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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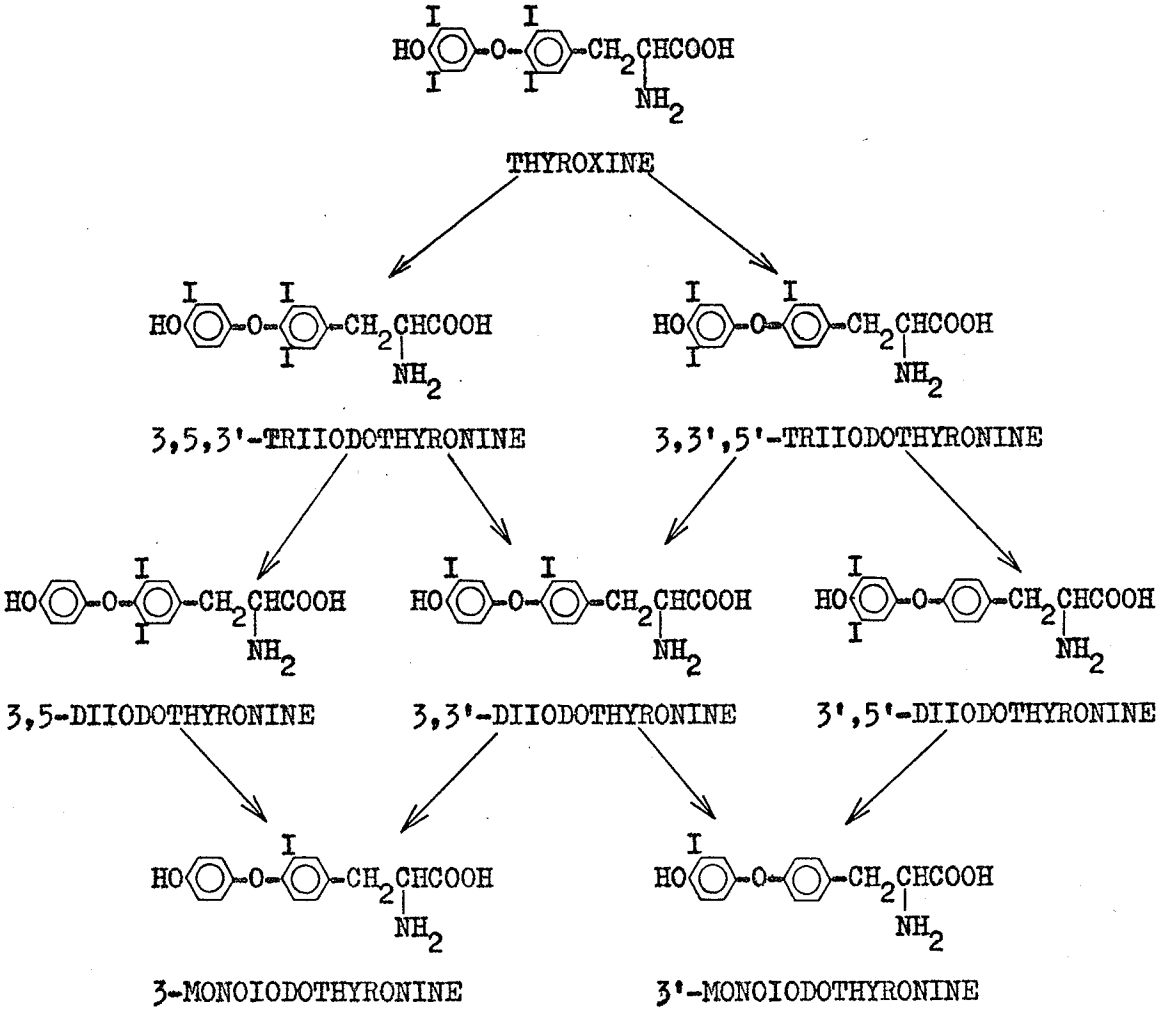
INTRODUCTION

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

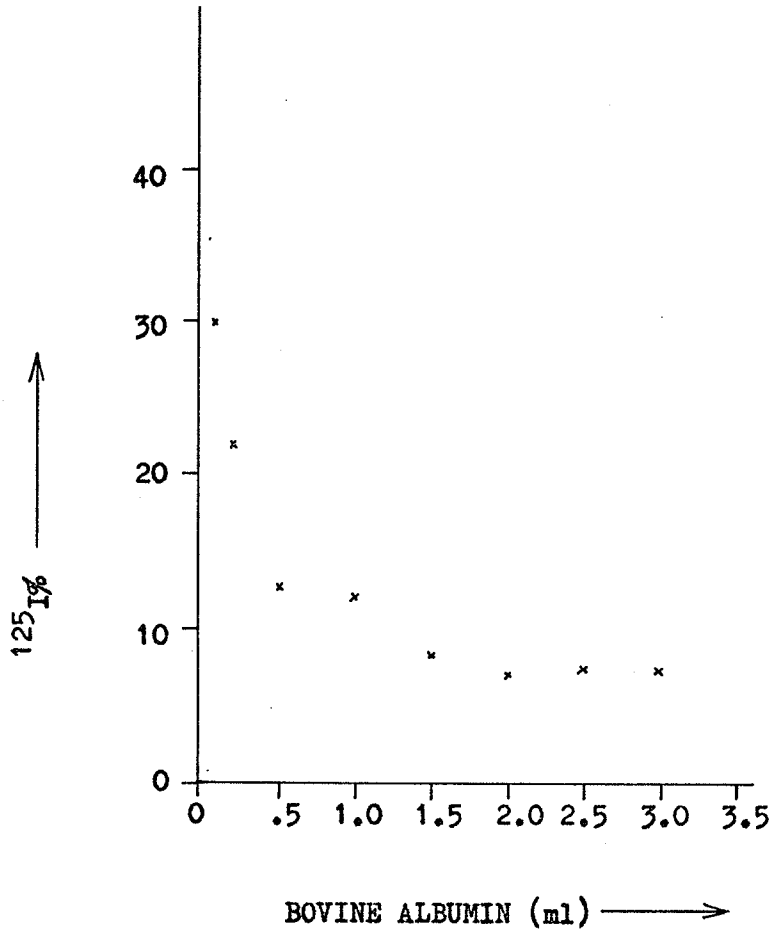
were incubated in a covered water bath with shaking at 1500 rpm for 1, 2 or 3 hours. The bath was set at a temperature corresponding to the acclimation temperature of the trout used. One set of tubes (Control, Boiled Homogenate, and Homogenate) was not incubated but was used immediately for analysis. 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

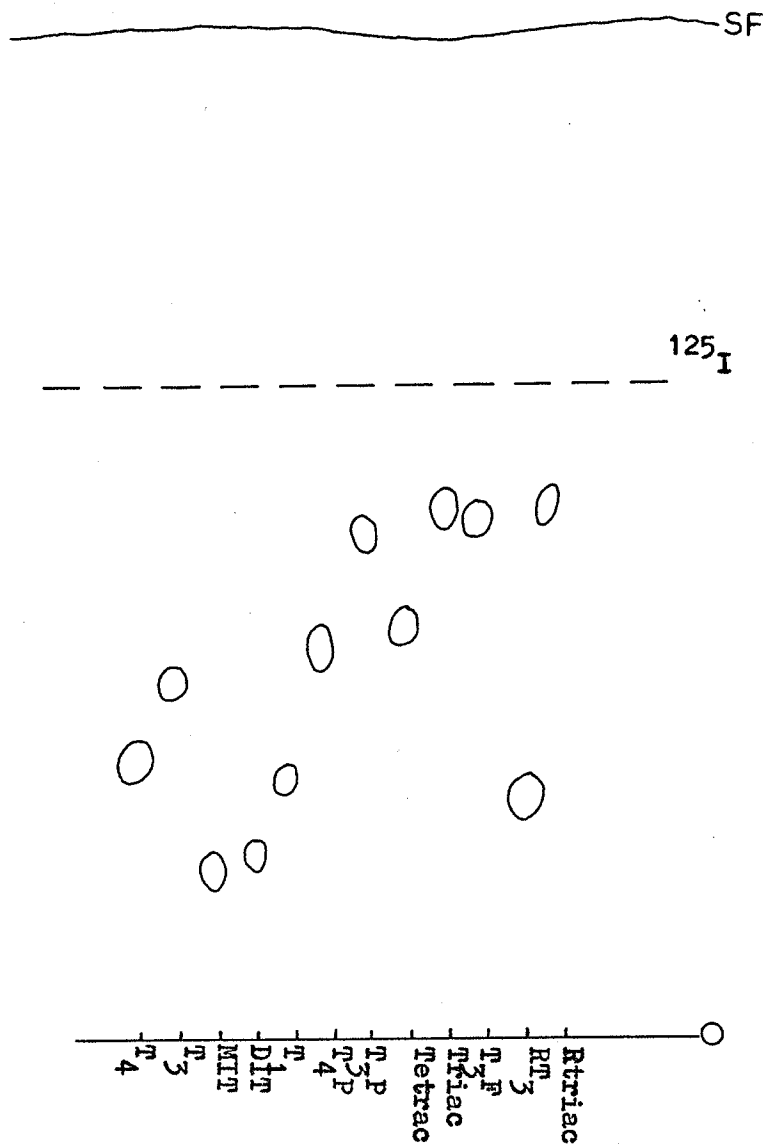
solvent systems (Appendix) among which two systems, BEA (1-butanol : ethanol : 6N ammonia, 5:2:1) and BMA (1-butanol : methabol : 6N ammonia, 5:2:1), were most satisfactory as seen from tracings under U/V (254 mμ) illumination (Figure 2).

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.

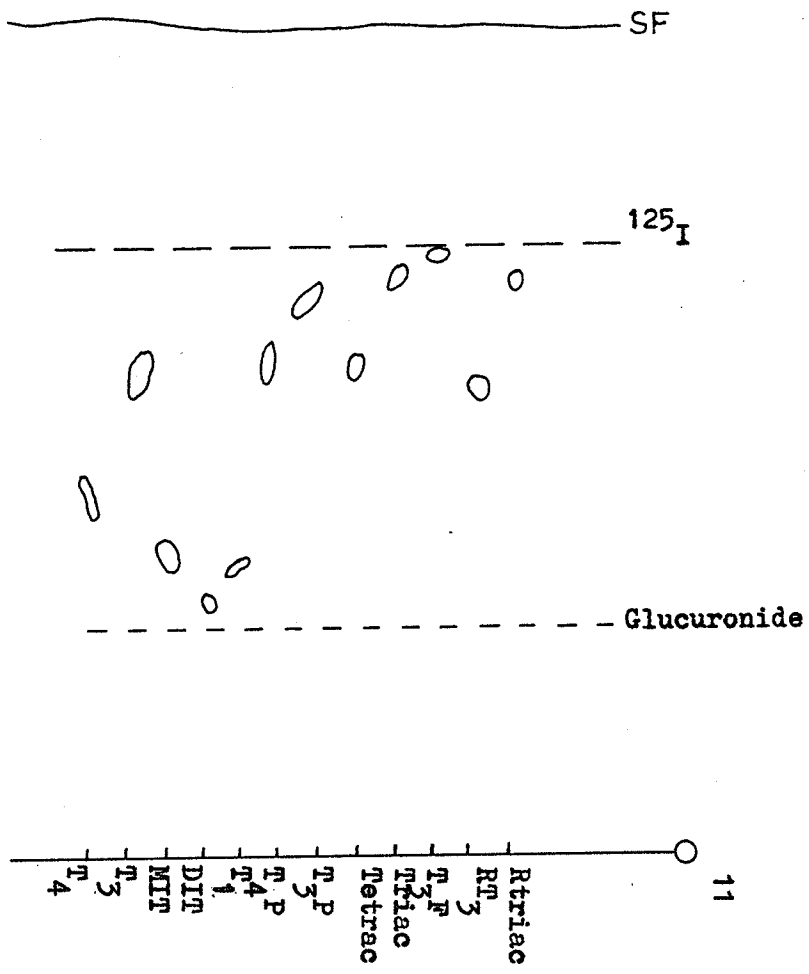
An approximately 10λ aliquot of extract in 10^{-3}M MMI was applied on a 2 inch line and developed in an ascending chromatographic tank. Each chromatogram was cut into strips of 1/8 **inch** wide and not longer than 1 inch. The strips were counted in glass vials. The counts were plotted on a graph to 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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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.

Table IV: Effect of incubation time on deiodination of thyroxine by trout tissue homogenates incubated at acclimation temperatures (12 - 14 C).
Deiodination is indicated by the percentage of free iodide in the homogenates which is not precipitated by TCA and which appears as the radioiodide peak in paper radiochromatograms.
Solvent: BAW. C = control; B = boiled homogenate; H = homogenate.

