A STUDY ON THE SITES OF THYROXINE DEIODINATION IN BROOK TROUT, SALVELINUS FONTINALIS, (MITCHILL).

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TO

MY PARENTS

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ABSTRACT

Trout tissue homogenates were incubated *in vitro* with radiothyroxine (T_4*) at acclimation temperature for up to 3 hours. Deiodination (measured by the production of radioiodide using protein-precipitation and chromatography) occurred in brain, gill, stomach, duodenum, intestine, heart, kidney and muscle but not in liver. Chromatography shows that iodide is the only detectable radioactive deiodination product. A thermostable deiodinating system is present in brain and heart.

GLOSSARY

DIT = diiodotyrosine

FMN = flavin mononucleotide

$^{125}I\%$ = percentage free radioiodide

MIT = monoiodotyrosine

MMI = methyl mercaptoimidazole

$PB^{125}I$ = protein-bound radioiodide

RT_3 = reverse triiodothyronine

Rtriac = reverse triiodothyroacetic acid

T_1 = monoiodothyronine

T_2 = diiodothyronine

T_3 = triiodothyronine

T_3F = triiodothyroformic acid

T_3P = triiodothyropropioninc acid

T_4 = thyroxine

$T_4^* = {}^{125}I\text{-}T_4$ = radiothyroxine

TCA = trichloroacetic acid

Tetrac = tetraiodothyroacetic acid

TLC = thin-layer chromatography

W = amount (μg) of thyroxine deiodinated per mg of dry tissue weight

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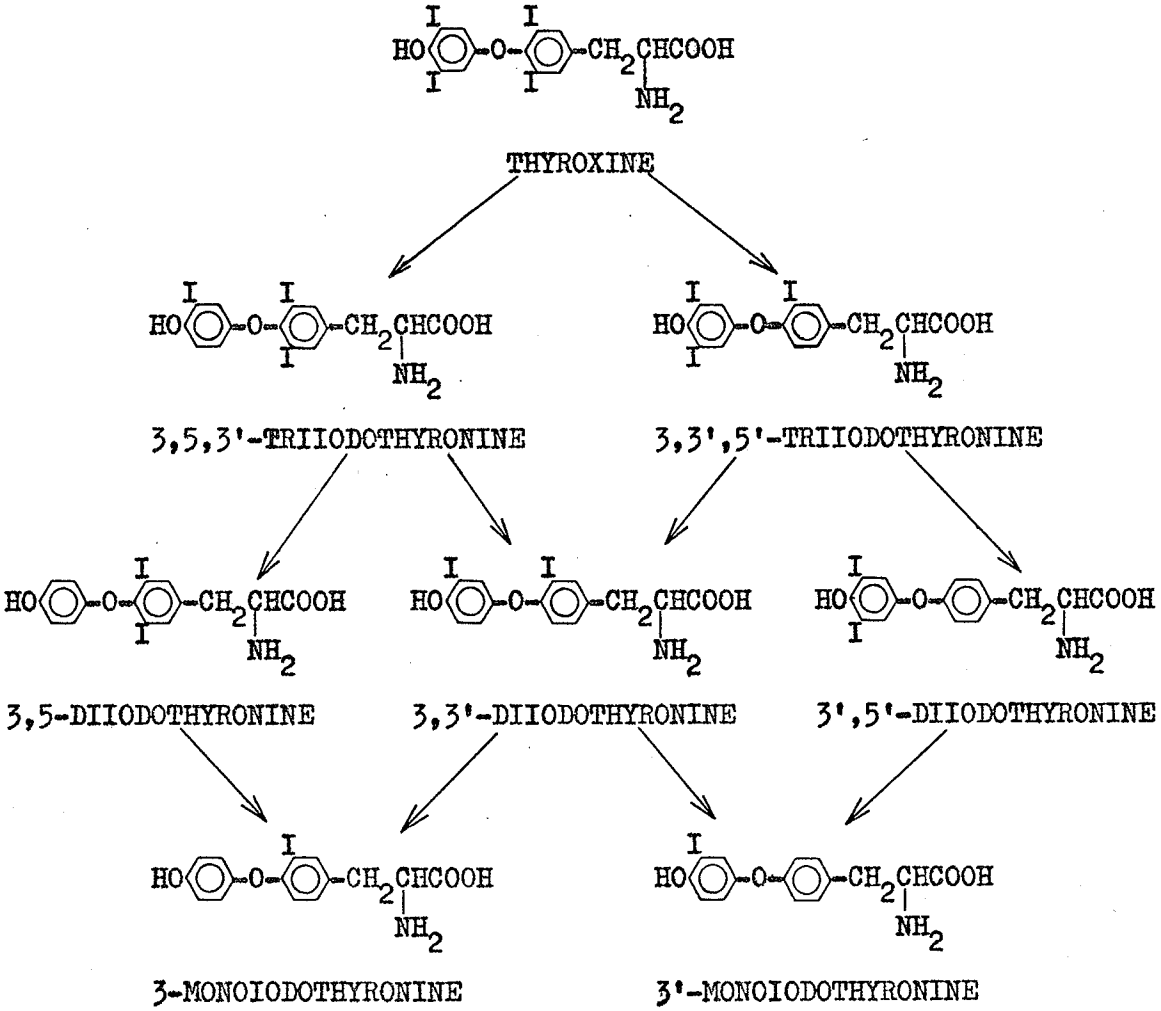
INTRODUCTION

