IDENTIFICATION, GASTROINTESTINAL ABSORPTION, AND PERIPHERAL METABOLISM OF BILIARY EXCRETED IODOTHYRONINES IN THE BROOK TROUT, SALVELINUS FONTINALIS (MITCHILL)

A Thesis

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by

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T O M O M A N D D A D

ABSTRACT

Twenty-four hours following intraperitoneal injection of L-thyroxine (T_4*) or 3, 5, 3' - triiodo-L-thyronine (T_3*) labelled with ^{125}I in the 3' or 5' positions into starved brook trout at 12 to 13 C, approximately 60 percent of bile radioactivity was identified by paper chromatography as thyronine glucuronide and 14 to 25 percent as free thyronines. For T_4*-injected fish, T_4*-glucuronide (T_4*-gl) was found to be the main product by thin layer chromatography (TLC); for T_3*-injected fish, T_3*-glucuronide was the main product, but other derivatives may be present. Paper and TLC differed in the relative proportions of free thyronines and glucuronides in bile separated by these methods.

Limited enterohepatic cycling of both T_4* and T_4*-gl was demonstrated. Approximately 12 to 33 percent of radioactivity from T_4* or radioactive bile introduced transintestinally was absorbed from the gut lumen with no clear differences in uptake of T_4* and T_4*-gl. Maximum absorption took place within 2 hours, with little subsequent uptake.

Absorbed T_4*-gl rapidly entered the liver and occurred in bile as such. T_4*, although occurring in bile, seemed more prone to deiodination. These differences may be partly due to T_4*-gl binding less strongly than T_4* to plasma proteins.

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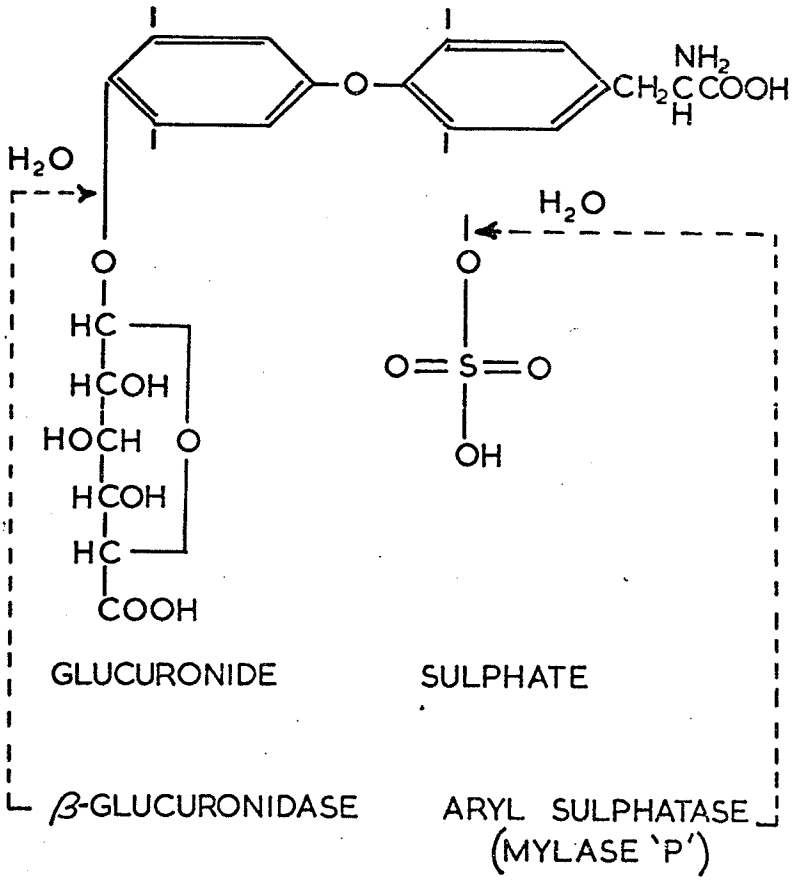
INTRODUCTION

Thyroid hormones and their derivatives have been identified in the bile of several homeotherms (Tata, 1964) including the rat, (Taurog et al. 1951, 1952, 1954; Briggs et al. 1953; Roche et al. 1953, 1954; Flock et al. 1965), human (Blomstedt and Neujahr, 1964; Myant, 1956), monkey (Myant and Osorio, 1964; Osorio and Myant, 1965), chicken (Hutchins and Newcomer, 1965), cat (Myant and Osorio, 1966), dog (Flock et al. 1960; Furth et al. 1968) and sheep (Irvine, 1969). Thyroid hormones while existing as such in the bile are usually encountered as sulphate or in most cases glucuronide conjugates (Fig. 1).

In poikilotherms, biliary excretion of thyroxine (T_4) has been recently demonstrated in the plaice, Pleuronectes platessa (Osborn and Simpson, 1969) and the brook trout, Salvelinus fontinalis (Eales, 1969, 1970). Biliary excretion of T_3 has also been shown in the brook trout (Eales et al. 1971). Following radiothyroxine injection Osborn and Simpson demonstrated several radioiodinated sulphate or glucuronide conjugate derivatives in plaice bile. In the brook trout injected with T_4* or T_3* (Eales, 1970; Eales et al. 1971), approximately 75 percent of bile radioactivity existed as an unknown compound or compounds. One objective has been to

FIGURE 1. Structural representation of thyroxine glucuronide and thyroxine sulphate, and the proposed site of hydrolytic action of β-glucuronidase and arylsulphatase in the respective molecules.


NH2
CH2CCOOH
H
O
H2O
H2O
HC
HCOH
HOCH
O
HCOH
HC
COOH
O
O=S=O
OH
GLUCURONIDE
SULPHATE
β-GLUCURONIDASE
ARYL SULPHATASE
(MYLASE 'P')

identify the major radioiodinated derivatives in the bile of brook trout following injection of T_4* or T_3*.

Since the discovery of the liver-bile pathway for excretion of thyroid hormones (Taurog et al. 1951), the role of enterohepatic circulation (Fig. 2) of these hormones has been studied in several animals. Most work has been done with homeotherms, particularly rats (Table I). Generally the extent of enterohepatic circulation of thyroid hormone depends upon: (a) the overall physiological state of the animal (b) the diet (c) the physiological state of the gastro-intestinal tract (d) and the form of the hormone in the bile.

Glucuroconjugation may influence enterohepatic circulation and thus regulate thyroxinemia. Briggs et al. (1953) discussed this on discovering large amounts of thyroxine glucuronide (T_4-gl) in rat bile. Others suggesting this were Roche et al. (1954, 1954) and Taurog (1954). Subsequently, Pitt-Rivers and Tata (1959) mentioned differences in several mammalian species. Tata (1964), emphasized glucuroconjugation as a deactivation process which allows an animal to rid itself of excess hormone by altering entero-hepatic circulation. Flock and Bollman (1965) suggested glucuroconjugation as a mechanism for storage of thyroid hormones. Williams et al. (1965) and Jayle and Pasqualini

FIGURE 2. Schematic representation of generalised enterohepatic circulation of thyroid hormones in vertebrates. Arrows represent directional movement of free thyroid hormone.

