gill of rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 265: 637-645.

MacLatchy, D. L., Kawauch, H. and Eales, J. G. 1992. Stimulation of hepatic thyroxine 5'-deiodinase activity in rainbow trout (*Oncorhynchus mykiss*) by Pacific salmon growth hormone. Comp. Biochem. Physiol. 101A :689-691.

McCormick, S. D. and Saunders, R. L. 1990. Influences of ration level and salinity on circulating thyroid hormones in juvenile Atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 78: 224-230.

McEnroe, M. and Cech, J. J. 1994. *Measurements of thyroid hormones, T3 and T4, in juvenile white sturgeon over the annual cycle.* High Performance Fish Proceedings of an International Fish Physiology Symposium at the University of British Columbia, in Vancouver Canada. Ed. Machinlay, D. D., Vancouver BC – Canada Fish Physiology Association. Pp. 182-185.

McLeese, J. M., Waytiuk, A. and Eales, J. G. 1998. Factors influencing the steady-state distribution and exchange of thyroid hormones between red blood cells and plasma of rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 109: 259-268.

McLeese, J. M., Wright, G. M., Youson, J. H. and Eales, J. G. 2000. Deiodination activity in extrathyroidal tissues of the Atlantic hagfish, *Myxine glutinosa*. J. Exp. Zool. (in press)

McNabb, F. M. A. 1992. *Thyroid Hormones*. Englewood Cliffs, NJ: Prentice-Hall. 283 pp.

Melamed, P., Eliahu, N., Levavi-Sivan, B., Ofir, M., Farchi-Pisantry, O., Rentier-Delrue, F., Smol, J., Yaron, Z. and Naor, Z. 1995. Hypothalamic and thyroidal regulation of growth hormone in tilapia. Gen. Comp. Endocrinol. 97: 13-30.

Mol, K., Kaptein, E., Darras, V. M., de Greef, W. J., Kuhn E. R., and Visser T. J. 1993. Different thyroid hormone-deiodinating enzymes in tilapia (*Oreochromis niloticus*) liver and kidney. FEBS Lett. 321: 140-144.

Mol, K. A., Van Der Geyten, S., Darras, V. M. Visser T. J., and Kuhn, E. R. 1997. Characterization of iodothyronine outer ring and inner ring deiodinase activities in the blue tilapia, *Oreochromis Aureus*. Endocrinology. 138: 1787-1793.

Morin, P. P., Hara, T. J., and Eales, J. G. 1993. Thyroid hormone deiodination in brain, liver, gill, heart, and muscle of Atlantic salmon (*Salmo salar*) during photoperiodically-induced parr-smolt transformation. 1. Outer- and inner-ring thyroxine deiodination. Gen. Comp. Endocrinol. 90: 142-156.

Moyle, P. B. and Cech Jr, J. J. 1996. Fishes: An introduction to ichthyology. 3rd Edition. Pp590.

Morin, P. P., Hara, T. J., and Eales, J. G. 1995. T4 depresses olfactory responses to L-alanine and plasma T3 and T3 production in smoltifying Atlantic salmon. Am. J. Physiol. 269: R1434-R1440.

Nejad, I., Bollinger, J., Mitnick, M. A., Sullivan, P., and Reichlin, S. 1975. Measurement of plasma and tissue triiodothyronine concentration in the rat by radioimmunoassay. Endocrinology 96: 773-780.

Obregon, M. J., Morreale-de-Escobar, G., and Escobar-del-Rey, F. 1978. Concentrations of triiodo-L-thyronine in the plasma and tissues of normal rats, as determined by radioimmunoassay: comparison with results obtained by an isotopic equilibrium technique. Endocrinol. 103: 2145-2153.

Parker, S. J., and Specker, J. L. 1990. Salinity and temperature effects on whole-animal thyroid hormone levels in larval and juvenile striped bass, *Morone saxatilis*. Fish Physiol. Biochem. 8: 507-514.

Peter, R. E. 1970. Comparison of the activity of the pronephric thyroid and the pharyngeal thyroid of the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 14: 334-356.

Plisetskaya, E. M. and Sullivan, C. V. 1989. Pancreatic and thyroid hormones in rainbow trout (*Salmo gairdneri*): What concentration does the liver see? Gen. Comp. Endocrinol. 75: 310-315.

Redding, J. M., Schreck, C. B., Birks, E. K., and Ewing, R. D. 1984. Cortisol and its effects on plasma thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 56: 146-155.