The structure of thyroxine (T_4) and the occurrence of the derivatives: 3,5,3'-triiodothyronine (T_3), 3,3',5'-triiodothyronine (RT_3), 3,5-diiodothyronine (3,5-T_2), 3,3'-diiodothyronine (3,3'-T_2), 3',5'-diiodothyronine (3',5'-T_2), 3-monoiodothyronine (3-T_1), 3'-monoiodothyronine (3'-T_1), suggest that T_4 is probably catabolized in the following fashion to release free iodide and form thyronine derivatives:



This process is generally believed to be enzymatic. Tata (1960) has studied the purification of thyroxine dehalogenase, and work has also been done in relation to an enzyme, tyrosine deiodinase (Greer & Grimm 1968; Kozyreff et al 1970). Haibach (1971) reported another possible enzymatic mechanism whereby T_4 can be deiodinated.

In mammals, deiodination of T_4 has been reported by Flock et al 1957; Plaskett 1961; Wynn & Gibbs 1962; Bénévent et al 1963; Escobar del Rey & Morreale de Escobar 1964; Goodman & Gilman 1965; Pittman & Shimizu 1966; Reichlin et al 1966; Brown-Grant 1967; and Schwartz et al 1969. Liver, muscle and kidney are the main sites of deiodination. A number of deiodination products has been discovered: T_3 (Sprott & Maclagan 1955; Flock et al 1957; Flock et al 1961; Reichlin et al 1966); diiodotyrosine (DIT) (Wynn & Gibbs 1962); glucuroconjugates (Flock et al 1957); and a number of unknowns (Flock et al 1957; Wynn & Gibbs 1962; Pittman & Shimizu 1966) including one suspected to be 3'-hydroxy-3:5-diiodothyronine (Plaskett 1961). From comparison of the deiodination activity of tissues of animals that do or do not respond to thyroid hormones, Galton & Ingbar (1962 a,b) suggested that deiodination is a prerequisite for the action of the hormones. However, other findings (Anbar et al 1965) produced contrary opinions.

The non-deiodinated products, tetraiodothyroacetic acid

(tetrac) and T_4 glucuroconjugates were found in dogs and rats (Flock et al 1957; Flock et al 1961; Pittman & Shimizu 1966). Wynn & Gibbs (1962) reported a rupture of the diphenyl linkage in their in vitro experiments, but in vivo findings by Pittman & Shimizu (1966) showed the opposite result.