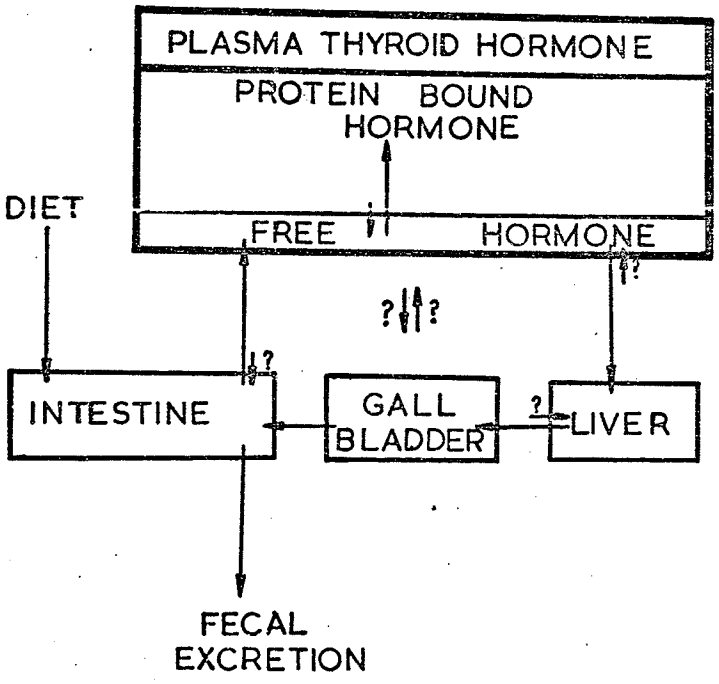

PLASMA THYROID HORMONE
PROTEIN BOUND HORMONE
FREE HORMONE
DIET
?
?
?
INTESTINE
GALL BLADDER
?
LIVER
FECAL EXCRETION

TABLE I

Various studies on absorption and enterohepatic circulation of thyroid hormones and their derivatives in several species.

Source	Animal	Injection Compound	Duration of Experiment	Method of Administration	Results	Miscellaneous Information
Clayton et al	Rat	$Na^{131}I$-T_4	0-96 hr.	Oral	(Oral) About 70% absorption.	
(1949)	Cat		0-6 hr.	Intraduodenum Subcutaneous Intravenous	(Duodenum) 25% absorption.	
Albert et al	Rat	$Na^{131}I$	0-2 hr.	Stomach	(Stomach) Very little absorption.	
(1952)			0-5 hr.	Samll Bowel Large Bowel	(Intestine) More than 90% absorption.	
		$Na^{131}I$-T_4	0-2 hr.	Stomach	(Stomach) Very little absorption.	
			0-5 hr.	Small Bowel Large Bowel	(Intestine) About 25% absorption.	
Albert and Keating (1952)	Rat	^{131}I-T_4	——	Intravenous Intraduodenum	(Duodenum) 50% absorption (97% participation in Enterohepatic Circulation.)	Bile ducts were ligated.
Briggs et al (1953)	Rat	^{131}I-T_4-Glucuronide (Compound 'U')	0-96 hr.	Intraduodenum Infusion	30% absorption of Endogenous bile.	Endogenous bile 62% ^{131}I-T_4Gl 36% ^{131}I-T_4
		^{131}I-T_4			58% absorption of T_4 in bile.	^{131}I-T_4 + bile

Table I continued

					24% to 40% absorption of T_4-gl in bile.	^{131}I-T_4gl + bile.
Myant (1957)	Rat	^{131}I	0-3 days	Intra Peritoneum	38 to 68% Biliary re-absorption	% reabsorbed = (Biliary - (Fecal Clearance) Clearance) X 100
Van Middlesworth (1957)	Rat	^{131}I-T_4	0-5 days	Intra Peritoneum	High bulk diets may cause goitre via general increase in fecal excretion of thyroid hormones.	
Intoccia and Van Middlesworth (1959)	Rat	^{131}I-T_4	0-4 days	———	More rapid fecal loss at 10 C	10 C acclimation 20 C acclimation
Kassenaar (1959)	Rat	^{131}I-T_4	24 hrs.	———	More rapid fecal loss at 4 C.	Antithyroid Drug: $KClO_4$ 4 C & 32 C - acclimation temperatures.
Herz et al (1961)	Rat	^{131}I Labelled Triac, Tetrac, Triprop, Tetraprop, L-T_4, L-T_3	120 min.	In Vitro Gut Sacs.	Glucuronides of analogues are not transported by Everted Gut Sacs. T_4 is passively absorbed by mucosa. Glucuronides may be manufactured by Gut Cells.	
Cottle and Veress (1964)	Rat	^{131}I-T_4	0-3 days	Intra Peritoneum	More rapid fecal loss occurred at 6 C, and with Purina diet - no difference between fecal excretion of 15 and 20% cellulose.	28 C 6C (Cellulose %) (a) 15% (b) 21% amphicel (c) Purina diet

Table I continued

Chung and Van Middlesworth (1964)	Rat	$^{131}I-T_4$	0-6 days	Intraduodenum Intraduodenum Jejunum Intraduodenum Ileum	20% to 50% cumulative absorption Ileum-highest Duodenum-lowest.	Essentially total absorption by first hour.
Cottle and Veress (1965)	Rat	$^{131}I-T_4$ + Bile	2 hr.	Intrajejunum	(L-T) 37% absorption at 5 C. 41% absorption at 25 C.	In vivo ligation of 5 cm Jejunal loops. -5 C acclimation of rat; 125 C acclimation of rat
		^{131}I Endogenous Bile after $^{131}I-T_4$ injection (75% T_4-gl)			(^{131}I Endog Bile) 16% absorption at 5C	-5C acclimation of rat
					15% absorption at 25C	-25C acclimation of rat.
		$^{131}I-D-T_4$			($D-T_4$) 43% absorption indicates absorption is passive rather than active.	
Baker et al (1965)	Rat	$^{131}I-T_4$	0-10 hr.	Oral	Dietary hemoglobin interfered with several aspects of T_4 action, and also intestinal reabsorption of T_4.	Diet supplements (a) hemoglobin (b) alkaline hydrolysate of hemoglobin.
Ruegamer and Wallace (1965)	Rat	$^{131}I-T_4$	0-60 hr.	Oral	Hemoglobin and other dietary factors interfered with T_4 absorption and therefore T_4 activity.	Effect of Different diets on Absorption of T_4.
Girard et al (1966)	Rat	^{131}I	0-17.5 weeks	Intra-Peritoneum	Cholesterol-Cholic Acid was goitrogenic increasing iodide excretion probably via interference with thyroid hormone reabsorption from bile.	Low iodine diet with various mixtures of cholesterol and cholic acid.