Riley, JR., W. W., and Eales, J. G. 1993a. Characterization of L-thyroxine transport into hepatocytes isolated from juvenile rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 90: 31-42.

Riley, JR., W. W., Higgs, D. A., Dosanjh, B. S., and Eales, J. G. 1993b. Influence of dietary amino acid composition on thyroid function of juvenile rainbow trout, *Oncorhynchus mykiss*. Aquaculture 112: 253-269.

Riley, JR., W. W. and Eales, J. G. 1994. Characterization of 3,5,3'-triiodo-L-thyronine transport into hepatocytes isolated from juvenile rainbow trout (*Oncorhynchus mykiss*), and comparison with L-thyroxine transport. Gen. Comp. Endocrinol. 95: 301-309.

Rivas, R. J., Grau, E. G., Nishioka, R. S., Dern, H. A., Dickhoff, W. W., Sullivan, C., Knutson, A. C. and Jochimsen, W. 1982. Abstracts. Effects of feeding triiodothyronine (T3) on thyroxine (T4) levels in the steel head trout, *Salmo gairdneri*. Am. Zool. pp. 854.

Santos, C. R. and Power, D. M. 1999. Identification of transthyretin in fish (*Sparus aurata*): cDNA cloning and characterization. Endocrinology 140: 2430-2433.

Sanders, J. P., Van Der Geyten, S., Kaptein, E., Darras, V. M., Kuhn, E. R., Leonard, J. L. and Visser, T. J. 1997. Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. Endocrinology 138: 5153-5160.

Saunders, R. L., McCormick, S. D., Henderson, E. B., Eales, J. G. and Johnston, C. E. 1985. The effect of orally administered 3,5,3'-triiodothyronine on growth and salinity tolerance of Atlantic salmon (*Salmo salar* L.). Aquaculture 45: 143-156

Schreiber, G., Aldred, A. R., Jaworowski, A., Nilsson, C., and Achen, M. G. and Segal, M. B. 1990. Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. Am. J. Physiol. 258: R338-345.

Scott, W. B. and Crossman, E. J. 1998. *Freshwater Fishes of Canada*. Galt House Publications Ltd. Oakville, Ont. Pp. 966.

Sefkow, A. J., DiStefano III, J. J., Himick, B. A., Brown, S. B. and Eales, J. G. 1996. Kinetic analysis of thyroid hormone secretion and interconversion in the 5-day-fasted rainbow trout, Oncorhynchus mykiss. Gen. Comp. Endocrinol. 101: 123-138.

Sharp, P. J., Klandorf, H. and Lea, R. W. 1984. Influence of lighting cycles on daily rhythms in concentrations of plasma tri-iodothyronine and thyroxine in intact and pinealectomized immature broiler hens (*Gallus domesticus*). J. Endocrinol. 103: 337-345.

Shi, Y. B. 1995. Cell-cell and cell-ECM interactions in epithelial apoptosis and cell renewal during frog intestinal development. Cell Biochem. Biophys. 27: 179-202.

Shields, C. A. and Eales, J. G. 1986. Thyroxine 5'-monodeiodinase activity in hepatocytes of rainbow trout, *Salmo gairdneri*: Distribution, effects of starvation, and exogenous inhibitors. Gen. Comp. Endocrinol. 63: 334-343.

Sinclair, D. A. and Eales, J. G. 1974. Enterohepatic cycling of thyroxine in starved and fed brook trout, *Salvelinus fontinalis* (Mitchill). Comp. Biochem. Physiol. 49A: 661-672.

Slebodzinski, A. B., Brzezinska-Slebodzinska, E. and Drews, R. 1982. Reciprocal changes in serum 3,3',5'-tri-iodothyronine concentration and the peripheral thyroxine inner ring monodeiodination during food restriction in the young pig. J. Endocr. 95: 349-355.

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

Specker, J. L., Brown, C. L. and Bern, H. A. 1992. Asynchrony of changes in tissues and plasma thyroid hormones during the parr-smolt transformation of coho salmon. Gen. Comp. Endocrinol. 88: 397-405.

Specker, J. L. and Richman III, N. H. 1984. Environmental salinity and the thyroidal response to thyrotropin in juvenile coho salmon (*Oncorhynchus kisutch*). J. Exp. Zool. 230: 329-333.