Deiodination of T_4 has also been demonstrated in several species of tadpoles and adults of frogs and toads (Tata 1960; Dowling et al 1964; Flock et al 1963; Yamamoto 1964; and Dowling & Razevska 1966) but could not be demonstrated in Necturus maculosus (Galton & Ingbar 1962b). Liver and muscle are the most potent sites but no deiodination product besides iodide was discovered. In most cases, a deaminated and decarboxylated product, tetrac, was found (Galton & Ingbar 1962a; Dowling et al 1964; and Dowling & Razevska 1966).

Little is known about the deiodination of T_4 in fish. Tata (1960) postulated the presence of thyroxine dehalogenase in muscle and liver of plaice (Pleuronectes platessa) and trout (Salmo fario). However, these findings were probably due to the effects of added ferrous ions (Fe^{++}) and flavin mononucleotide (FMN) which caused non-enzymatic deiodination. Osborn & Simpson (1969), in their in vivo experiments with adult plaice, recovered radioactivity from injected ^{125}I-labelled thyroxine in the form of 3,5,3'-triiodothyronine, 3,3',5'-triiodothyronine, and 3,3'-diiodothyronine (products of stepwise deiodination), together with tetraiodothyropyruvic acid, tetraiodothyrolactic

acid and tetraiodothyroacetic acid (products of deamination), and sulpho- and glucuro-conjugates (products of conjugation).

From *in vivo* finding Eales (1970) showed that deiodination does occur in brook trout.

However, prior to any studies on the enzymatic nature of deiodination, and possibly, a determination of the target sites of T_4, in brook trout, certain data are necessary. These include the organs or tissues responsible for deiodination of T_4, the activity of these organs or tissues and their deiodination products.

This thesis presents a study on these questions by *in vitro* methods. Trichloroacetic acid precipitation and chromatography were used to determine:

a) the sites of deiodination

b) extent of deiodination in each site

c) identification of products of deiodination.

MATERIALS AND METHODS

Brook trout (one- or two-year old) from the Province of Manitoba Trout Hatchery, West Hawk Lake were acclimated for 3 weeks to eight months at temperatures between 8.5 and 14.0 C. The fish were fed every alternate days with ground liver.

In each experiment, 5 fish were sacrificed and the same organs were pooled and homogenized. The homogenate was incubated in T_4 labelled with ^{125}I at 3' and 5' positions (Amersham/ Searle Corp.). By precipitating the thyronines with trichloro-acetic acid (TCA), deiodination was measured by comparing the amounts of radioiodide present in the supernatant before and after incubation. Chromatography was carried out to show the products of deiodination.

A. Preparation of reaction medium and incubation

Each of the five trout was killed by applying a blow at the head. Individual organs were removed and suspended in 30-50 ml of Hickman's saline (composition in grams/litre: NaCl 6.42; KCl 0.15; $CaCl_2$ 0.22; $MgSO_4$ 0.12; $NaHCO_3$ 0.084; NaH_2PO_4 0.06). The same organs from 5 fish were pooled. The suspension was homogenized with a Sorvall omnimixer in an ice bath and 26.4 ml of homogenate was added to 6.6 ml of 0.5M Tris buffer (pH 7.8) and 26.4 ml distilled water (Yamamoto 1964). This homogenate medium was pipetted into glass tubes in small portions; 4.4 ml for TCA precipitation and 0.9 ml for chromatography. The tubes