Table I continued

Chung and Van Middlesworth (1967)	Rat	$^{131}I-T_4$	0-5 hr.	Intra Ileum Intra Colon	(Ileum)37% absorption (Washed Loops) (colon) 56% absorption (washed loops). Ileum and Colon contents interfered with absorption. Fasting rats showed higher absorption than fed rats.	Washed Gut Loops Washed Gut Loops Fasting rats (24 hrs.)
Ruegamer et al (1967)	Rat	$^{131}I-T_4$	0-16 days	Oral	Hemoglobin increases fecal excretion (by decreasing absorption) probably via (1) increasing rate of food movement through gut (2) increasing fecal mass (3) interference in T_4 absorption via binding T_4.	10% hemoglobin in diet.
Hall and Hershman (1968)	Rat	$^{125}I-T_4$ $^{131}I-T_4$	0-6 days	$^{125}I-T_4$ oral $^{131}I-T_4$ Intraperitoneum	54 to 63% absorption (hypo) 60 to 65% absorption (normal)	-Hypothyroid rats - Normal rats
Galton and Nisula (1969)	Rat	^{125}I ^{127}I	———	———	Net intestinal absorption of endogenously metabolized ^{131}I compounds referred to as nil at all temperatures.	4 C acclimation 22 C acclimation
Heroux and Petrovic (1969)	Rat	$^{125}I-T_4$	0-48 hr.	Intravenous	T_4 clearance rate faster at 4C for all diets. T_4 turnover faster at 4 C for Hi bulk diet. In general a demonstration of diet dependence for changes in T_4 turnover in cold acclimation of rats.	High bulk diet Low bulk diet Both at 4C 28 C acclimation
Linazasoro et al (1970)	Rat	$^{131}I-T_4$	0-48 hr.	Gastric Incubation	20-25% absorption (walnut diet) 80-90% absorption (control)	Walnut diet and Control diet with ligated bile ducts

Table I continued

Cottle and Veress (1971)	Rat	^{131}I-T_3-gl (a)+ T_3 (stable)	0-2 hr.	Intrajejunum	(Jejunum) a. 5% absorption (Unwashed) b. 40% absorption (Unwashed) c. 9% absorption (Washed)	Isolated gut loop lumens Unwashed Unwashed Washed
		(b) ^{131}I-T_3 + gl (stable)		Intracolon	(Colon) a. 19% absorption (Unwashed) b. 19% absorption (Unwashed) Washing Lumens increased absorption.	Unwashed Unwashed
		(c) ^{131}I-Endog. bile + T_3 (stable)				
Sinha and Van Middles-Worth (1971)	Rat	^{125}I-T_4 ^{125}I-T_3	0-4 hr.	Intrajejunum (2 hr. and 4 hr. Injection)	(T_4) 37% absorption (without bile) (T_3) 69% absorption (without bile) Addition of bile produced no significant difference.	Isolated Jejunum loops without bile without bile
Bergman et al (1966)	Hamster	^{131}I-T_4	0-14 days	Intravenous	Cholestyramine decreased absorption and increased fecal excretion of T_4.	cholestyramine in diet.
Bergman et al (1967)	Hamster	$Na^{131}I$ ^{131}I-T_4	0-12	Intravenous	Charcoal increased fecal excretion of T_4 but not of iodide. Cholestyramine increased fecal excretion of both.	Charcoal in diet. Cholestyramine in diet.
Mixner and Lennon (1960)	Cows	^{127}I-T_4 Thyroprotein Thyroglobulin	0-120	Oral	12 to 16% absorption	Cows were lactating
Yatvin et al (1965)	Calves	^{131}I-T_4	0-24 hrs.	Intravenous	Enterohepatic circulation of T_4 with variable absorption.	

Table I continued

Miller et al (1971)	Calves	^{125}I ^{131}I	0-25 min.	Intragastric Intravenous	16 to 28% absorption of iodide	Double isotope method with dual ligation of obomasum.
Irvine (1969)	Sheep	^{125}I-T_4	0-4 days	Intravenous	Enterohepatic circulation although present is not too important.	
Furth et al (1968)	Dog	^{131}I-Endogenous Bile	___	Intraduodenum	Less than 15% absorption of ^{131}I derivatives.	
Myant & Pochin (1950)	Man	^{131}I-T_4	0-25 hr.	Oral	Almost complete reabsorption although caution in interpretation suggested.	
Johnson et al (1953)	Man	^{131}I-T_4	0-6 days	Oral	Absorption not quite total, but blood to gut secretion suggested as explanation	
Myant (1956)	Man	^{131}I ^{131}I-T_4	0-16 days	Intravenous	About 50% enterohepatic recycling of ^{131}I-T_4.	
Myant (1956)	Man	^{131}I Endogenous Bile (24 hours)	0-7 days	Upper Intestinal Infusion	30% Enterohepatic recycling.	
Van Middlesworth (1960)	Man	^{131}I-T_4	0-3 days	Oral	About 60 - 70% absorption.	
Hiss and Dowling (1961)	Man	^{131}I-T_4	0-9 days	Intravenous	Disorder resulted in higher organic iodine excretion.	Metabolic disorder pancreatic steatorrhea.
Hays (1966)	Man	^{125}I-T_4 ^{131}I-T_4	0-72	Oral Intravenous	Both sexes showed 40-50% absorption effectively within 2 hours.	Double Isotope Method.

Table I continued

Hays (1968)	Man	^{125}I-T_4 ^{131}I-T_4 ^{131}I-T_4-gl with ^{125}I-T_4	0-72 hrs. a few hours	Oral Intravenous Oral	42% absorption (capsule fed) 74% absorption (liquid fed) Greater absorption of T_4 than T_4-gl, rapid urinary excretion of T_4-gl suggested.	Double Isotope
Hays (1970)	Man	^{125}I-T_3 ^{131}I-T_3	0-72 hrs.	Oral Intravenous	95% absorption within 4 hours	Double Isotope
Read et al	Man	^{125}I-T_4 ^{131}I-T_4	0-24 days	Oral Intravenous	75 ± 17% absorption (Hypo) 68 ± 13% absorption (normal)	Double Isotope Hypothyroid Normal
Hutchins and Newcomer (1966)	Chicken	^{131}I-T_4 ^{131}I-T_3	0-4 hrs.	Intravenous	Importance of Biliary excretion mentioned.	
Monroe and Turner (1949)	Chicks	T_4, mono- and di-sodium salts of T_4	0-21	Oral	Crystalline T_4 - 20% absorption; sodium salts of T_4 - 45% absorption.	Indirect Measurement via prevention of thyroid hypertrophy.
Osborn and Simpson (1969)	Fish (Plaice)	^{131}I ^{125}I-T_4	___	Introduced into gut	Enterohepatic circulation of T_4 referred to as extensive.	
De Los Reyes and Jones (1970)	Frog (a) Salamander (b) Turtle (c)	^{131}I-T_4	0-120 min.	(a) Intragastric Small Intestine Large Intestine	(a) 78 ± 6% absorption (stomach) 62 ± 4% absorption (S. Intestine) 67 ± 3% absorption (L. Intestine)	

Table I continued

(b) Small Intestine	(b) 58 ± 3%	absorption (small Intestine)
(c) Intragastric	(c) 37 ± 4%	absorption (Stomach)
Small Intestine	45 ± 6%	absorption (S. Intestine)
Large Intestine	25 ± 5%	absorption (L. Intestine)

absorption occurred rapidly and was probably hindered by binding substances in gut.

(1966) emphasized that this process is associated with regulation of thyroxinemia. Most of the work indicated that in homeotherms, the thyroid hormone glucuronides are not absorbed from the gut as readily as the free hormone.