Tissue	Dry Tissue Weight (mg)	Incubation (hour)	TCA Precipitation $^{125}I\%$			TCA Precipitation W		Paper Chromatography $^{125}I\%$		
			C	B	H	B	H	C	B	H
Brain	8.8[1]	0	7.65	21.56	8.99	.0630	.0060	5.05	7.47	5.49
		1	7.05	67.42	16.15	.2740	.0410	4.67	15.36	7.68
		2	5.25	67.32	21.51	.2820	.0740	5.16	40.16	11.49
		3	4.83	70.56	21.40	.2980	.0750	3.56	44.82	16.96
Gill	14.7	0	4.43	4.49	5.53	.0002	.0024	9.04	16.26	11.17
		1	5.24	4.53	21.86	-	.0450	3.35	5.62	14.03
		2	4.59	5.15	34.52	.0015	.0810	4.38	4.33	27.20
		3	4.23	4.55	45.46	.0009	.1120	3.53	7.84	34.40
Stomach	16.2	0	4.99	4.52	6.31	-	.0033	9.64	5.55	15.91
		1	5.53	6.78	23.32	.0031	.0440	9.22	4.26	25.02
		2	5.81	6.32	24.90	.0013	.0470	5.17	3.03	27.40
		3	5.66	6.38	25.66	.0018	.0490	4.98	4.34	28.60
Duodenum	77.7	0	16.44	8.64	10.70	-	-	5.05	5.77	7.88
		1	10.81	7.85	10.64	-	-	4.67	4.71	10.98
		2	7.75	9.61	14.97	.0010	.0040	5.16	5.36	13.75
		3	8.92	15.99	21.51	.0036	.0060	3.56	5.82	17.11

Table IV continued

Intestine	10.2[2]	0	5.61	6.90	8.57	.0050	.0120	3.89	6.00	11.18
		1	5.25	8.29	34.60	.0120	.1150	5.89	5.60	21.85
		2	4.62	6.62	43.51	.0080	.1530	4.52	5.58	27.44
		3	5.67	6.36	43.63	.0220	.1490	4.01	7.08	29.58
Heart	6.5	0	4.60	6.33	8.85	.0110	.0260	10.41	14.62	18.71
		1	4.45	66.15	75.52	.3800	.4370	4.18	52.51	58.59
		2	4.09	69.25	73.63	.4010	.4280	7.88	44.17	57.15
		3	4.76	69.37	76.53	.3980	.4420	8.67	38.80	59.16
Kidney	12.6[3]	0	4.82	5.09	4.89	.0009	.0002	10.08	16.09	8.21
		1	5.96	4.64	8.53	-	.0008	4.35	7.63	8.63
		2	6.02	4.69	8.78	-	.0088	2.76	3.68	9.81
		3	5.22	5.56	10.17	.0011	.0160	3.23	3.53	14.04
Liver	31.7	0	4.96	4.88	4.98	-	-	6.43	2.86	4.88
		1	4.88	6.17	5.47	.0016	.0007	5.31	2.28	3.04
		2	5.37	5.82	5.68	.0006	.0004	4.57	2.57	2.79
		3	5.31	5.81	5.36	.0006	.0001	2.51	2.30	2.47
Muscle	9.1	0	4.76	5.03	8.60	.0012	.0170	9.43	5.48	11.80
		1	4.90	6.12	8.40	.0054	.0150	4.26	2.82	6.49
		2	5.05	7.12	11.24	.0091	.0270	5.63	3.71	6.35
		3	4.80	8.30	10.94	.0150	.0270	5.83	4.35	7.71

[1] Dry tissue weight for TCA ppt. was 8.8 mg; and that for paper chromatography was 16.5 mg.

[2] Dry tissue weight for TCA ppt. was 10.2 mg; and that for paper chromatography was 11.5 mg.

[3] Averaged dry tissue weight from other experiments.

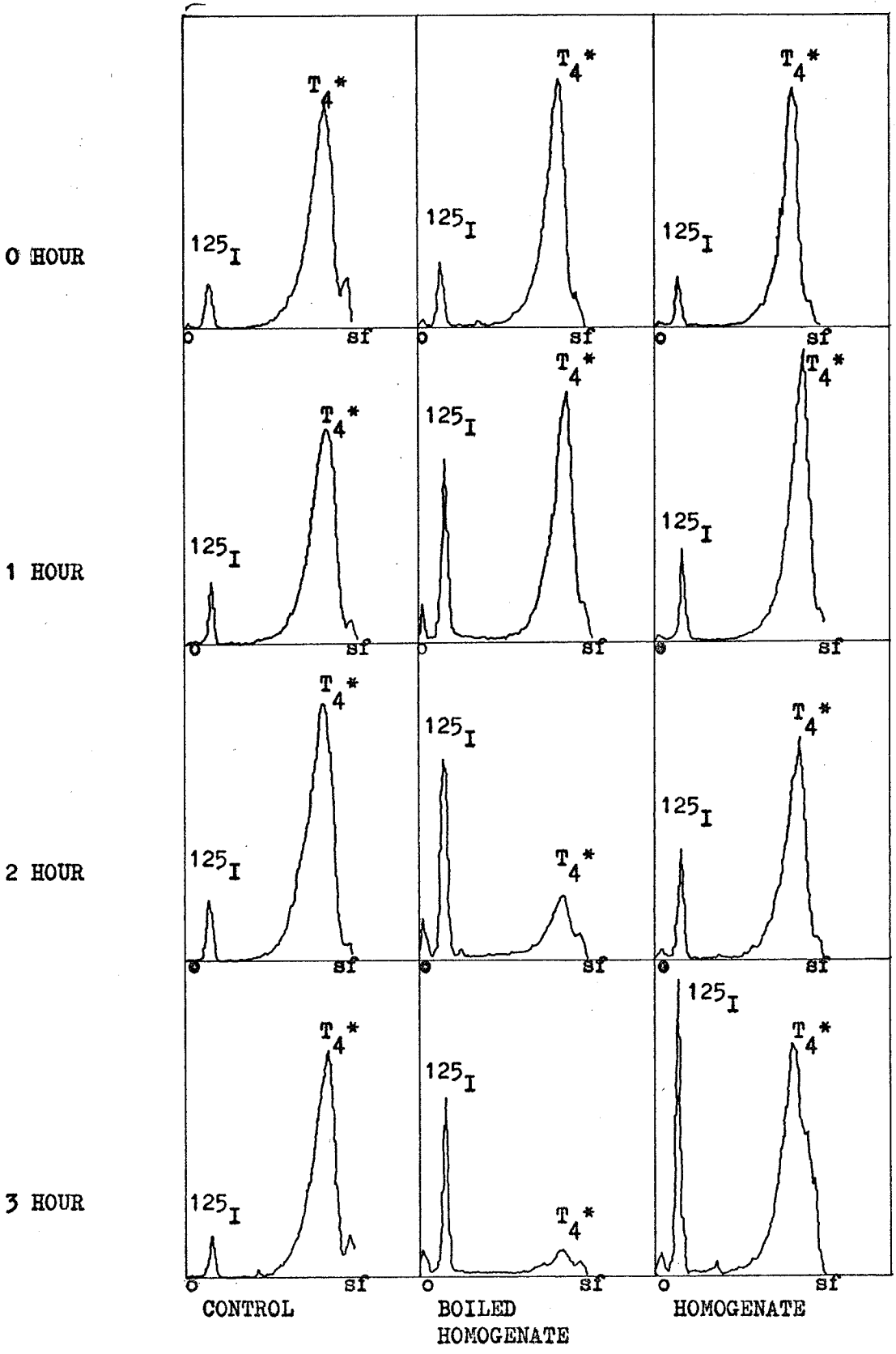
Kidney and muscle showed slight deiodination only after the third hour of incubation. Liver did not deiodinate.

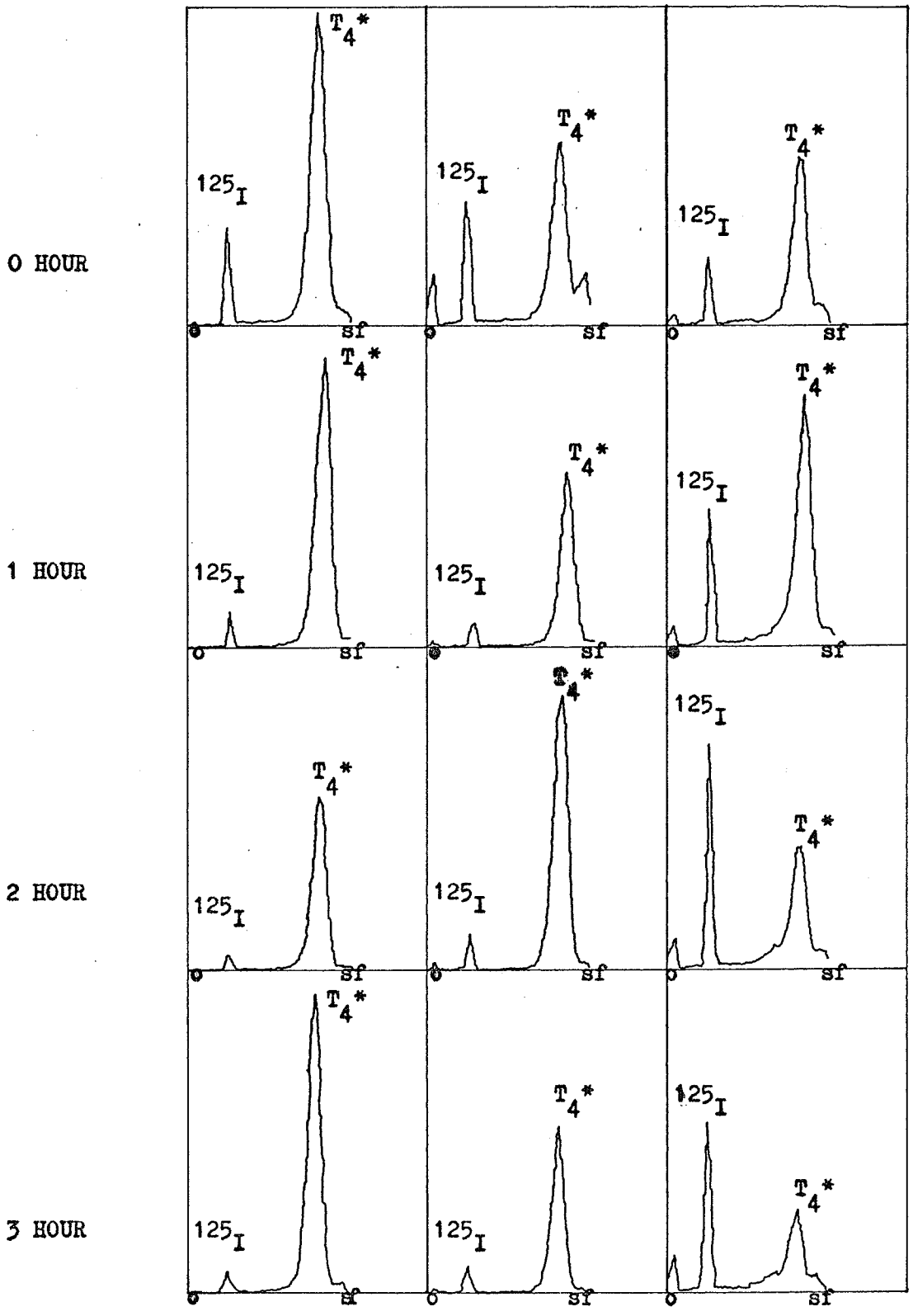
Paper chromatograms revealed that in all cases two peaks were obtained: a sharp peak at R_f .10 corresponding to ^{125}I, and an extended peak at R_f .65-.85 representing radioiodothyronines (Figures 3-11). Percentage free radioiodide obtained by this method showed increases with time for brain, gill, stomach, duodenum, intestine, heart and kidney corresponding to those obtained from TCA precipitation.