St. Germain, D. L., Schwartzman, R. A., Croteau, W., Kanamori, A., Wang, Z. and Brown, D. D., and Galton, V. A. 1994. A thyroid hormone-regulated gene in *Xenopus laevis* encodes a type III iodothyronine 5-deiodinase. Proc. Natl. Acad. Sci. USA. 91: 11282.

St. Germain, D. L. 1986. Hormonal control of a low Km (Type II) iodothyronine 5'-deiodinase in cultured NB41A3 mouse neuroblastoma cells. Endocrinology 119: 840-846.

Stock, M. J., Chapman, C., Stirling, J. L. and Campbell, I. T. 1978. Effects of exercise, altitude, and food on blood hormone and metabolite levels. J. Applied Physiol. 45: 350-354.

Sullivan, C. V., Darling D. S. and Dickhoff, W. W. 1987. Nuclear receptors for L-triiodothyronine in trout erythrocytes. Gen. Comp. Endocrinol. 65: 149-160.

Sweeting, R. M. and Eales, J. G. 1992a. HPLC analysis of *in vitro* hepatic deiodination products of thyroid hormones in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 85: 367-375.

Sweeting, R. M. and Eales, J. G. 1992b. The acute influence of ingested thyroid hormone on hepatic deidination pathways in the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 85: 376-384.

Sweeting, R. M. and Eales, J. G. 1992c. Thyroxine 5'-monodeiodinase activity in microsomes from isolated hepatocytes of rainbow trout: Effects of growth hormone and 3,5,3'-triiodo-L-thyronine. Gen. Comp. Endocrinol. 88: 169-177.

Sweeting, R. M. and Eales, J. G. 1993. The effects of fasting on hepatic thyroxine monodeiodination activity in the rainbow trout, *Oncorhynchus mykiss*. Can. J. Zool. 70: 1516-1525.

Sweeting, R. M., Alexander, G. P., Eales, J. G. and McKeown, B. A. 1994. Hepatic and branchial thyroid hormone deiodinase activities associated with the parr-smolt transformation of coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. 94: 171-181.

Tanabe, Y., Ishii, T. and Tamaki Y. 1969. Comparison of thyroxine-binding plasma proteins of various vertebrates and their evolutionary aspects. Gen. Comp. Endocrinol. 13: 14-21.

Timiras, P. S. and Nzekwe, E. U. 1989. Thyroid hormones and nervous system development. Biol. Neonate. 55: 376-385.

Valverde, C. R., Croteau, W., Lafleur, G. J., Orozco, A. and St. Germain, L. 1997. Cloning and expression of a 5'-iodothyronine deiodinase from the liver of *Fundulus heteroclitus*. Endocrinology 138: 642-648.

Van Der Hayden, J. T. M., Docter, R., Van Toor, H., Wilson, J. H. P., Henneman, G. and Krenning, E. P. 1986. Effects of caloric deprivation on thyroid hormone tissue uptake and generation of low-T3 syndrome. Amer. J. Physiol. 251: E156-E163.

Van Der Kraak, G. J. and Eales, J. G. 1980. Saturable 3,5,3'-triiodothyronine-binding sites in liver nuclei of rainbow trout (*Salmo gairdneri* Richardson). Gen. Comp. Endocrinol. 42 : 437-448.

Van Doorn, J., Roelfsema, F. and Van der Heide, D. 1985. Concentrations of thyroxine and 3,5,3'-triiodothyronine at 34 different sites in euthyroid rats as determined by an isotopic equilibrium technique. Endocrinology 117 : 1201-1208.

Veronikis, I. E., Braverman, L. E., Alex, S., Fang, S. L., Norvell, B. and Emerson, C. H. 1996. Comparison of the effects of propylthiouracil and selenium deficiency on T3 production in the rat. Endocrinology 137 : 2580-2585.

Visser, T. J. 1990. Importance of deiodination and conjugation in the hepatic metabolism of thyroid hormone. In: Green M. A., editor. The Thyroid Gland. New York: Raven Press, Pp. 255-283.

Whittaker, A. L. and Eales, J. G. 1993. Comparison of 3,5,3'-triiodo-L-thyronine and L-thyroxine absorption from the intestinal lumen of the fasted rainbow trout, *Oncorhynchus mykiss*. Fish Physiol. Biochem. 10: 431-441.

Yamauchi, K., Nakajima, J., Hayashi, H., Hara, A. and Yamauchi K. 1999. Purification and characterization of thyroid-hormone-binding protein from masu salmon serum – A homolog of higher-vertebrate transthyretin. Eur. J. Biochem. 265: 944-949.