Two papers relate to poikilotherms, Osborn and Simpson (1969) mentioned the existence of enterohepatic cycling of T_4* following transintestinal injection; while De Los Reyes and Jones (1970) compared absorption of ^{131}I - T_4 in different areas of the gut in the frog, salamander and turtle. Thus the other objective of this study was to compare the absorption of T_4* and T_4*-gl from the gastrointestinal tract of the brook trout, and to determine the magnitude of enterohepatic circulation of biliary derivatives of T_4* in this fish.

MATERIALS AND METHODS

Fish Maintenance

One to three-year-old brook trout (26 to 543 g) from Province of Manitoba Trout Hatchery, West Hawk Lake, were acclimated for at least one week prior to injection at 10 to 13 C in circulating, aerated, dechlorinated Winnipeg City water in covered tanks. Most fish were starved for more than 2 weeks prior to injection although some were fed ground liver on alternate days, but this was withheld 48 hours prior to and 24 hours following injection.

Injection

T_3* (specific activity (s.a.) 40 m Ci/mg) or T_4* (s.a. 28.6 to 62.5 m Ci/mg) labeled in the 3 or 5 positions (Amersham-Searle), in 50% propylene glycol or a bile vehicle, or carrier-free $Na^{125}I$ (Atomic Energy of Canada) in distilled water, were injected intraperitoneally, into the stomach, or into the intestine from a 1.0 ml syringe. Fish were anaesthetized in MS 222 (Sandoz) 1:30,000. Standards were prepared which contained the injected dose or an aliquot of it. These were made up to the same geometry and in the same type of tubes as samples to which they would be compared.

Intraperitoneal injections were made with a 27-gauge needle either just above the lateral line level with the dorsal

fin, or ventrally, anterior to the pelvic fins.

Stomach injections were made using a 5-inch Yale needle (R 76 BD 20) blunted with a piece of plastic tubing, and a 1.0 ml. glass syringe (Hamilton-Gastight). The needle was introduced orally into the stomach and the dose expressed, holding the anaesthetized fish, head up vertically.

In vivo operations were necessary for transintestinal injection and were performed on anaesthetized fish held intermittently out of water for 5 to 7 minutes. The operation and injection of each fish went as follows: a posterior-ventrolateral incision was made 3 to 4 cm anterior to the anus, the intestine exposed and tightly ligatured with silk thread. The incision was then sutured using a 3/8 circle stainless steel surgeon's needle and silk thread. The fish was placed in the anaesthetic for 2 to 3 minutes to relieve exaquatic stress. Subsequently an anterior-ventromedial incision was made either slightly anterior to the pelvic fins or above the liver, the intestine exposed, and tightly ligatured (in the latter case, just posterior to the pyloric sphincter). The injection was accomplished by obliquely piercing the intestinal wall with the needle, and posteriorly expressing the syringe contents. After suturing, the fish was replaced in the holding tank at 13 C and revived by gently moving water over the gills.

Counting

This was done using one of two automatic gamma systems (Nuclear Chicago), for two consecutive 5 - minute periods or 900,000 counts. Either plastic or glass counting tubes were used. The background was regularly checked and accounted for. Also all physical decay of the radioactivity was accounted for.

Blood and Tissue Sampling

Fish were killed by a high dose of MS 222 and a blow to the head. Two methods for collecting blood were used: (i) the tail was severed and blood from the caudal artery allowed to drip into individual heparinized foil cups (ii) *in vivo* collections were made by removing 0.3 to 0.5 ml of blood from the caudal artery of anaesthetized trout using a 1 - ml heparinized syringe (25-gauge needle) and applying slight negative pressure.

Plasma was collected by centrifuging each whole blood sample for 5 minutes in 1 - ml disposable polyethylene microcentrifuge tubes at 15,000 g (International Microcapillary Centrifuge Model MB slanted head), and removing the supernatant. The plasma samples and intact carcasses were frozen at -20 C.

Trichloroacetic acid (TCA) precipitation (Higgs 1971) was performed on thawed plasma samples thus: (i) 0.1 to 0.2 ml

of the sample was placed in a 15-ml nalgene centrifuge tube (ii) 2 ml of 12.5 percent TCA were added twice with vigorous shaking and subsequently the tube was centrifuged at 2200 g, and the supernatant removed. This was repeated using 2.5 percent TCA. The two, 4 - ml supernatants were combined and a 4 - ml aliquot removed. (iii) The precipitate was suspended in 1.9 (or 1.8) ml of distilled water and 2 ml of 4 N NaOH was used to dissolve the suspension. (iv) The TCA-soluble and insoluble aliquots were counted against a standard and the radioactivity in both bound and unbound plasma fractions was expressed as percent injected dose per ml of plasma correct for a body weight of 100 g thus: $\frac{\text{percent dose}}{\text{ml}} \times \frac{\text{body weight}}{100\ \text{g}}$.

Bile was obtained by removing the frozen gallbladder from the carcass and collecting the bile. The bile was pooled for several fish and centrifuged in siliconized glass tubes (siliconization was accomplished by dipping the tubes in 5 percent dichloro-dimethyl silane), for 20 minutes at 2200 g (Sorvall centrifuge GLC1 swing out head).

Frozen tissues including liver, gallbladder, in some cases total gut less isolated intestinal loop, stomach and gastric caeca (together), total intestine (in some cases isolated intestinal loop), in some cases gonad (testis or ovary), kidney, gill and thyroid were collected for analysis. All tissues were counted in plastic counting tubes and percent

dose for each tissue was determined.

Treatment of Bile For Identification of Radioactive Constituents

Up to 4 replicate samples of the bile supernatant were incubated for 24 hours with shaking in darkness at 37 ± 0.5 C (Metabolyte Shaker New Brunswick Instrument), with either 500 units of β - glucuronidase (bacterial Sigma Chemical Corporation) or with 0.005 g of Mylase 'P' (Arylsulphatase; Nutritional Biochemicals) at pH 5.0 (adjusted with a sodium acetate buffer). These samples were then compared chromatographically with a control (0.25 ml of bile supernatant alone under identical conditions), on either paper or thin layer systems following centrifugation for 20 minutes at 2200 g. Tests establishing arylsulphatase activity and inhibition by saccharolactone of residual β - glucuronidase activity in Mylase 'P', were performed (Appendix I).

Chromatography

Descending paper chromatography was performed using either Whatman. No. 1 or 3 paper, with butanol; acetic acid (glacial); water; 4:1:1, V/V (BAW) as solvent, with development for 16 hours in darkness at room temperature. Half-inch sections of the sheets were counted for determinations of radiochromatograms.

Ascending thin-layer chromatography (TLC) was performed on either Polygram silica gel (0.25 mm, UV 254, Macherey-Nagel and Co., Duren) or preferably Bakerflex silica gel (0.25 mm 1B-F) media, using BEA (butanol; ethanol; 6N ammonia, 5:4:1 V/V) or BMA (butanol; methanol; 6N ammonia 5:4:1 V/V) as solvents. For both paper chromatography and TLC, pre-equilibration of the solvent in the tank was allowed for at least one hour. One-eighth-inch sections of the TLC media were counted for determination of radiochromatograms. Both stable and radioactive standards were used for identification, the former being detected by UV light at a wavelength of 250 mu.