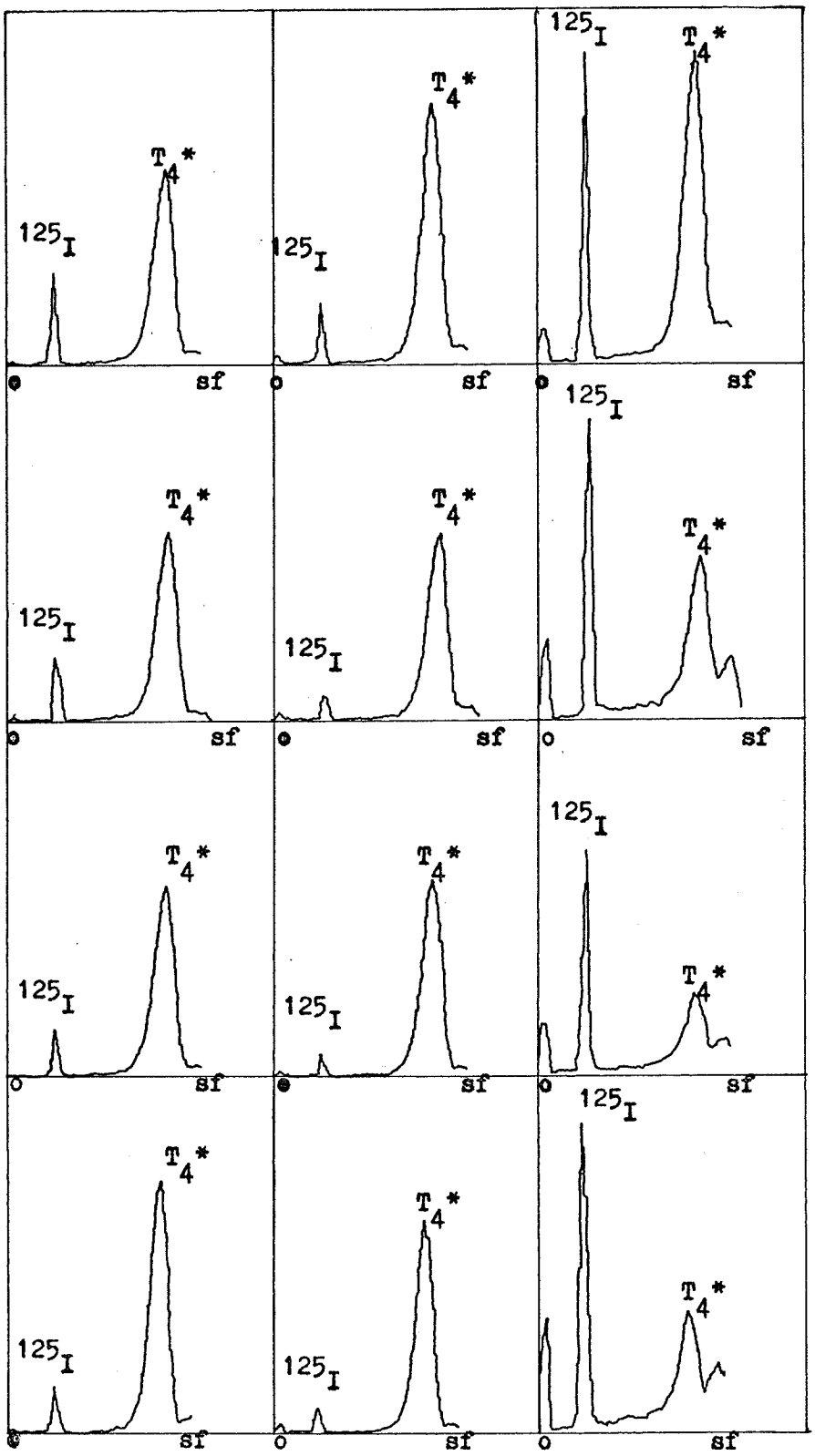
Both TCA precipitation and chromatography showed that brain and heart homogenates deiodinate even after boiling. For brain, deiodination is more vigorous in boiled homogenate. Boiled homogenates of gill, stomach, duodenum, intestine, kidney, liver and muscle did not show deiodination.

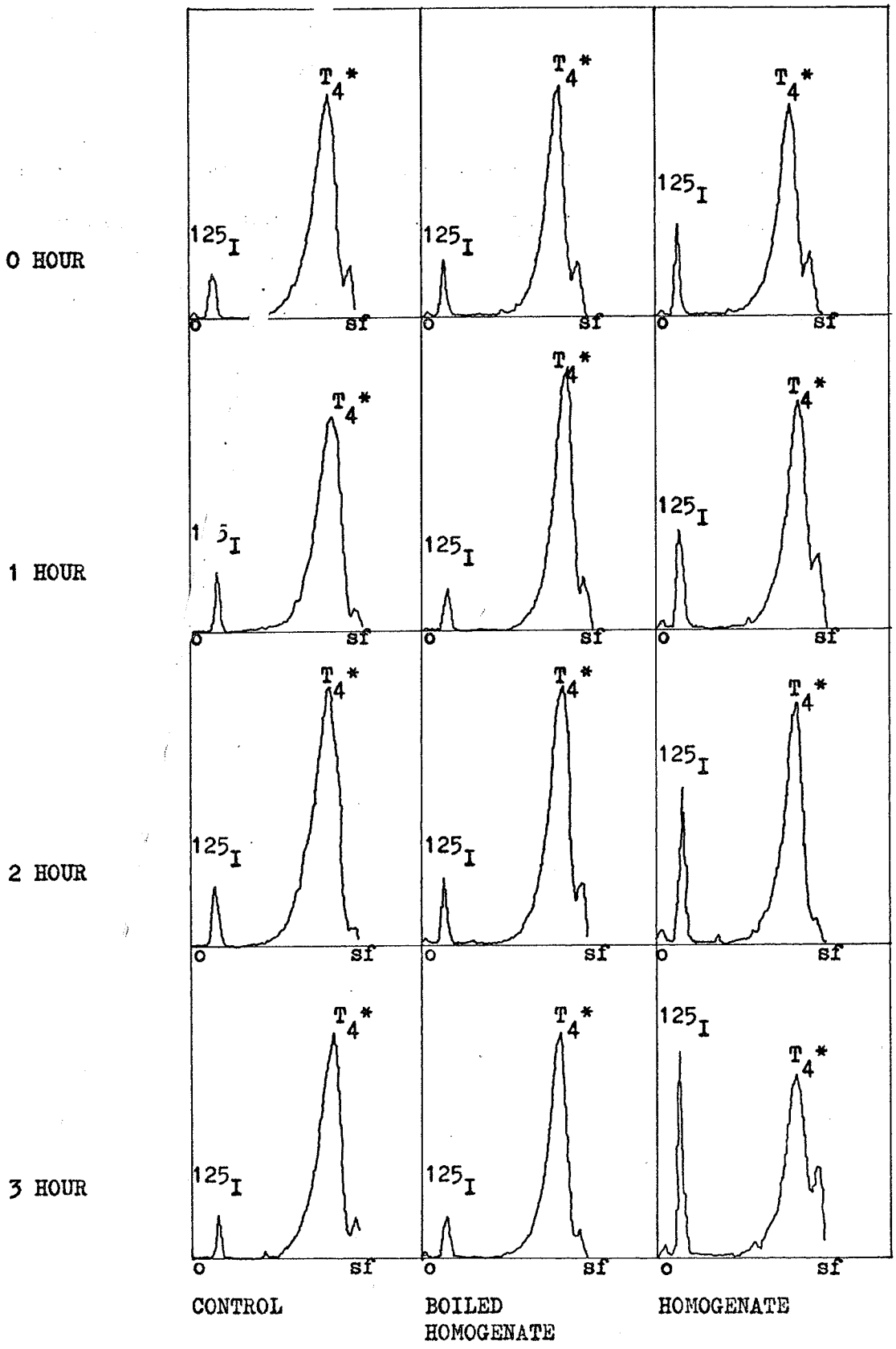
C. Identification by TLC

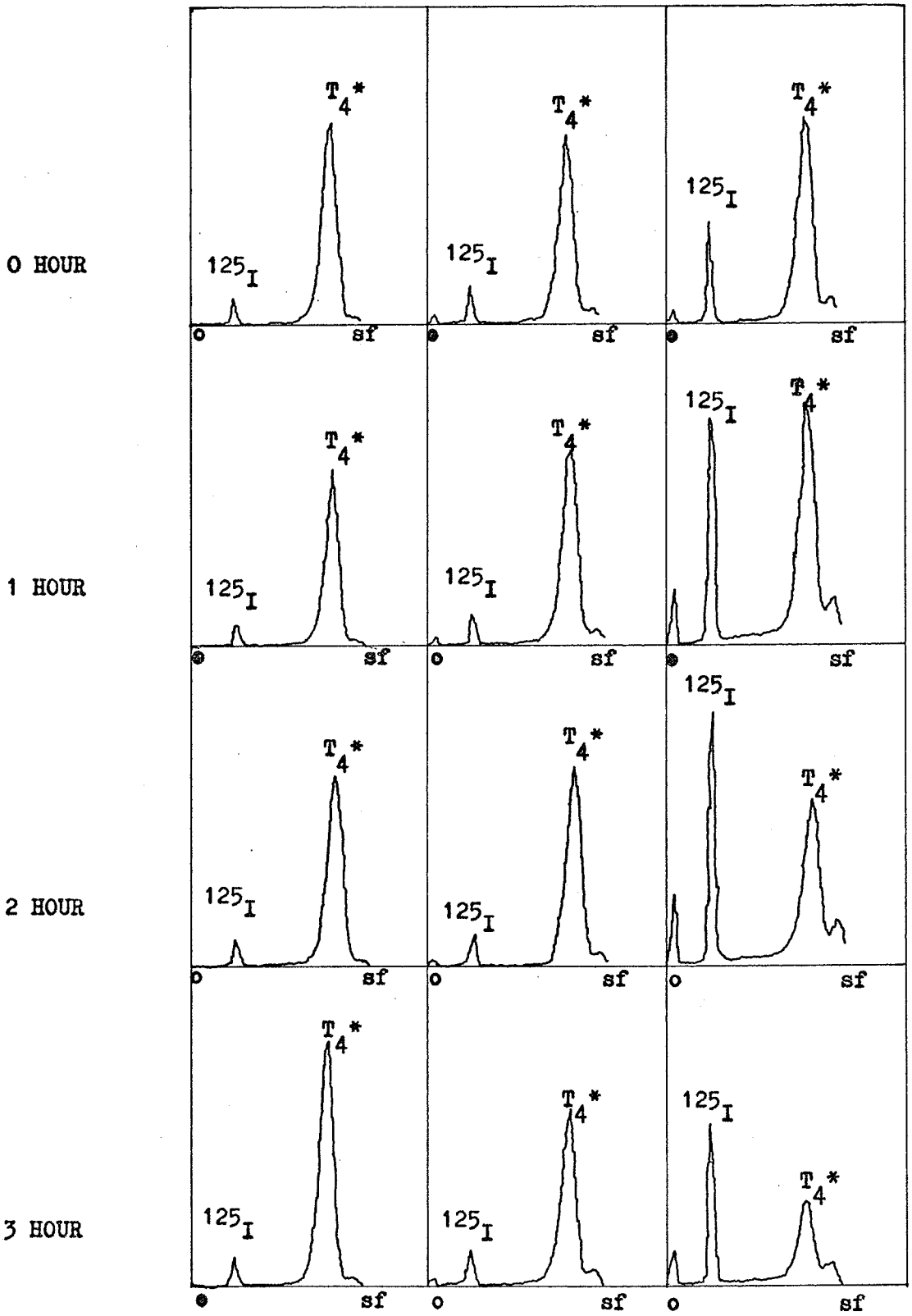
To further characterize the products, TLC was used. In BEA, besides the two labelled peaks for ^{125}I (R_f .74) and ^{125}I-T_4 (R_f .25), a peak was found at the origin (Figure 12). The height of this peak varied with that for ^{125}I, and was probably iodide bound to protein. Another possibility was ^{125}I-T_4 glucuronide conjugates which occur at this low rf in this solvent system. Another solvent system, BMA, was used to check for glucuronides because these conjugates migrate from the origin in this system and are produced in brook trout (Eales 1970, 71).