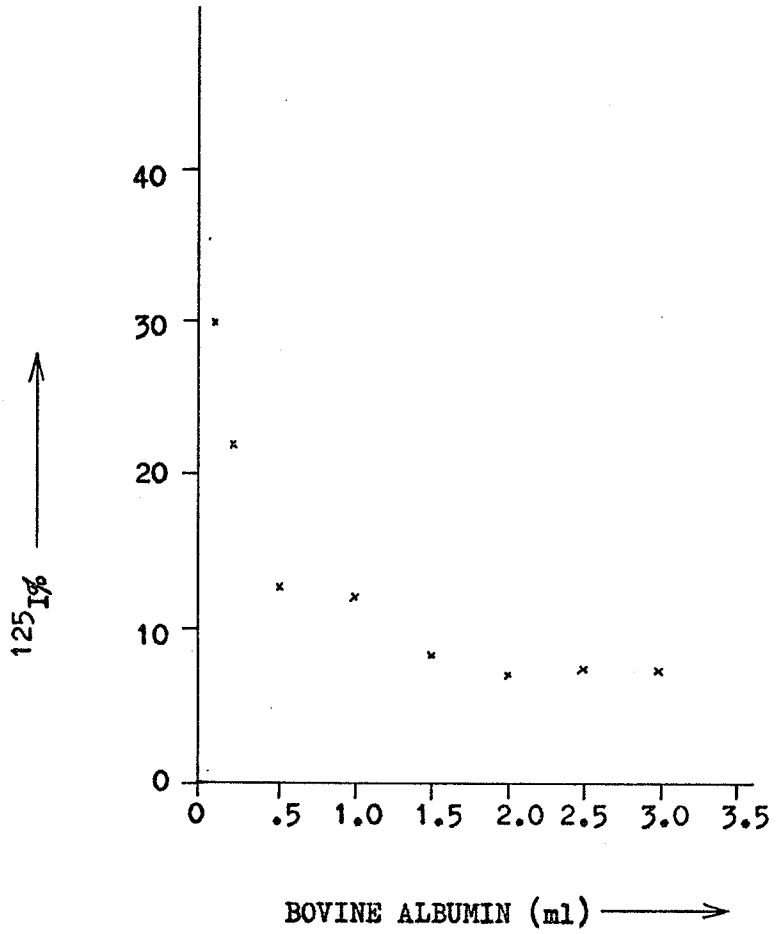
were incubated in a covered water bath with shaking at 1500 rpm for 1, 2 or 3 hours. The bath was set at a temperature corresponding to the acclimation temperature of the trout used. One set of tubes (Control, Boiled Homogenate, and Homogenate) was not incubated but was used immediately for analysis. This set represented 0 hour. In the control tube, Hickman's saline was used in place of homogenate. In the boiled homogenate tube, the homogenate medium was boiled for 3 minutes and then chilled in the refrigerator.

All the tubes were allowed to equilibrate in the water bath before the introduction of $^{125}I\text{-}T_4$. Tubes for TCA precipitation received 0.01 ml of a $^{125}I\text{-}T_4$ solution made up of 1 mg of sodium salt of dehydrated $L\text{-}T_4$ dissolved in a minimum amount of 0.1N NaOH, to which was added approximately 5 μCi of $^{125}I\text{-}T_4$ (specific activity 30-50 mCi/mg), and the total volume was made up to 25.0 ml with distilled water. Tubes for chromatography received about 0.2 μCi $^{125}I\text{-}T_4$ (in 50% propylene glycol).

Two ml of Hickman's saline and 2.0 ml of homogenate medium were dried in the oven (80 C) to constant weight in separate aluminum dishes. Dry tissue weight was calculated by substracting the dry weight of the Hickman's saline from the dry weight of the homogenate medium.

B. Deiodination by % free radioactivity after TCA precipitation

Preliminary tests (Figure 1) showed that 2.5 ml of 7% aqueous


40
30
20
10
0
^{125}I%
0
.5
1.0
1.5
2.0
2.5
3.0
3.5
BOVINE ALBUMIN (ml)

solution of Bovine Albumin (Sigma Chemical Co.) was sufficient to bind with the T_4 in the reaction medium within 1½ hours of refrigeration. Protein-bound radiothyroxine (PB^{125}I) was precipitated by 3.0 ml 50% TCA containing 2×10^{-3}M KI and a trace of ascorbic acid. After centrifugation for 15 minutes at 1050 x G, the residue was washed by 1.9 ml of 50% TCA. The volume of the supernatant was then taken as 12.0 ml and an aliquot of 4.0 ml was counted in a glass vial in a well-type counter with a 2¼ inch by 2¼ inch crystal (Nuclear Chicago, DS-202). The residue was broken up in 2.0 ml distilled water, dissolved by adding 2.0 ml 1N NaOH in a glass vial and its radioactivity was counted.