Samples of centrifuged bile or plasma were spotted (25 µl, paper; 5 or 10 µl, TLC). In some cases ethanol extracts of bile, plasma, or tissue samples were spotted. The extracts were obtained by adding 2 volumes of absolute ethanol to 1 volume of tissue in a 15-ml nalgene tube. This was vigorously mixed, centrifuged (at 2200 g for 30 minutes) and the supernatant removed and evaporated to dryness under air streams in a water bath at 40 C. The residues were dissolved in a small volume of methanolic ammonia (99:1, V/V).

Binding of T_4* and T_4*gl to Plasma Proteins

One ml of T_4* in bile or radioactive bile (24 hours

after T_4* injection), was extracted with ethanol and the residue mixed with 1 ml of whole plasma from trout acclimated at 10 to 13 C. The two plasma samples were then incubated at 13 $\pm$ 0.5 C in darkness for 24 hours with shaking (Falkner 1971). After incubation four 0.2-ml aliquots were removed from each sample and analysed by TCA precipitation. The soluble fractions were also examined by TLC (Bakerflex BEA).

RESULTS

Identification of Bile Radioactive Compounds after Intraperitoneal Injection of T_3* and T_4*

One-year-old brook trout (51 to 203 g), acclimated at 12 C and fed ground liver on alternate days until 48 hours prior to injection, were anaesthetized and injected intraperitoneally with 1 to 5 µCi of T_4* or T_3* (in 50% propylene glycol) in the lateral region. Twenty-four hours later they were killed.

At 24 hours p.i. in trout injected with either T_3* or T_4*, 70 to 80 percent of bile radioactivity occurred at Rf 0.2 to 0.3 and about 20 to 30 percent corresponded to free thyronines on paper chromatograms (Table II and III; Fig. 3). Negligible radioiodide was detected. Treatment with Mylase 'P' did not alter significantly the distribution of radioactivity along the chromatogram. This negative result was not due to lack of sulphatase activity in the Mylase 'P' preparation, for this activity was established. It is concluded that negligible quantities of thyronine-sulphate esters occur in trout bile.

Treatment with β - glucuronidase displaced approximately 60 percent of the total chromatogram radioactivity from the region of Rf values 0.2 to 0.3, to that of thyronines. It

TABLE II

Percentage of bile radioactivity on paper chromatograms (BAW) corresponding to thyronines and unidentified peaks following incubation with Mylase 'P' and β-glucuronidase. Bile was sampled 24 hours after T_3* injection.

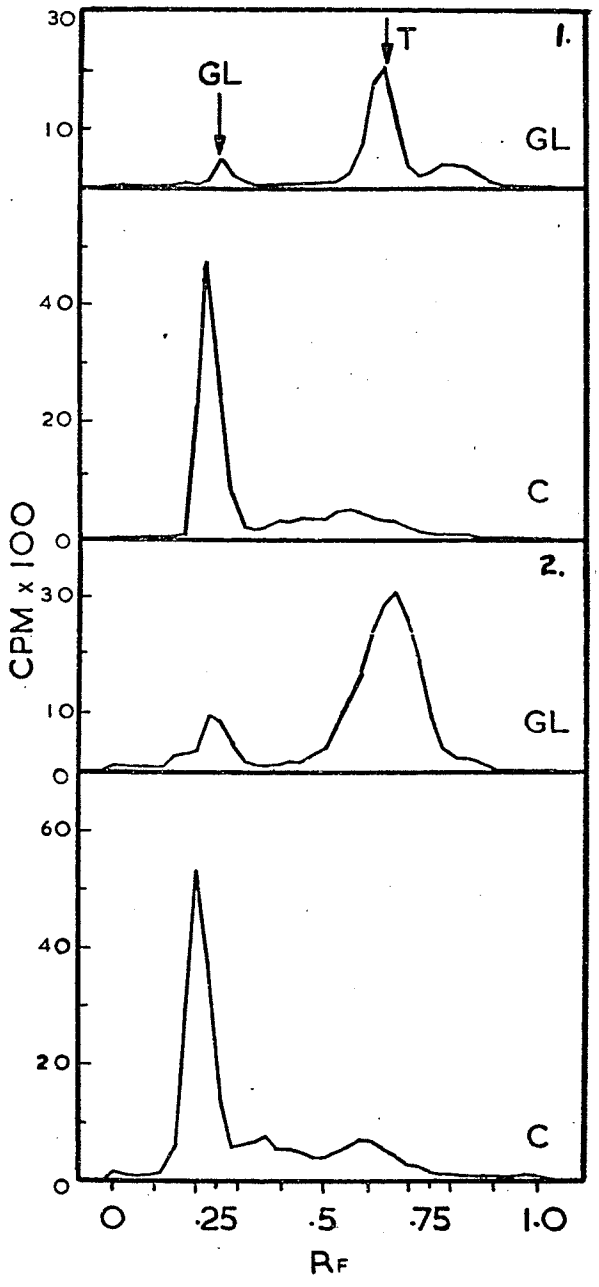
Treatment of Bile	n	Unidentified Peaks (Rf 0.2-0.3) $\bar{x}$	range	Thyronines TH $\bar{x}$	range	Remainder $\bar{x}$	range
Control	8	72.0	67.2-75.3	25.0	23.7-28.5	3.0	2.0-5.5
Mylase 'P'	2	72.3	72.0-72.6	24.7	24.3-25.1	3.0	2.9-3.1
Control	8	72.0	67.2-75.3	25.0	23.7-28.5	3.0	2.0-5.5
β-glucuronidase	8	11.2	10.1-12.8	84.2	82.2-86.2	4.5	1.8-6.1

TABLE III

Percentage of bile radioactivity on paper chromatograms (BAW) corresponding to thyronines and unidentified peaks following incubation with Mylase 'P' and β-glucuronidase. Bile was sampled 24 hours after T_4* injection.

Treatment of Bile		Unidentified Peaks (Rf 0.2-0.3)		Thyronines (TH)		Remainder	
	n	$\bar{x}$	range	$\bar{x}$	range	$\bar{x}$	range
Control	6	76.6	74-82.3	22.0	17.1-24.5	1.4	0.6-2.3
Mylase 'P'	2	76.9	76.5-77.4	21.6	21.6-21.7	1.4	0.9-1.9
Control	4	79.9	71.9-85.9	17.7	11.1-25.8	2.4	0.8-3.4
β-glucuronidase	4	16.1	15.7-16.9	80.2	79.1-81.2	3.6	2.4-5.1

FIGURE 3. Paper radiochromatograms (BAW) of bile collected at 24 hours after T_3* (1), or T_4* (2) intraperitoneal injection and then incubated alone (C) or with β-glucuronidase (GL) (GL indicates Rf of large peak while T indicates Rf of thyronines).


1.
GL
T
GL
C
2.
GL
C
CPM x 100
30
10
40
20
0
30
10
0
60
40
20
0
0
.25
.5
.75
1.0
RF

appears by paper chromatography that about 20 to 30 percent of bile radioactivity occurs as free thyronines and about 60 percent as thyronine glucuronide conjugates. About 10 to 20 percent of the radioactivity at Rf 0.2 to 0.3 was not degraded by β-glucuronidase.