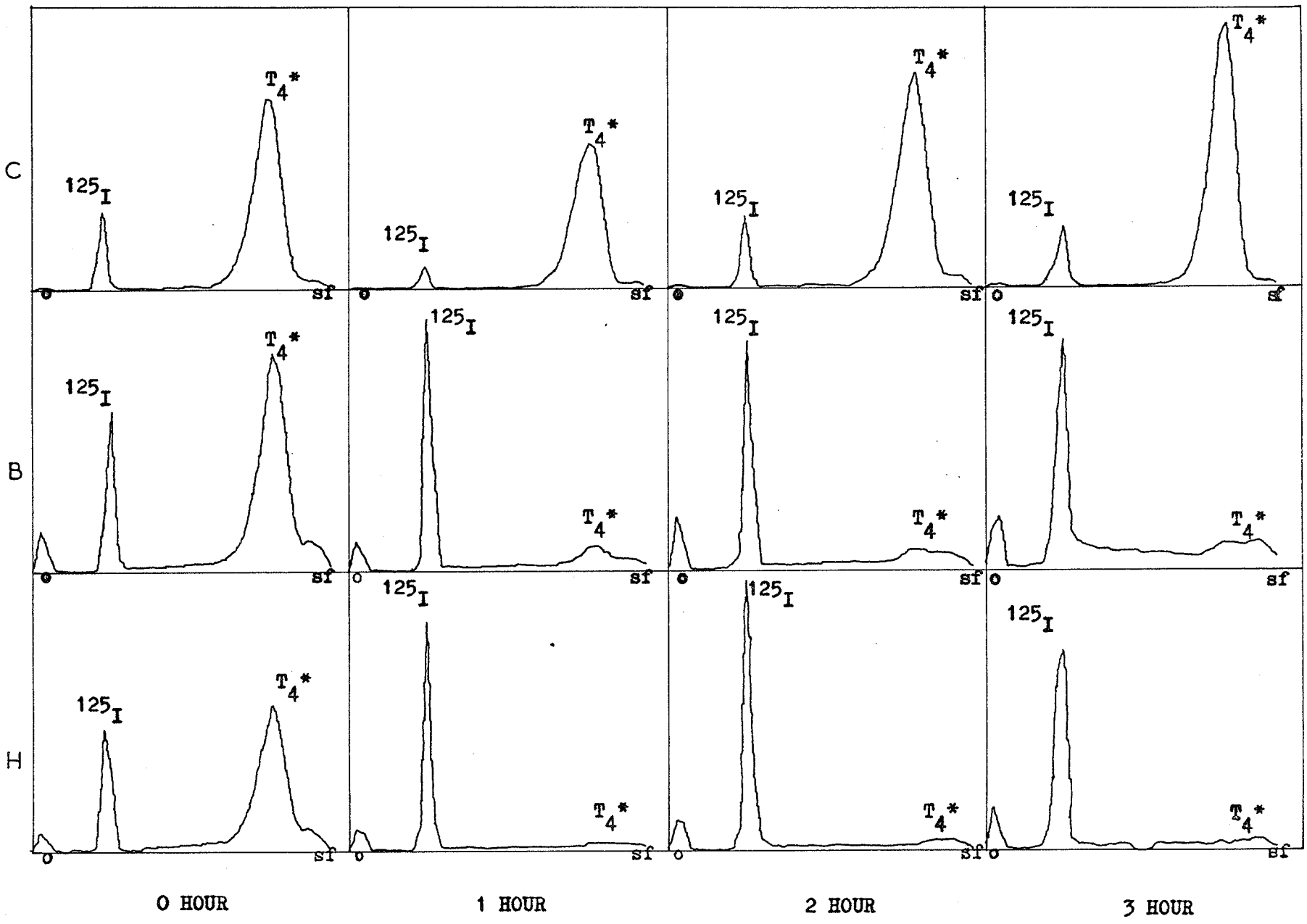

0 HOUR
1 HOUR
2 HOUR
3 HOUR
T_4*
^{125}I
o
sf
CONTROL
BOILED HOMOGENATE
HOMOGENATE

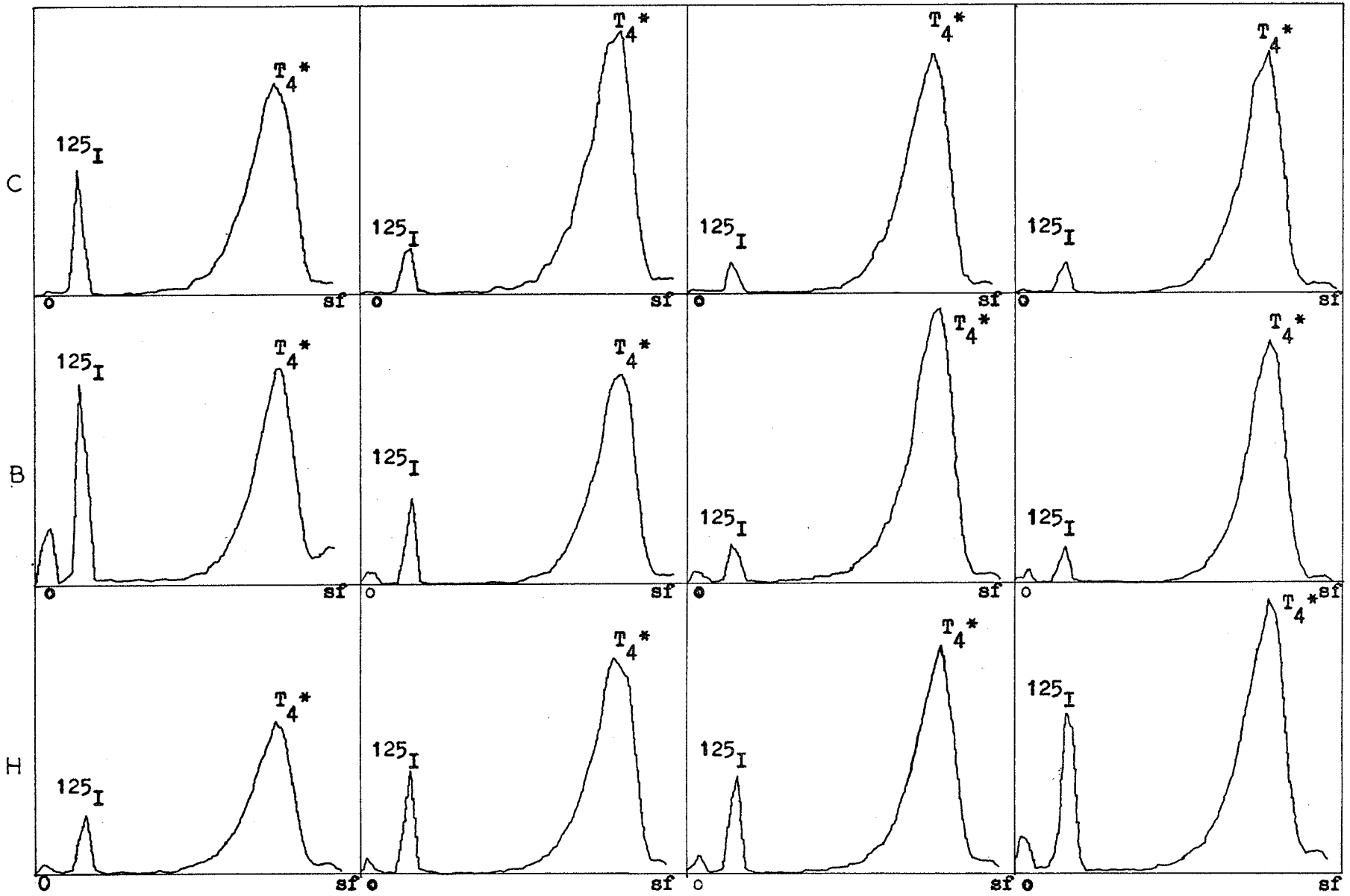

0 HOUR
1 HOUR
2 HOUR
3 HOUR
T_4*
^{125}I
sf
CONTROL
BOILED HOMOGENATE
HOMOGENATE

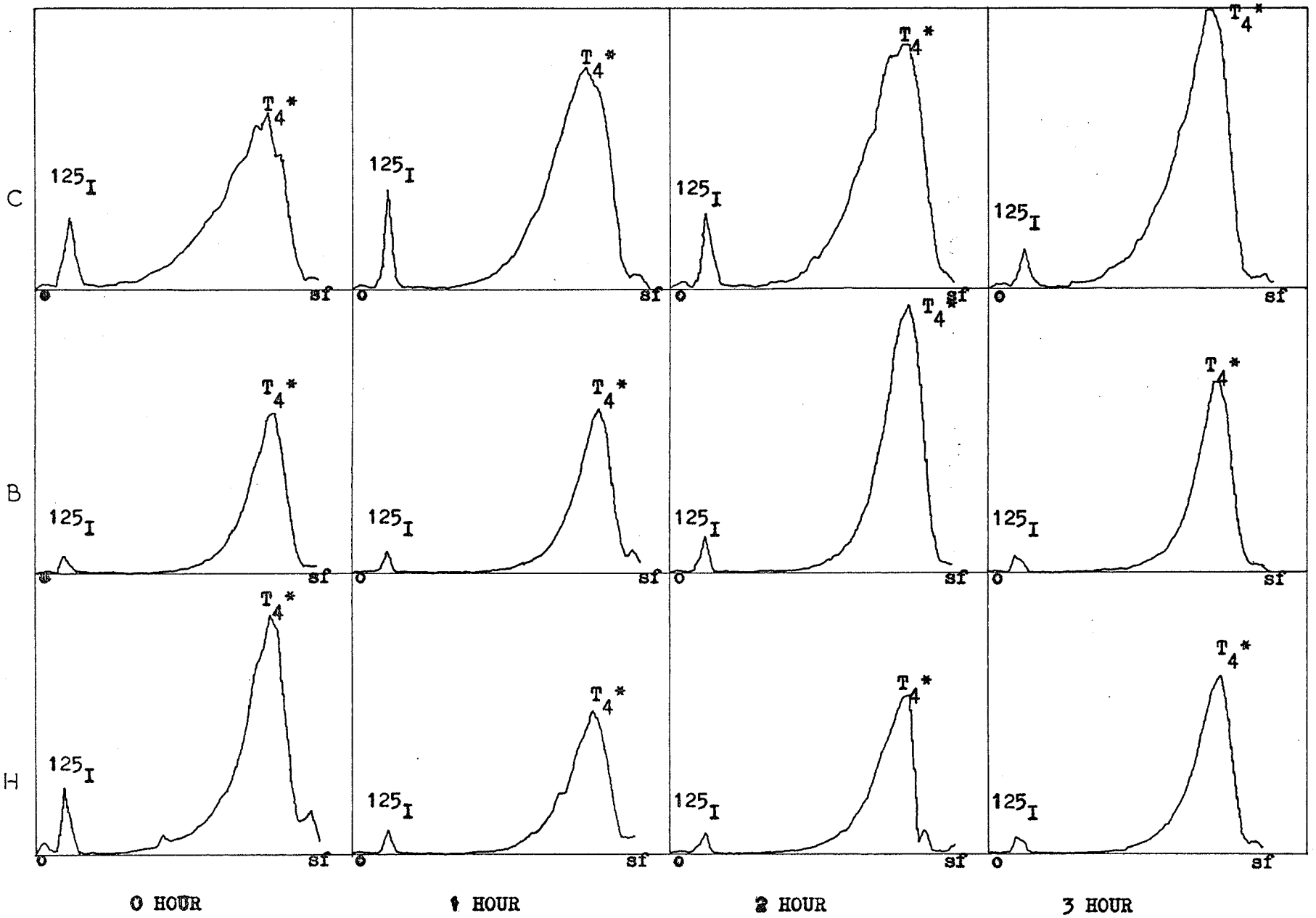

0 HOUR
1 HOUR
2 HOUR
3 HOUR
^{125}I
T_4*
o
sf
CONTROL
BOILED HOMOGENATE
HOMOGENATE

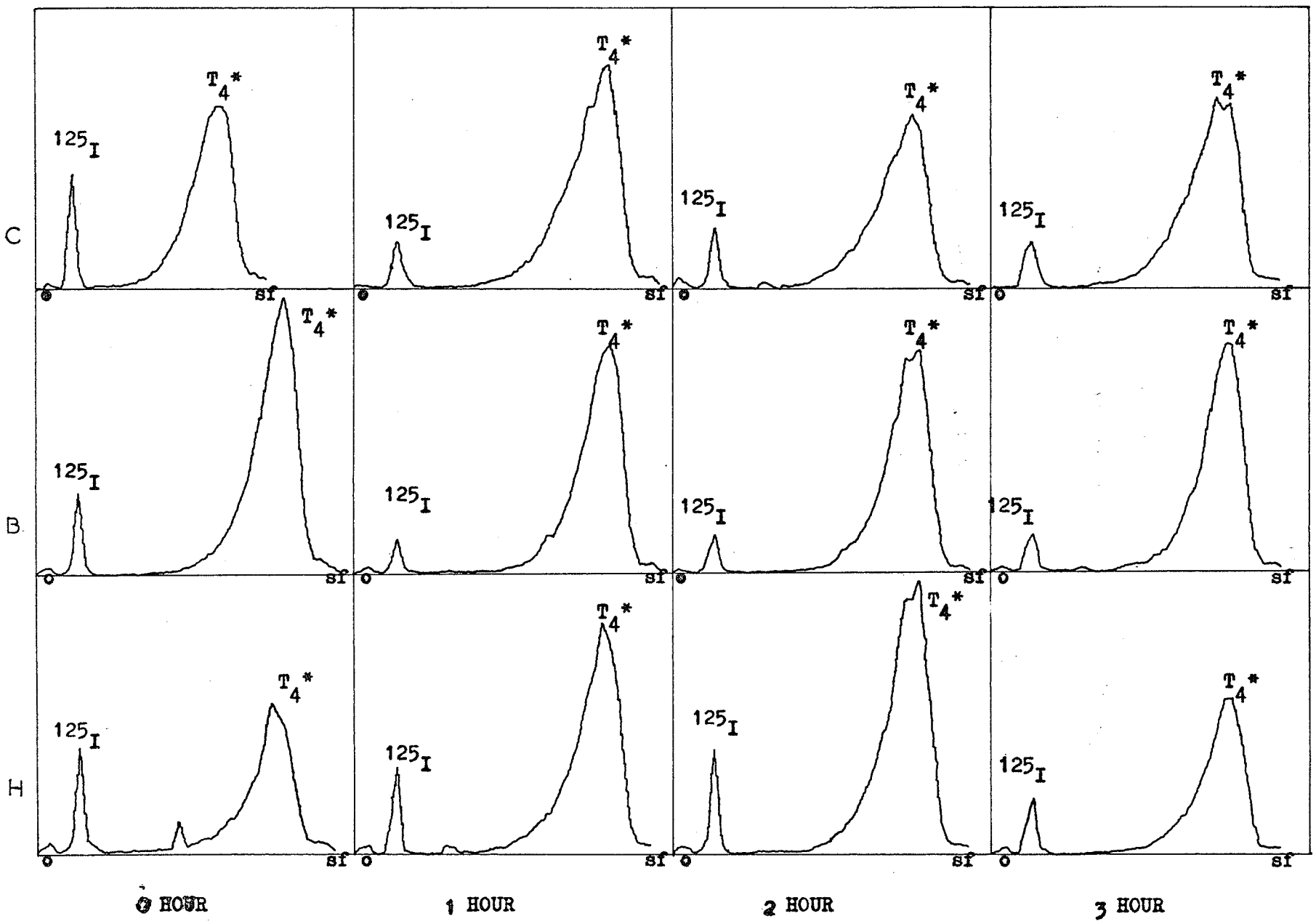

0 HOUR
1 HOUR
2 HOUR
3 HOUR
T_4*
^{125}I
o
sf
CONTROL
BOILED HOMOGENATE
HOMOGENATE

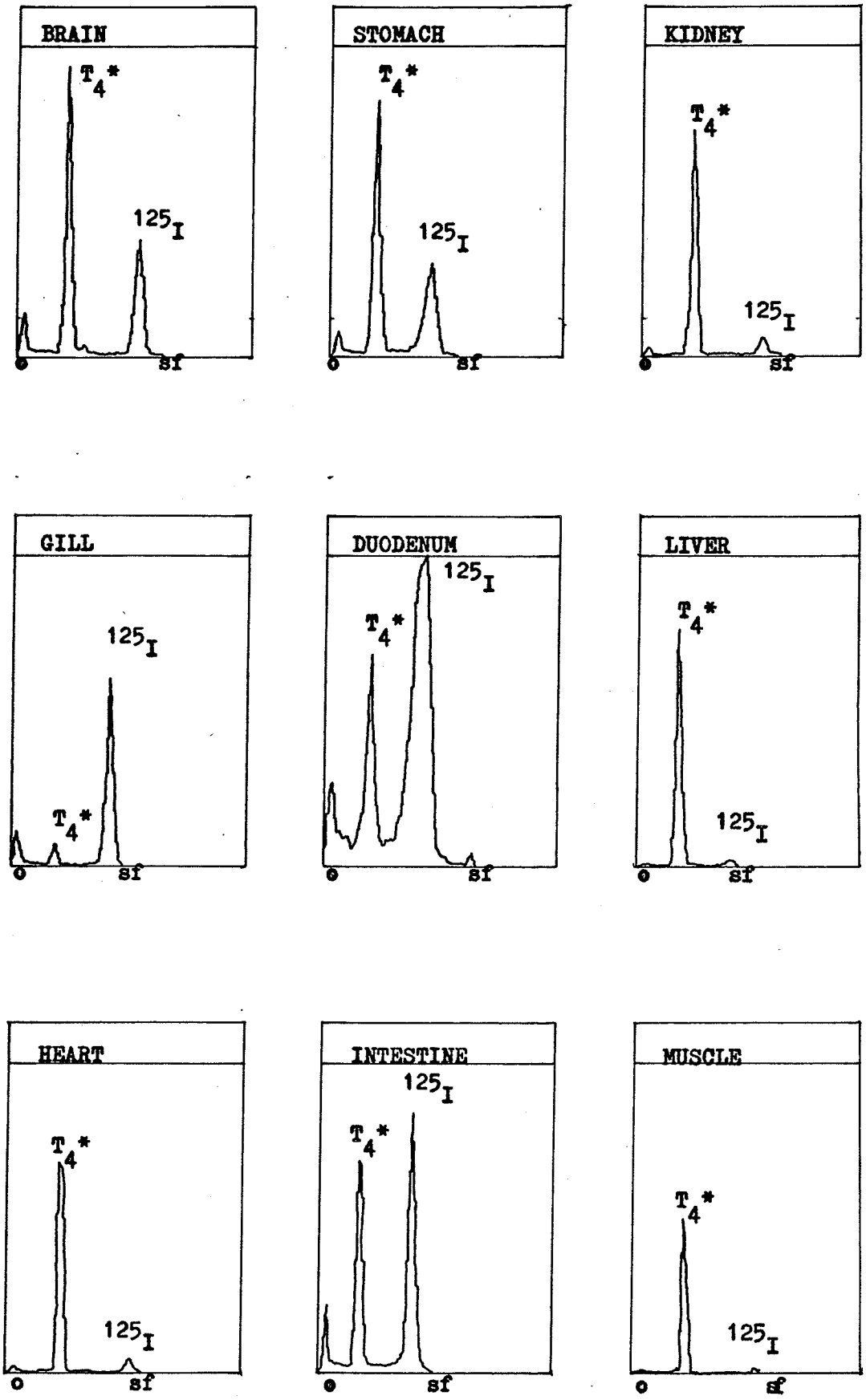

0 HOUR
1 HOUR
2 HOUR
3 HOUR
T_4*
^{125}I
o
sf
CONTROL
BOILED HOMOGENATE
HOMOGENATE


C
B
H
^{125}I
T_4*
o
sf
0 HOUR
1 HOUR
2 HOUR
3 HOUR


C
B
H
^{125}I
T_4*
o
sf
0 HOUR
1 HOUR
2 HOUR
3 HOUR

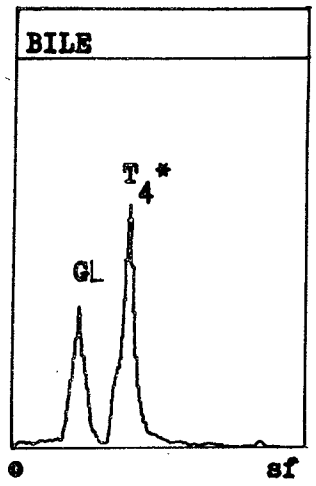

C
B
H
^{125}I
T_4^*
o
sf
0 HOUR
1 HOUR
2 HOUR
3 HOUR

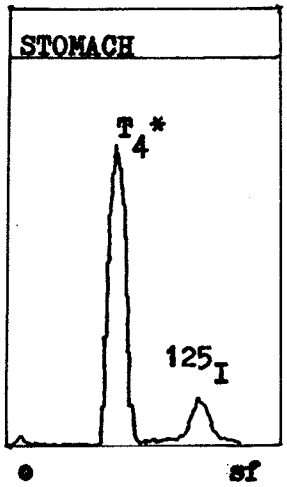

C
B
H
^{125}I
T_4*
o
sf
0 HOUR
1 HOUR
2 HOUR
3 HOUR

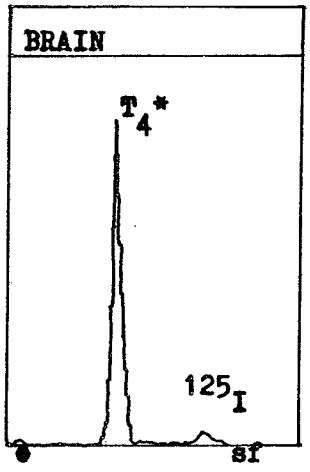

BRAIN
T_4*
^{125}I
o
sf
STOMACH
T_4*
^{125}I
o
sf
KIDNEY
T_4*
^{125}I
o
sf
GILL
^{125}I