Percentage of free inorganic radioiodide (^{125}I%) was calculated as

$$\frac{(\text{cpm for 4.0 ml supernatant} \times 3)}{(\text{cpm for 4.0 ml supernatant} \times 3) + (\text{cpm for residue})} \times 100$$

The extent of deiodination, weight (W) in µg of T_4 deiodinated/mg dry weight, was calculated as

$$\frac{(^{125}\text{I\%})_{\text{homogenate}} - (^{125}\text{I\%})_{\text{control}}}{\text{Dry Tissue Weight}} \times 0.04\ \mu g$$

since 0.04 µg is the amount of T_4 in 0.01 ml of the ^{125}I-T_4 solution used for TCA precipitation analysis.

C. Chromatographic Analysis

1. Paper Chromatography

Approximately 25 λ of the sample, containing 10^{-3}M methyl

mercaptoimidazole (MMI) to prevent iodination due to formation of iodine, was applied on a 10.0 cm line on Whatman Paper #1. The sample was dried by a stream of cool air. Authentic substances (T_4, T_3 and ^{125}I) were spotted on both sides. The chromatogram was developed in a descending system (1-butanol : glacial acetic acid : water, 4:1:1 v/v; BAW) for 14-16 hours. Each chromatogram was cut into strips 1.0 cm wide which were rolled up and counted in individual glass vials.

The counts were plotted on a graph to make up the radiochromatogram. The position of the peaks was compared with that of the authentic substances.

Percentage of free inorganic radioiodide ($^{125}I\%$) was calculated as

$$\frac{\text{Total cpm for } ^{125}I \text{ peak}}{\text{Total cpm for the chromatogram}} \times 100$$

2. Thin-layer Chromatography (TLC)

Thirteen authentic substances: L-thyroxine, tetraiodothyropropionic acid, tetraiodothyroacetic acid, 3,5,3'-triiodothyronine, sodium salt of 3,5,3'-triiodothyronine, 3,3', 5'-triiodothyronine, 3,5,3'-triiodothyropropionic acid, 3,5,3'-triiodothyroacetic acid, 3,3',5'-triiodothyroacetic acid, 3,5,3'-triiodothyroformic acid, 3'-monoiodothyronine, diiodotyrosine (DIT), and monoiodotyrosine (MIT), were spotted on Baker silica gel F (thickness 0.25 mm) with spots not more than 5 mm wide and not less than 5 mm apart. Chromatograms were developed in 9

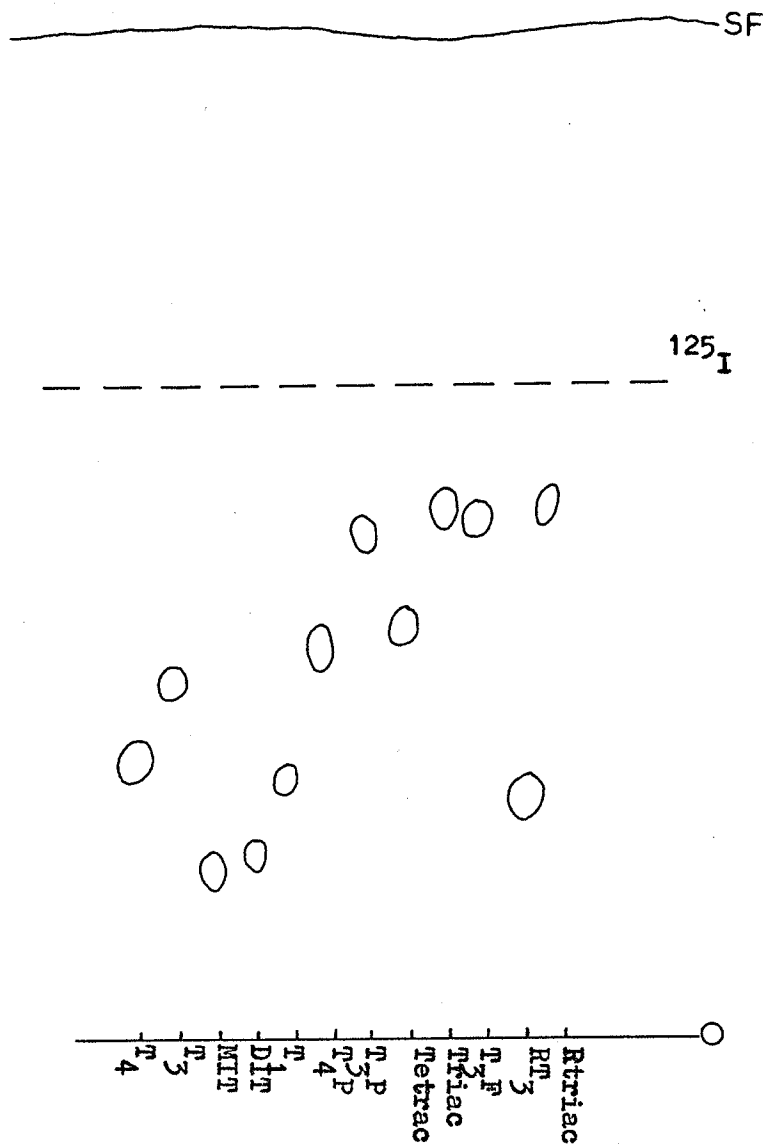
solvent systems (Appendix) among which two systems, BEA (1-butanol : ethanol : 6N ammonia, 5:2:1) and BMA (1-butanol : methabol : 6N ammonia, 5:2:1), were most satisfactory as seen from tracings under U/V (254 mμ) illumination (Figure 2).