Thin layer chromatograms of untreated and β-glucuronidase-hydrolysed bile on Polygram silica gel, suggested that free thyronines and their glucuronide conjugates were the main radioactive constituents in bile from T_3* - or T_4* - injected trout (Figs. 4 and 5). In contrast to paper separation, treatment with β-glucuronidase on thin layer media eliminated almost entirely the peak at lower Rf values. T_4* was the only detectable product of β-glucuronidase hydrolysis of bile from T_4* - injected fish. While T_3* was the main product of β-glucuronidase hydrolysis of bile from T_3* - injected fish, lesser quantities of radioactivity occurred at slightly higher Rf values and suggest other degradation products.

A comparison of paper chromatography and TLC (Bakerflex BEA) of endogenous bile (from fish 24 hours after intraperitoneal injection of T_4), showed that with paper 83 percent of the radioactivity existed as glucuronide with 17 percent as thyronines; while for TLC, 27 percent of the radioactivity

FIGURE 4. Thin-layer radiochromatograms (BEA) of bile collected at 24 hours after T_3* or T_4* intraperitoneal injection and then incubated alone (C) or with β-glucuronidase (GL). The positions of authentic materials are shown by arrows (T_4 = thyroxine, T_3 = triiodothyronine, GL = glucuronide).

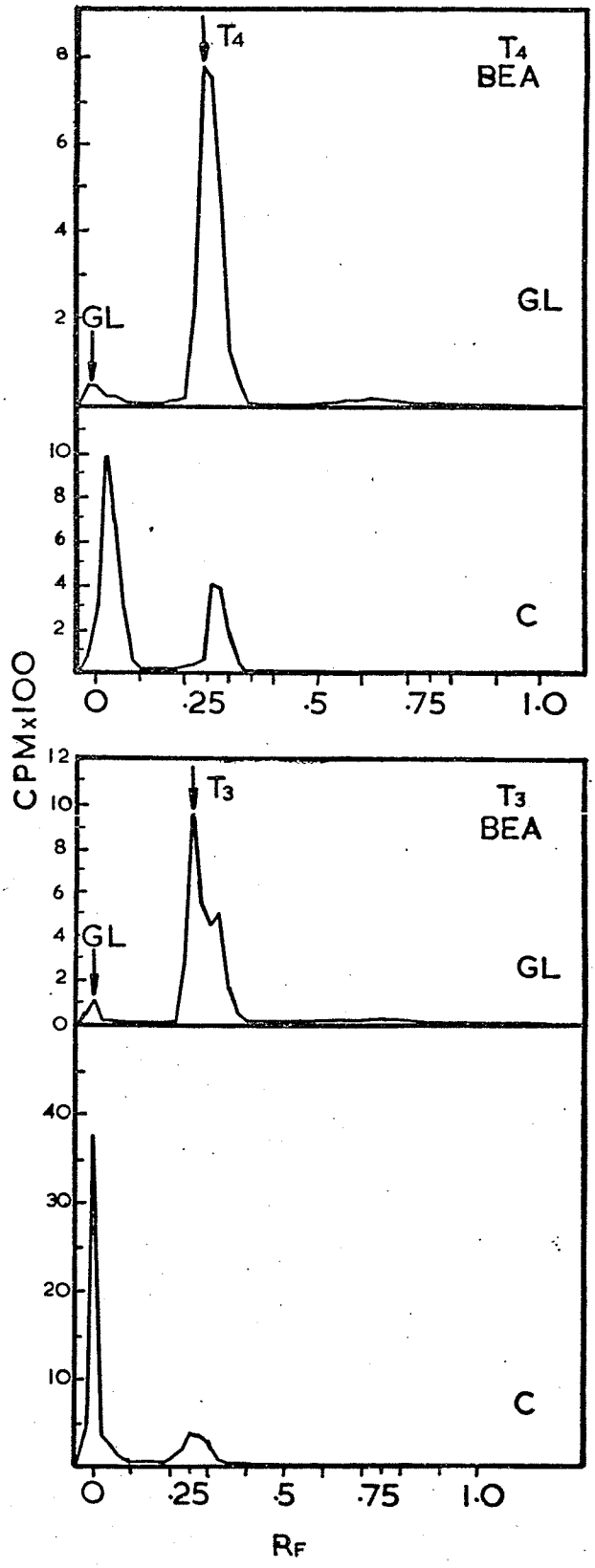
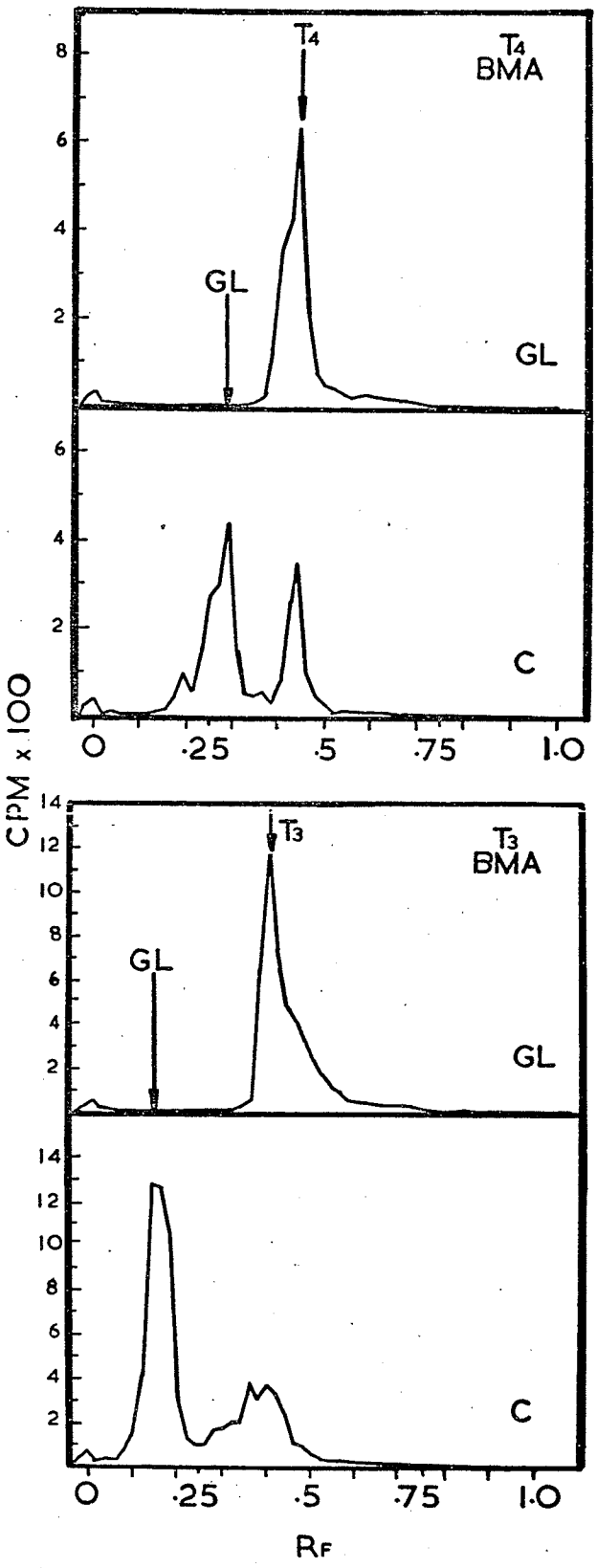

T4
T4
BEA
GL
GL
C
T3
T3
BEA
GL
GL
C
CPMx100
0
.25
.5
.75
1.0
RF

FIGURE 5. Thin-layer radiochromatograms (BMA) of bile collected at 24 hours after T_3* or T_4* intraperitoneal injection and then incubated alone (C) or with β-glucuronidase (GL). The positions of authentic materials are shown by arrows (T_4 = thyroxine, T_3 = triiodothyronine). GL = glucuronide


T4
T4
BMA
GL
GL
C
CPM x 100
T3
T3
BMA
GL
GL
C
0
.25
.5
.75
1.0
RF

existed as glucuronide and 72 percent as T_4.