T_4*
o
sf
DUODENUM
^{125}I
T_4*
o
sf
LIVER
T_4*
^{125}I
o
sf
HEART
T_4*
^{125}I
o
sf
INTESTINE
^{125}I
T_4*
o
sf
MUSCLE
T_4*
^{125}I
o
sf

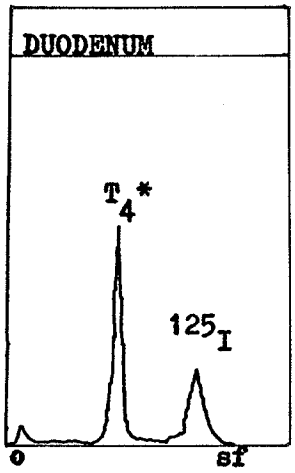
Compared with the radiochromatograms obtained for bile, there were no glucuronides in the samples and the peak at the origin disappeared (Figure 13). This suggests that it was radioiodide, and eliminated the probability of iodinated proteins as suggested by Tata (1960).

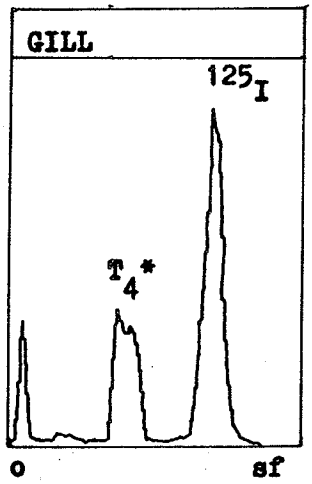
There was suspicion that some intermediate compounds of deiodination might be detected within a shorter period of incubation. TLC in BEA and BMA were done for gill and intestine homogenates at 10, 30, 60 and 90 minutes of incubation. Only the ^{125}I and $^{125}I\text{-}T_4$ peaks were found in intestinal homogenate in both systems (Figure 14). Gill homogenate gave an additional small peak at R_f .25 in BMA (Figure 15). This substance does not resemble any of the authentic thyroxine analogues or glucuronide conjugates in migration and remains unidentified.