There were a few modifications for TLC analysis experiments. Samples of two fish were used and the fish were bled before their organs were removed. Also the tubes were incubated for two hours **only** except for short time-sequence analysis done on gill and intestine.

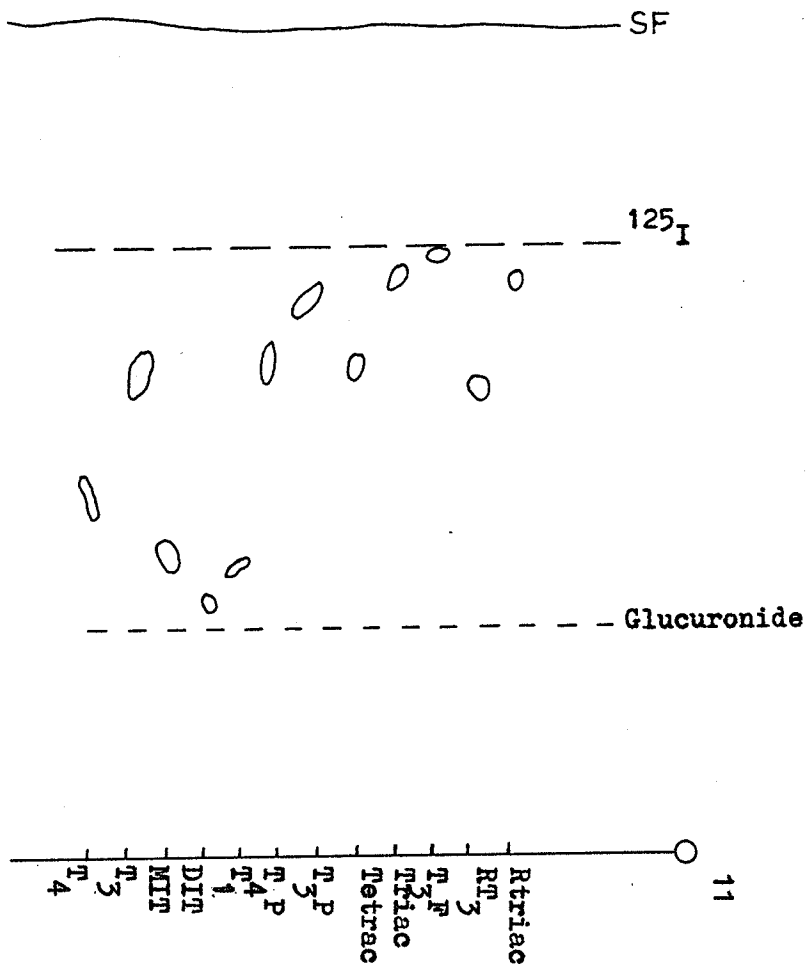
An approximately 10λ aliquot of extract in 10^{-3}M MMI was applied on a 2 inch line and developed in an ascending chromatographic tank. Each chromatogram was cut into strips of 1/8 **inch** wide and not longer than 1 inch. The strips were counted in glass vials. The counts were plotted on a graph to make up the radiochromatogram and the position of the peaks were compared with that of the authentic substances and ^{125}I spotted on the sides of the chromatogram.