Uptake of Bile Iodothyronines Following Stomach Injection

One hundred thirty trout (35 - 75 g) were acclimated at 10 - 13 C and starved for over 2 weeks and their skin marked with a number by electrodesiccation (Owens and Gebhardt 1968). Fifty six trout were each injected into the stomach with T_4* containing 90,000 cpm in 0.1 ml of brook trout bile (T_4 group), and 56 with radioiodinated thyroxine glucuronide conjugate (T_4* -gl; T_4 -gl group) preparation containing the same amount of radioactivity in 0.1 ml of trout bile (see Appendix IV for preparation of the latter). Several paper and TLC radiochromatograms were run to test the purity of these substances. The T_4* contained on paper 96 percent as thyronines and 4 percent as iodide, and by TLC, 86 to 89 percent as T_4, 1 to 3 percent as iodide, and the remainder as origin material. The T_4* -gl in bile contained on paper, 11 to 15 percent as thyronines, 85 to 89 percent as gl, and on TLC 25 to 31 percent as T_4 and 69 to 75 percent as gl. After injection each fish was held for exactly 2 min. in 2 l of clean water. An aliquot of this was taken and later counted to test for initial leakage of radioactivity. All fish were held at 11 C after injection. Usually 8 fish were killed and bled in both groups at 2, 5, 10, 24, 48, 72, and 96 hours

p.i. The percentage of injected dose in plasma bound and unbound fractions, and various organs was measured. Some chromatography was performed on bile and serum.

Table IV shows the initial loss to the water at the sampling intervals for fish of the two treatments. Corrected standards were calculated from these individual losses.

Changes in plasma bound and free radioactive fractions during the experimental period are shown in Fig. 6. The plasma bound levels were low for both groups and their validity beyond 24 hours is questionable due to low counts. Because of the low radioactivity and considerable variation, it is difficult to compare fish of different treatments, although the levels were higher for T_4* - injected fish at all intervals.

The plasma unbound levels for both groups were much higher than the bound levels, and the values for the T_4 group (maximum 0.25 percent per ml at 48 hours) were higher than those for the T_4 -gl group (maximum 0.13 percent per ml at 2 hours), at all intervals.

Paper chromatography of plasma of the T_4 group at 48 hours p.i. (Fig. 7), revealed mostly radioiodide. The 48 hour plasma radioactivity for the T_4 -gl group was too low for detection on radio chromatograms.

TABLE IV

Percentage of injected radioactivity lost ($\pm$ standard errors) within 2 minutes of stomach injection of brook trout with T_4* or T_4* -gl.

Sampling Time Hr. (P.I.)	Percent Loss in 2 Min.	
	T_4*	T_4* gl
2	19.2 $\pm$ 4.1	29.2 $\pm$ 3.7
5	21.6 $\pm$ 3.9	28.0 $\pm$ 4.0
10	20.6 $\pm$ 3.9	27.3 $\pm$ 5.6
24	17.1 $\pm$ 5.6	27.9 $\pm$ 4.2
48	22.7 $\pm$ 5.4	25.8 $\pm$ 6.0
72	18.0 $\pm$ 5.0	33.1 $\pm$ 1.5
96	28.7 $\pm$ 8.4	17.0 $\pm$ 5.6
Total Sample Size	55	53
Mean Loss	20.7 $\pm$ 5.2	26.9 $\pm$ 4.4

FIGURE 6. Relationship between plasma bound and unbound levels of radioactivity (percent dose per ml plasma, standardized for a fish of 100 g), at various times after injection of T_4* (-o-) or T_4*-gl (-□- -□-) into brook trout stomach. The unbound levels for radioiodide (-△-.-.) stomach injection are also shown. Each point represents a mean of values from 5-8 fish (± 2 standard errors). The point in parenthesis at 48 hours represents mean if one aberrant fish injected with T_4* is included.