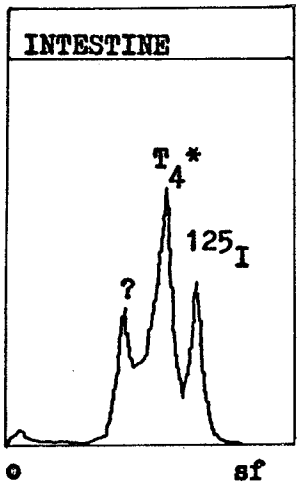

BILE
GL
T4*
o
sf

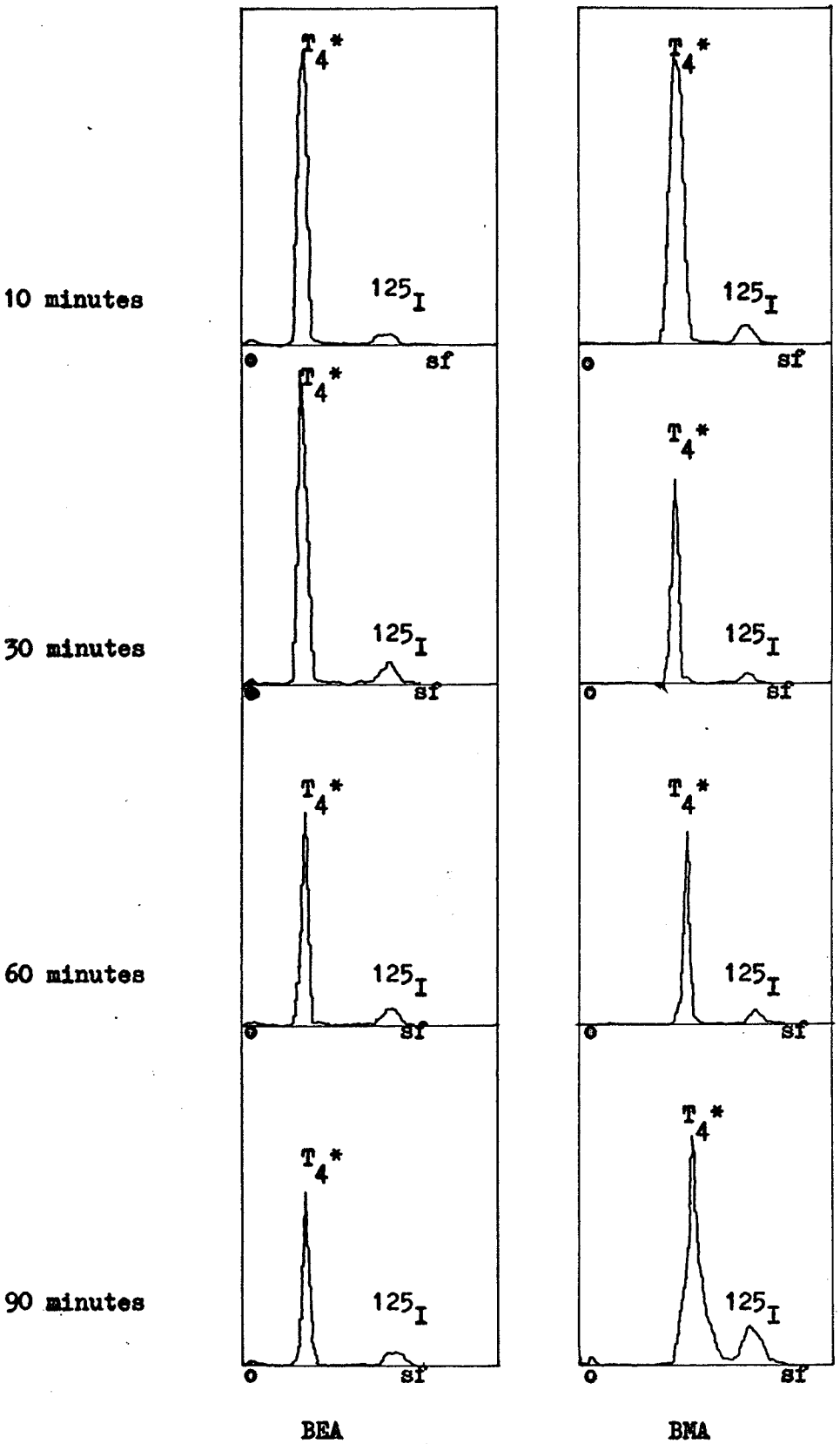

STOMACH
T4*
125I
o
sf

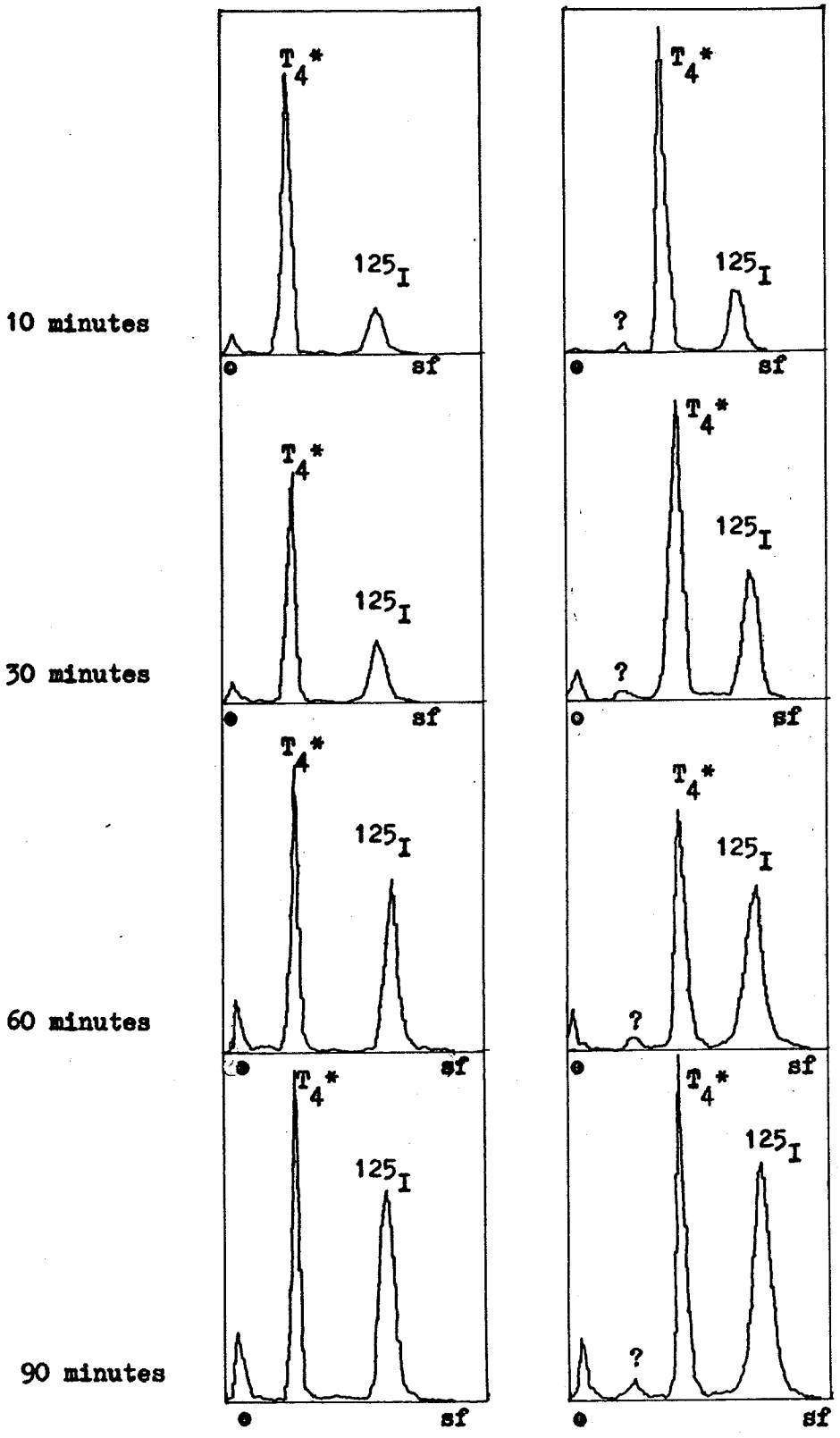

BRAIN
T4*
125I
o
sf


DUODENUM
T4*
125I
o
sf


GILL
125I
T4*
o
sf


INTESTINE
T4*
125I
?
o
sf


10 minutes
30 minutes
60 minutes
90 minutes
T_4*
^{125}I
sf
BEA
BMA


10 minutes
30 minutes
60 minutes
90 minutes
T_4*
^{125}I
?
sf
BEA
BMA

DISCUSSION

A number of tissues were shown to be capable of deiodinating T_4; a few, including liver, did not show deiodination. Though each tissue deiodinates to a different extent, it seems that in trout, deiodination of T_4 is complete at least at the 3' and 5' positions.

A. Sites and products of T_4 deiodination

1. Brain

Trout brain homogenate is rather moderate in deiodinating T_4. No intermediate products were identified. However, Sprott and Maclagan (1955) had shown that T_4 was converted into T_3 in rat brain. Reichlin, Volpert and Weiner (1966) and Brown-Grant (1967) reported monodeiodination of T_4 by pituitary of mammals.

2. Gill

Like the brain, trout gill homogenate showed a steady increase in deiodination with time. Intact gill filaments showed a lower percentage deiodination than homogenate. Since in these experiments thyroid tissues were removed from the gill, deiodination could not have been due to such tissues though Maayan and Rosenberg (1963) had reported deiodination by rat thyroid.

Gill is the only structure which has no counterpart in mammals and adult amphibians, its role in deiodination of T_4 will be of much interest.

3. The gut - stomach, duodenum and intestine

The gut seems to play an important role since it was shown to carry out up to 40% of deiodination of T_4 in three hours.

The process is likely to be intracellular since the gut had been rinsed thoroughly of its contents before homogenization. Labelling T_4 with ^{14}C would enable detection of any rupturing of the diphenyl linkage, or deamination.

The time-series experiments on duodenal homogenates may not be completely representative because of the high dry tissue weight and the abnormally high free radioiodide percentage in the controls.

Deiodination levels off after one to two hours of incubation in the gut. This is probably due to the action of proteolytic enzymes in the gut tissue destroying the deiodinase enzyme.

No specific mention in the literature could be found for *in vitro* deiodination by the rat gut. Flock *et al* (1956) worked with 'dehepatized' dogs and found a decrease in T_4 deiodination. Their dogs were in fact eviscerated and it was very probable that other than the liver, the gut played an important role in mammalian deiodination of T_4.

4. Heart

Results obtained in the preliminary tests showed less deiodination by trout heart homogenates than in later experiments. The reason for this is not certain, but the fact that the later

fish were kept at a more constant and slightly higher temperature (12-13 C) might have contributed to such discrepancies.

Previous experiments on deiodination by heart are few. Galton and Ingbar (1962b) reported the complete absence of deiodinating ability in tissue homogenates of Necturus maculosus which included heart homogenate.

5. Kidney

Trout kidney homogenate deiodinates T_4 rather slowly over the three hours of incubation. The use of slices did not enhance the process. But these findings are very far from the potent deiodinating kidney in mammals. Flock et al (1961) cited the conversion of T_4 to T_3 by rat kidney slices against the deamination product, tetraiodothyroacetic acid, found in kidney homogenate.

In vivo study on variation in ^{125}I excretion in brook trout might reveal how well this in vitro finding represents the true state.

6. Liver

Trout liver homogenate did not deiodinate T_4, nor was any product from other processes, e.g. deamination, decarboxylation or conjugation, found.

The use of slices did not lead to deiodination though Plaskett (1961) obtained different results when liver slices were used instead of extracts. Also Galton and Ingbar (1966)

hypothesized the co-existence of a heat-stable and a heat-labile deiodinating system in rat liver, and the former could be destroyed by leaching of the slices. Neither of the systems was proved to exist in trout liver.

The negative result was probably not due to technique as extensive deiodination was achieved by the same method in mouse liver at 37 C.

Data from *in vivo* experiments shows that in bile following T_4 injection, T_4, T_4-glucuronide and negligible iodide were found (Eales 1970). This suggests negligible deiodination by liver which agrees with findings in the *in vitro* experiments.

In view of the extensive deiodination by mammalian liver (Flock *et al* 1956; Tata 1960; Plaskett 1961; Wynn & Gibbs 1962; Benevent *et al* 1963; Galton & Ingbar 1966; and Kobayashi *et al* 1966) the absence of deiodination in trout liver was surprising. This should be looked at in more detail since it may give some insight into the difference in roles played by the liver of fish from that in higher animals.

7. Muscle

Trout muscle did not show any deiodination of T_4 in the form of slices and very slightly in homogenates. In their paper, Flock *et al* (1961) mentioned that Tata in 1957 reported active monodeiodination of T_4 in mammalian muscle. Data obtained by Kobayashi *et al* (1966) showed a more extensive deiodination

in muscle homogenate over slices.

8. Skin

Trichloroacetic acid precipitation has shown that trout skin did not deiodinate T_4. But Dowling and Razevska (1966) reported tetrac as a deamination product in frog skin. It is possible that though trout skin does not deiodinate T_4, another process, deamination of T_4, takes place in this tissue.

B. Thermal stability of deiodinating systems

From data shown in Table IV, there seemed to be two deiodinating systems in trout: a thermolabile and a thermostable system. Homogenates of gill, stomach, duodenum, intestine, kidney, liver and muscle showed no deiodination after boiling. However, deiodination was slightly accelerated in boiled brain and heart homogenates. Reports on deiodinating activity of boiled homogenates varied with investigators. Galton and Ingbar (1961, 1965) reported boiling destroyed deiodinating ability of tadpole, frog and toad liver homogenates while Dowling and Razevska (1964, 66) found that frog and toad liver homogenates deiodinated even after boiling. Mammalian muscle (Tata 1957, Kobayashi et al 1966), liver (Kobayashi et al 1966), pituitary (Escobar del Rey & Morreale de Escobar 1964), brain (Tata 1957) and frog skin (Dowling et al 1964) all contain thermolabile deiodinating systems. But Galton and Ingbar (1961) reported evidence for a thermostable system in mouse liver.

Sprott and Maclagan (1955), Etling and Barker (1959) and Stanbury et al (1960) have cited evidences for thermostable systems.

Very little is known of the thermostable mechanism of deiodination. One speculation may be drawn from this experiment. If deiodination plays an important role in the overall metabolism of the tissues, and since brain and heart are the relatively delicate organs requiring very stable environmental conditions for functioning, it is probable that in these two organs an inhibitor is present to bind with the deiodinating enzyme and energy (probably in the form of ATP on cellular level) is required to unlock the enzyme at times of need.

It is also probable that there are two deiodinating mechanisms in trout. One is represented by the thermolabile system in gut and other tissues, and the other represented by that in the brain and the heart.

C. Role of deiodination in brook trout

As can be seen from the data of this thesis, T_4 is deiodinated by several tissues in brook trout and there are at least two types of deiodinases present.

Despite the lack of T_4 derivatives recovered, it is still too early to say that deiodination is the only route for T_4 catabolism in brook trout. A search for such derivatives in in vivo experiments would be required.

However, it is obvious that the gut ministers most of the

deiodination of T_4 in brook trout. Probably this is how T_4 is removed from the body of the fish.

CONCLUSION

1. Over the three hours, brain and gill show a steady increase of deiodination of T_4 with time while stomach, duodenum, intestine and heart climb to a maximum after the first hour of incubation.
2. Kidney and muscle do not show much effect on T_4. Deiodination, if present, is very slow.
3. Liver does not show any effect on T_4: deiodination, deamination or decarboxylation. No conjugation is formed with T_4 after three hours of incubation.
4. Brain and heart deiodinate actively after boiling.
5. Deiodination of T_4 is complete at at least the 3' and 5' positions because only ^{125}I and $^{125}I\text{-}T_4$ were detectable after incubation.