D. Tissues Examined

Homogenates from brain, gill (mid-ventral thyroid-containing tissues removed), stomach, duodenum (including pyloric caecae), intestine, heart, kidney, liver (gall bladder excluded), and muscle were examined with all three methods. TCA precipitation was also done on blood, skin and spleen.


SF
125I
T4
T3
MIT
DIT
T1
T3P
T4P
T2P
Tetrac
T4F
T3F
RT3
Rtriac

BEA


SF
125I
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T4
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MIT
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T1
T4P
T3P
T2P
Tetrac
T4F
T3F
RT3
Rtriac
11

BMA

RESULTS

A. Preliminary survey of tissues

Homogenates of 12 tissues were incubated for 3 hours at temperatures corresponding to the acclimation temperatures of the fish (8.5-11 C) (Table I). Brain, gill, stomach, duodenum, intestine, heart and kidney homogenates showed more free radioiodide than their controls suggesting deiodination. Muscle showed very slight deiodination. Liver, skin and spleen homogenates gave no evidence of deiodination, and neither did whole blood, eliminating the possibility of deiodination by blood itself in any of the tissues.

Factors suspected to affect deiodination were examined. The possibilities of homogenization or temperature inhibiting deiodination in the non-deiodinating tissues were partly ruled out by experiments run with the above procedure but using tissue slices and intact gill filaments instead of homogenates (Table II), and incubating homogenates at different temperatures (Table III). Kidney, liver and muscle slices all failed to show deiodination. Even in the case of gill filaments, deiodination was extremely slow as compared with its homogenate. Variation in incubation temperature did not induce deiodination in liver or spleen. When mouse liver homogenate was incubated at 37 C under the same procedure, 36-43% deiodination of T_4 was measured, suggesting that lack of deiodination in trout liver was not due to a fault in

Table I: Extent of thyroxine deiodination in trout tissue homogenates incubated at acclimation temperatures (8.5-11.0 C) for 3 hours. Deiodination is indicated by the percentage of free iodide in the homogenate which is not precipitated by TCA. Each experiment consists of 5 replicates.

Tissue	No. of Experiments	Dry Tissue Weight (mg)		$^{125}I\%$			
				Control		Homogenate	
		$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE
Whole blood	1	3.10	-	4.79	-	5.78	-
Brain	3	5.43	.88	5.50	.75	10.60	1.34
Gill*	6	5.83	.84	4.81	.55	14.24	4.70
Stomach	3	7.10	2.27	5.22	.31	11.75	2.63
Duodenum	3	28.50	5.34	5.22	.31	43.73	5.22
Intestine	3	13.17	7.03	5.22	.31	29.28	3.60
Heart	3	4.43	.19	5.23	.51	6.80	.91
Kidney*	5	12.68	2.42	5.03	.62	10.03	.84
Liver	4	34.28	9.89	5.51	.67	5.08	.60
Muscle	3	18.03	8.06	5.39	1.00	6.01	.93
Skin	1	3.90	-	4.93	-	5.21	-
Spleen	2	2.00	.10	5.04	.06	5.39	.13

* One experiment consisted of 10 tubes of each sample.

Table II: Extent of thyroxine deiodination in trout tissue slices incubated at acclimation temperatures (8.5-9.5 C) for 3 hours. Deiodination is indicated by the percentage of free iodide in the reaction medium which is not precipitated by TCA. Each value is the mean of 5 replicates.