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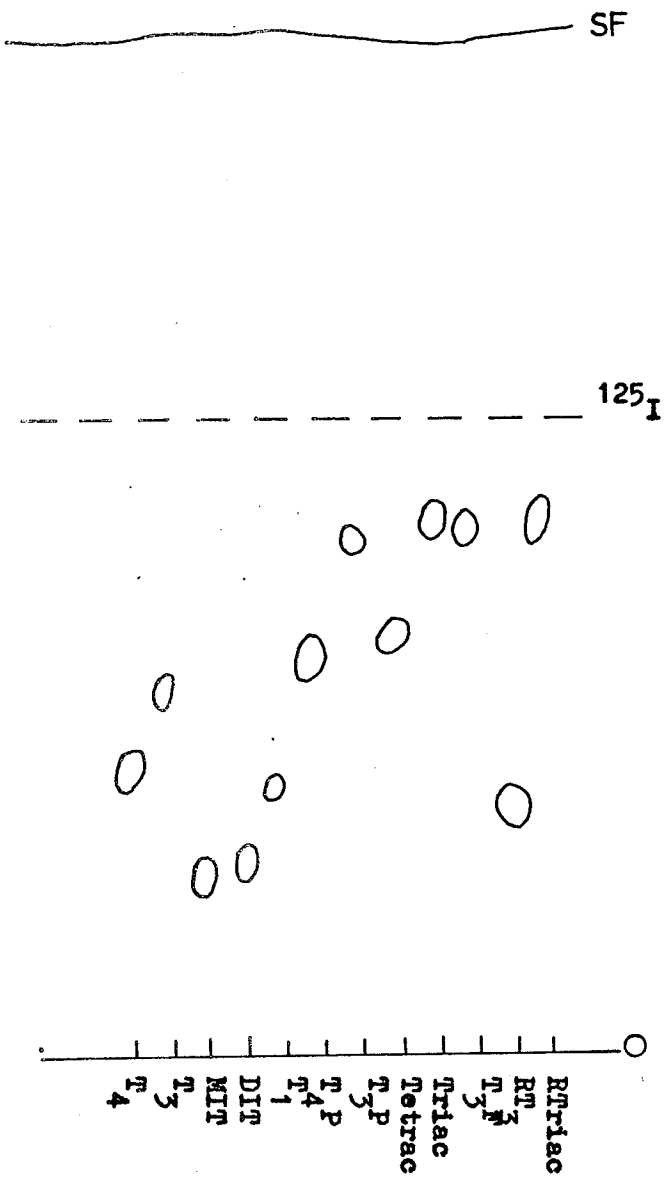

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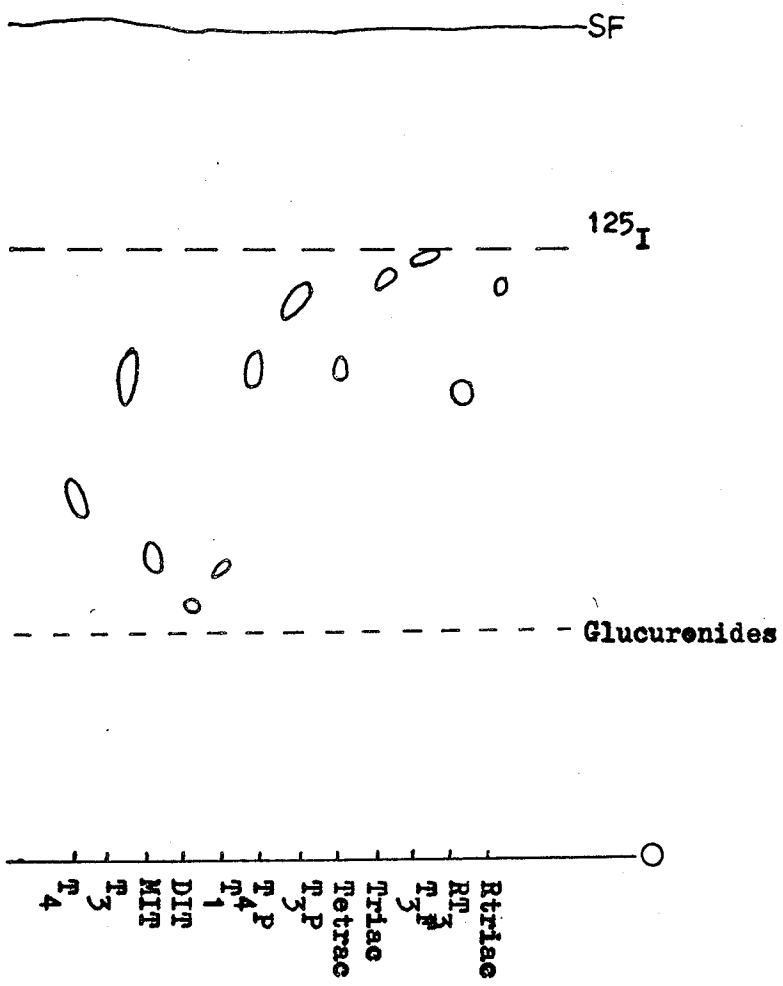
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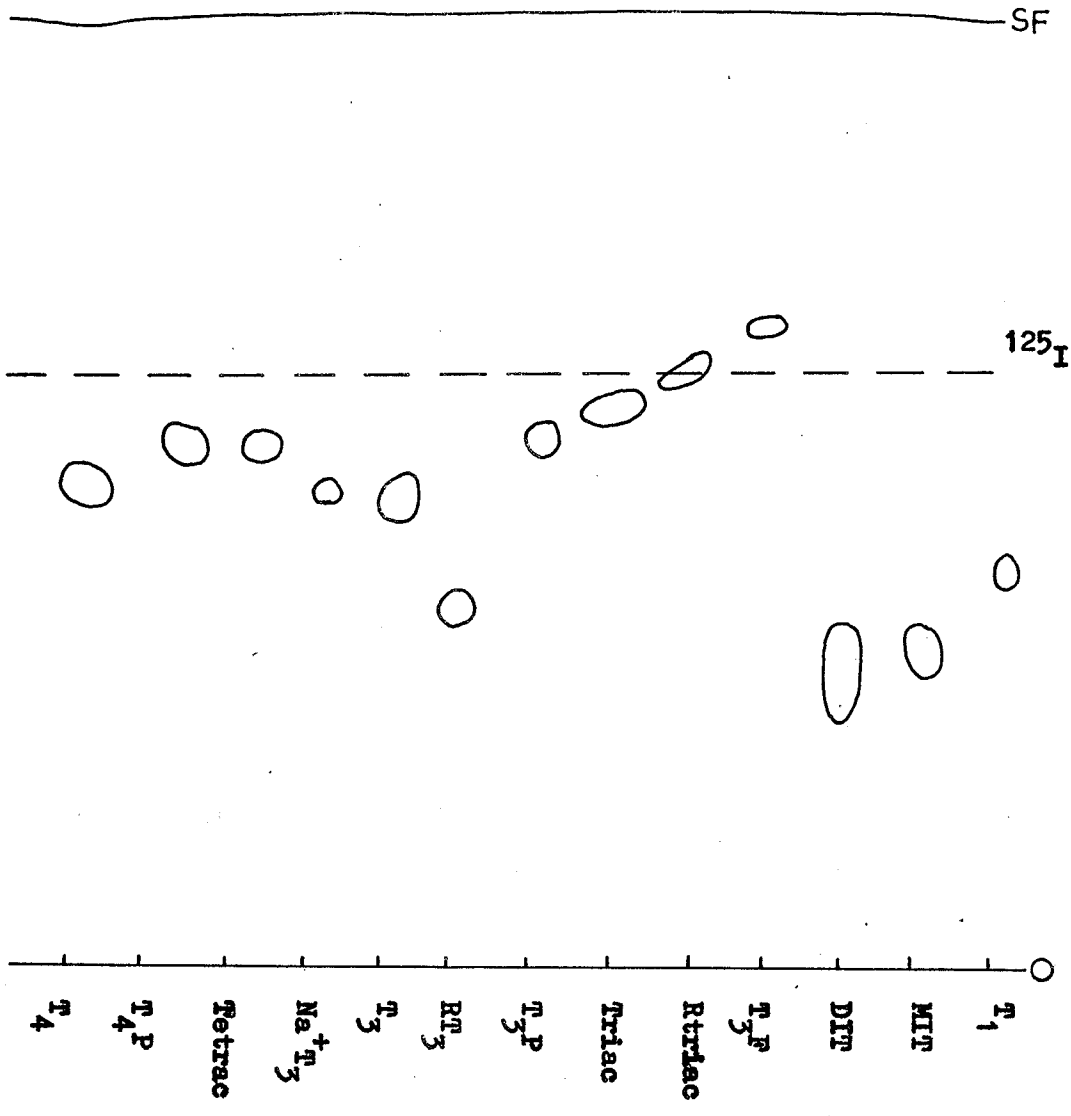
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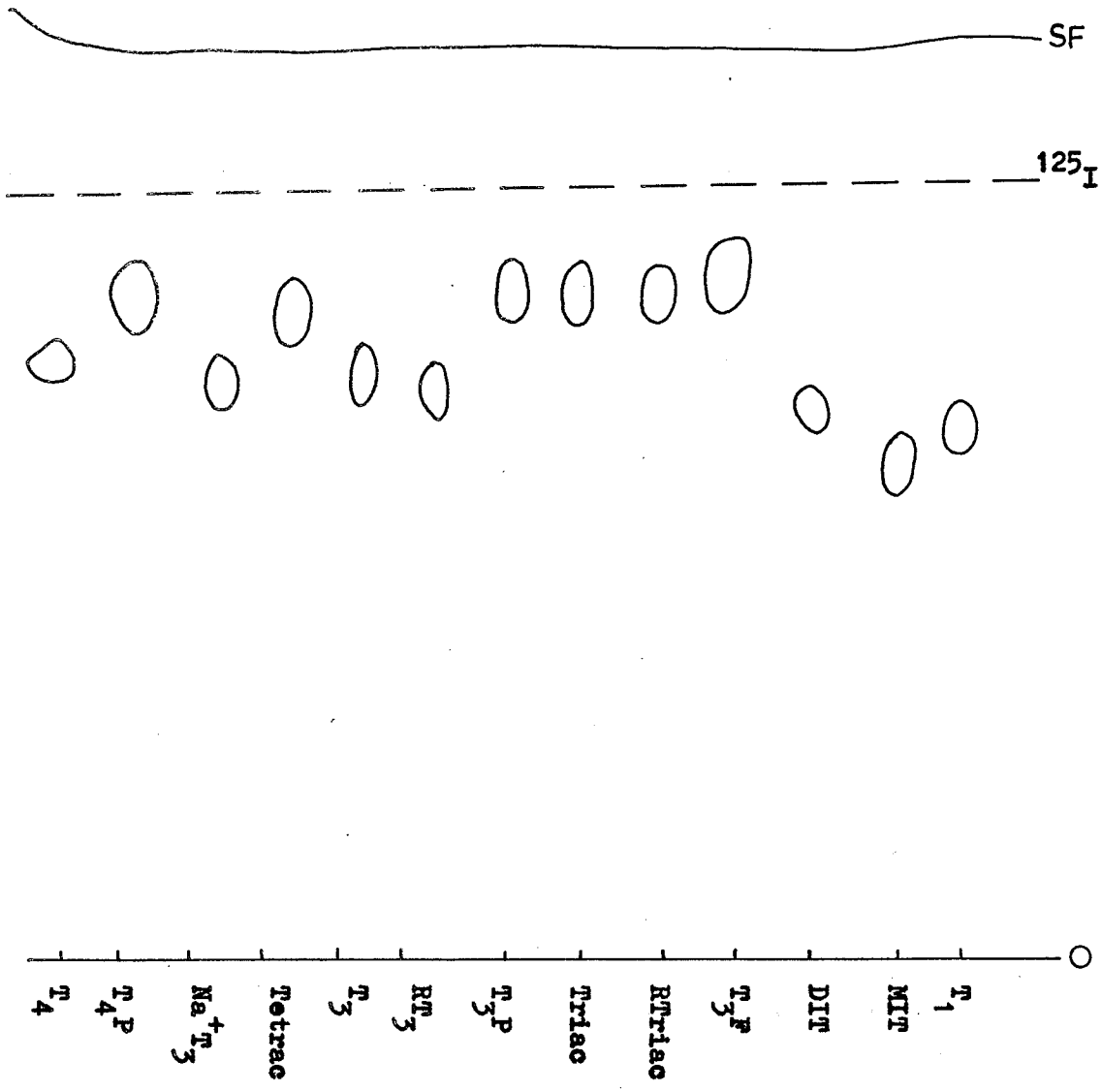
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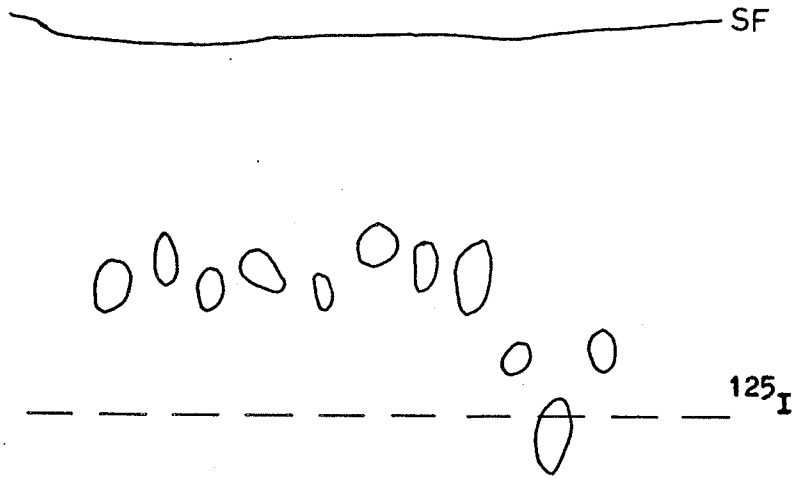
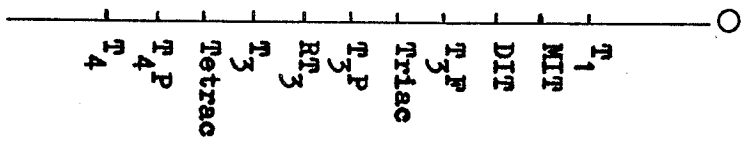
BAW1





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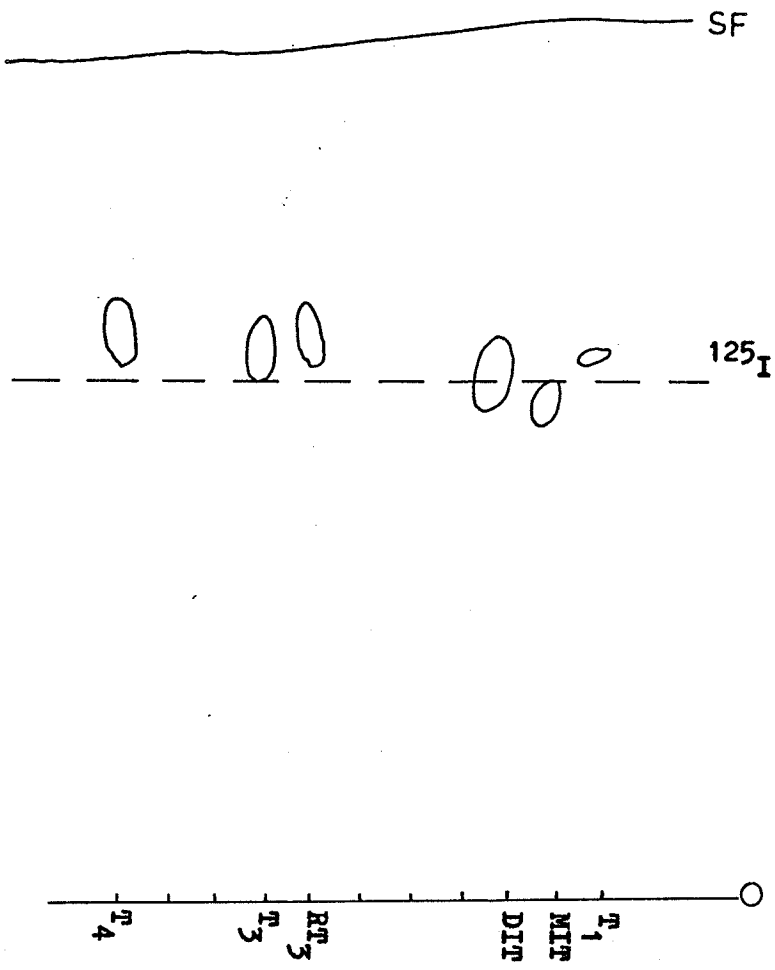

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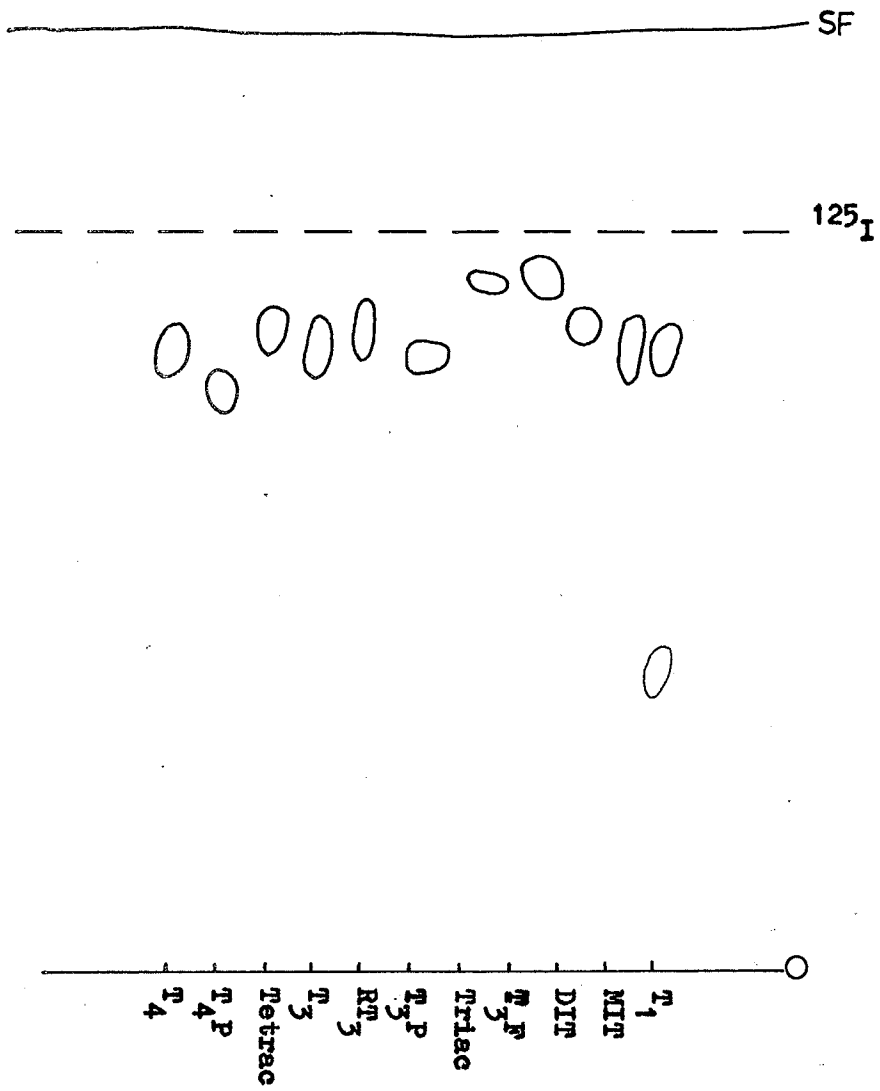
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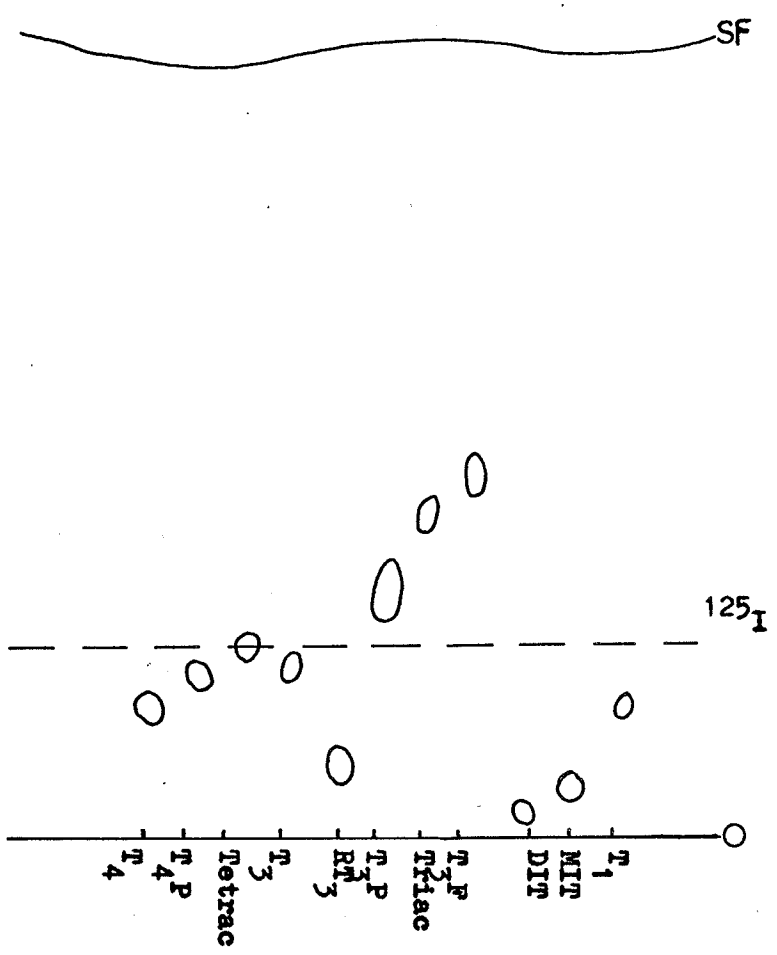

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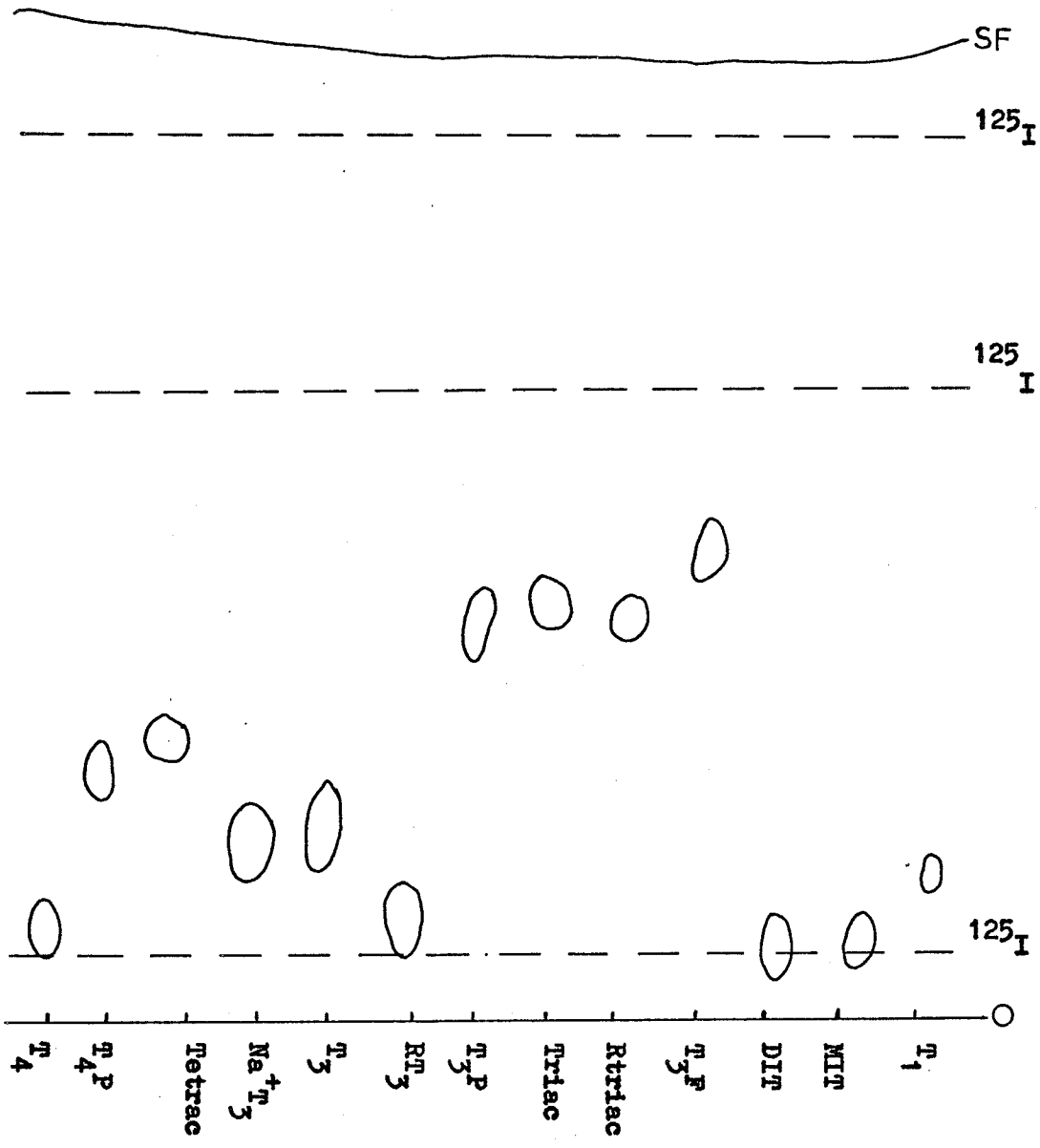
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T1

CMA