Tissue	Dry Tissue Weight (mg)	$^{125}I\%$			
		Control $\bar{X}$	SE	Slices $\bar{X}$	SE
Gill filaments	27.3	3.98	.17	4.88	.32
Kidney	5.3	4.13	.24	5.15	.35
Liver	23.4	4.57	.26	4.70	.23
Muscle	5.6	4.39	.31	4.66	.17

Table III: Effects of temperature on deiodination of thyroxine by trout tissue homogenates incubated for 3 hours. Fish were acclimated at temperatures between 9-10 C. Deiodination is indicated by the percentage of free iodide in the homcgenate which is not precipitated by TCA, and amount of thyroxine deiodinated per mg of dry tissue weight. Each value is the mean of 5 replicates.

Tissue	Incubation Temp. (C)	Dry Tissue Weight (mg)	^{125}I% Control $\bar{X}$	SE	^{125}I% Homogenate $\bar{X}$	SE	W ug T_4/ mg dry tissue
Brain	5	2.6	6.23	.09	8.15	.23	.0300
	15	10.0	6.95	.17	12.74	.33	.0230
	20	12.9	7.77	.20	22.30	.71	.0450
Gill	5	7.3	6.61	.32	16.87	1.32	.0560
	15	12.7	6.81	.25	13.34	.79	.0210
	20	6.3	7.21	.39	21.47	.93	.0910
Stomach	5	6.2	4.42	.21	7.75	.30	.0210
	15	5.5	7.42	.24	10.31	.39	.0210
	20	7.1	6.82	.24	10.95	.99	.0230
Duodenum	5	30.9	4.42	.21	35.95	.63	.0410
	15	27.3	7.42	.24	46.11	.92	.0570
	20	17.1	6.82	.24	42.83	.34	.0840
Intestine	5	9.6	4.42	.21	7.17	.26	.0110
	15	8.8	7.42	.24	10.27	.32	.0130
	20	9.0	6.82	.24	12.80	.51	.0270
Heart	5	5.7	6.23	.09	6.92	.48	.0050
	15	11.0	6.95	.17	10.30	.52	.0120
	20	7.1	7.77	.20	10.22	.35	.0140
Kidney	5	9.9	6.61	.32	9.59	.75	.0130
	15	13.9	6.81	.25	10.88	.18	.0120
	20	15.4	7.21	.39	12.57	.40	.0140
Liver	5	46.0	6.61	.32	6.81	.22	.0002
	15	51.4	6.81	.25	6.42	.30	-
	20	31.8	7.21	.39	7.59	.22	.0005
Spleen	5	1.8	6.23	.09	6.46	.31	.0051
	15	6.7	6.95	.17	8.88	.26	.0120
	20	11.5	7.77	.20	8.19	.33	.0015

the method.

Trout liver homogenate did not deiodinate T_4 when sucrose was used in place of Hickman's saline in the reaction medium. This disagreed with Yamamoto's (1964) claim that liver homogenate showed a higher deiodinase activity when prepared with sucrose solution and incubated with addition of tris buffer than when saline solution and phosphate buffer were used. Other conditions: buffer at pH 7.4 and T_4^* solution without the unlabelled T_4 substrate were tried without success in bringing about deiodination in liver.

Artificial deiodination as described by Morreale de Escobar et al (1962), Reinwein & Rall (1966), and Reinwein, Rall and Durrer (1968) was avoided by incubating the tubes in darkness without the addition of FMN, H_2O_2, Fe^{++}, or chelated ions.

B. Time Series and Boiling

The progress of deiodination with time was followed for each tissue homogenate by arresting deiodination of thyroxine at 0 (actually 5 minutes after introduction of ^{125}I-T_4), 1, 2 and 3 hours of incubation at acclimation temperatures (12-14 C). The proportion of free radioiodide was assessed both by precipitation of $PB^{125}I$ and by paper chromatography (Table IV).

Gill and heart homogenates showed a rapid rise in free iodide with time. Brain, stomach, duodenum and intestine showed a gradual levelling off of deiodination within the three hours.