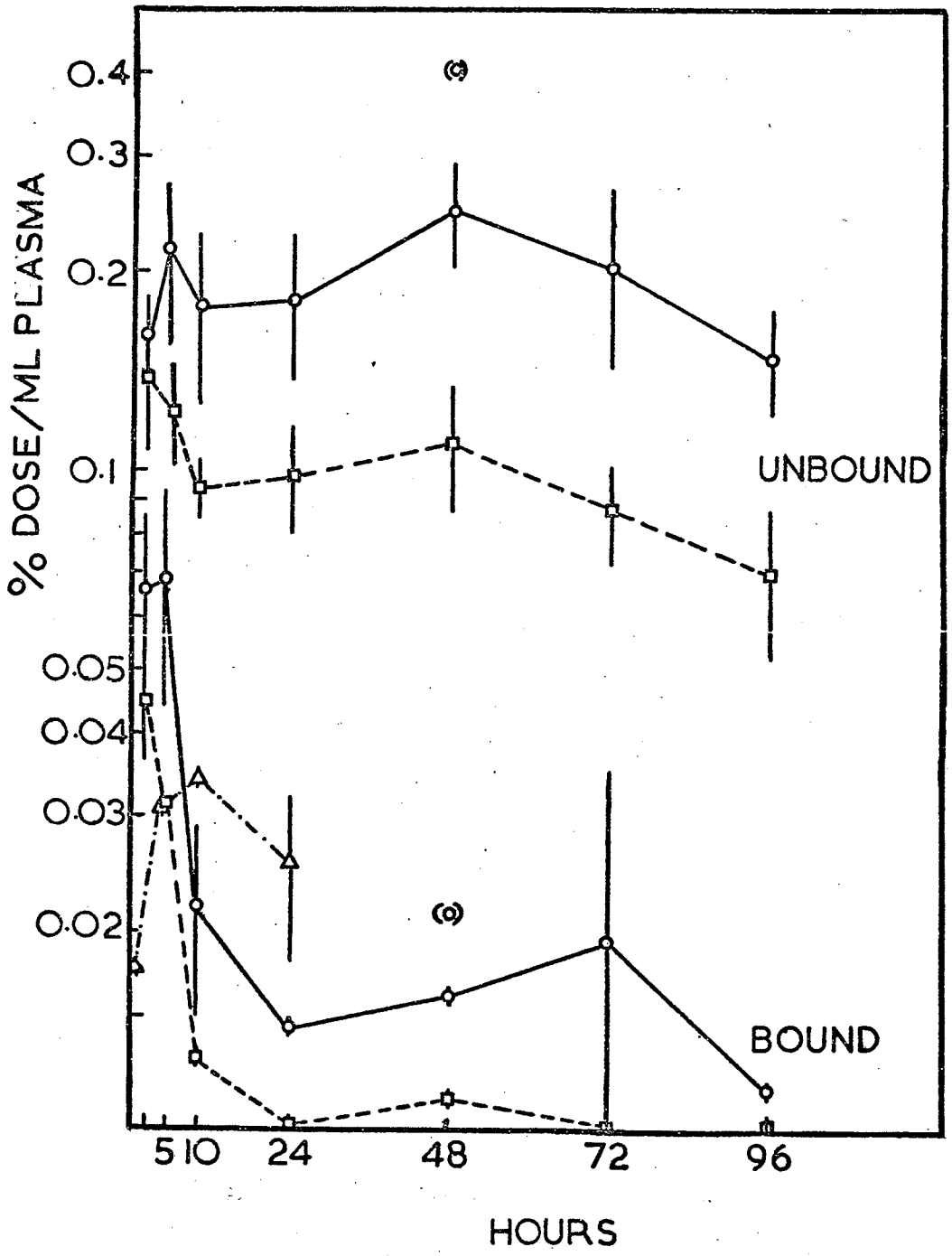
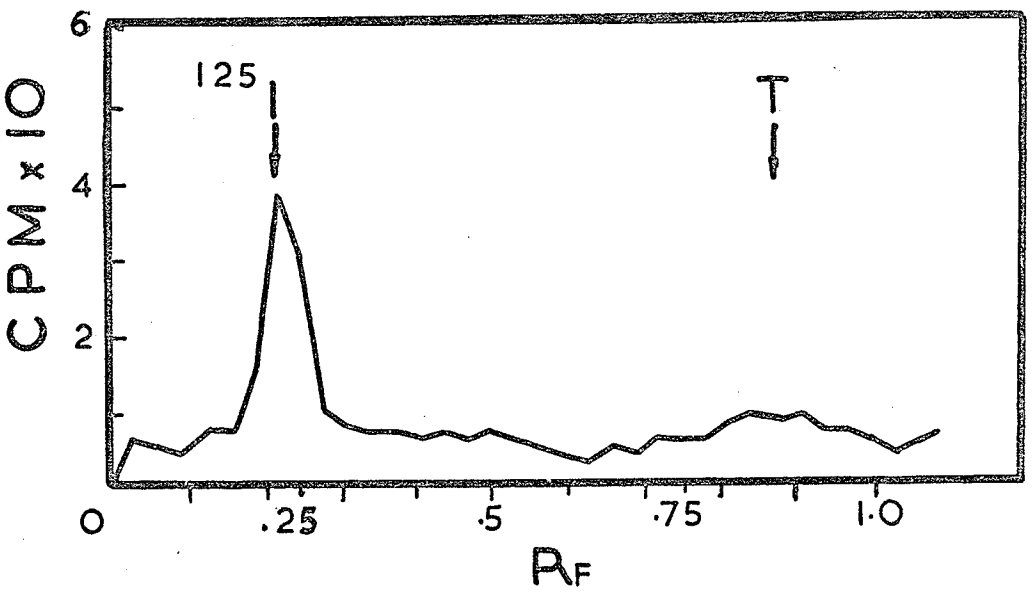

0.4
0.3
0.2
0.1
0.05
0.04
0.03
0.02
% DOSE/ML PLASMA
UNBOUND
BOUND
5
10
24
48
72
96
HOURS

FIGURE 7. Paper radiochromatogram (BAW) of plasma at 48 hours after T_4* injection into brook trout stomach. The positions of authentic materials are shown by arrows (^{125}I = radioiodide, T = thyronines).

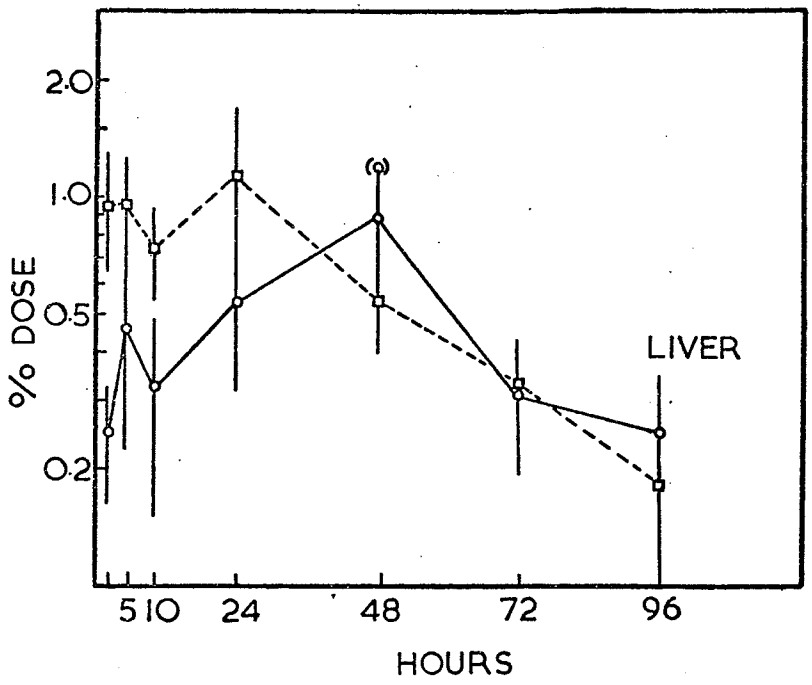

6
4
2
0
CPM x 10
125
T
.25
.5
.75
1.0
RF

Maximum uptake of radioactivity by the livers and gallbladders of both groups coincided (Fig. 8). For T_4, maximum liver (1.0 percent) and gallbladder (0.70 percent) levels occurred at 48 hours with minor peaks at 5 hours; while for T_4 -gl the major peak occurred at 24 hours for liver (1.1 percent) and gallbladder (1.2 percent), with minor peaks at 2 hours. The overall gallbladder levels for T_4 -gl appeared higher than for T_4, while the liver levels for the two groups were similar.

The radioactivity levels in gut components are shown in Fig. 9. The difference in 2 hour-stomach levels of T_4 (30 percent) and T_4 -gl (20 percent), may be due to different losses of radioactivity following the initial measured leakage. Both groups showed a steady decline in stomach radioactivity from 24 to 96 hours, after an initial steep decline (2 to 24 hours). Thus about 24 hours were required for the stomach to empty most of its radioactive contents into the intestine.

The decline in gastric radioactivity between 2 and 24 hours coincided with an increase in intestinal radioactivity to 24 hours (T_4 group 20.5 percent, T_4 -gl 18.4 percent). These levels then gradually declined to 72 hours and showed a more rapid decrease at 72 to 96 hours. Both groups showed that the bulk of the radioactivity remained in the intestine for 48 hours.

FIGURE 8. Percentage of radioactivity in liver and gallbladder of brook trout at various times after T_4* (-O-) or T_4*-gl (-□-) stomach-injection. Each point represents a mean ($\pm$ 2 standard errors) for 5 - 8 fish. The point in parenthesis at 48 hours represents mean if one aberrant fish injected with T_4* is included.


2.0
1.0
0.5
0.2
% DOSE
LIVER
5 10
24
48
72
96
HOURS

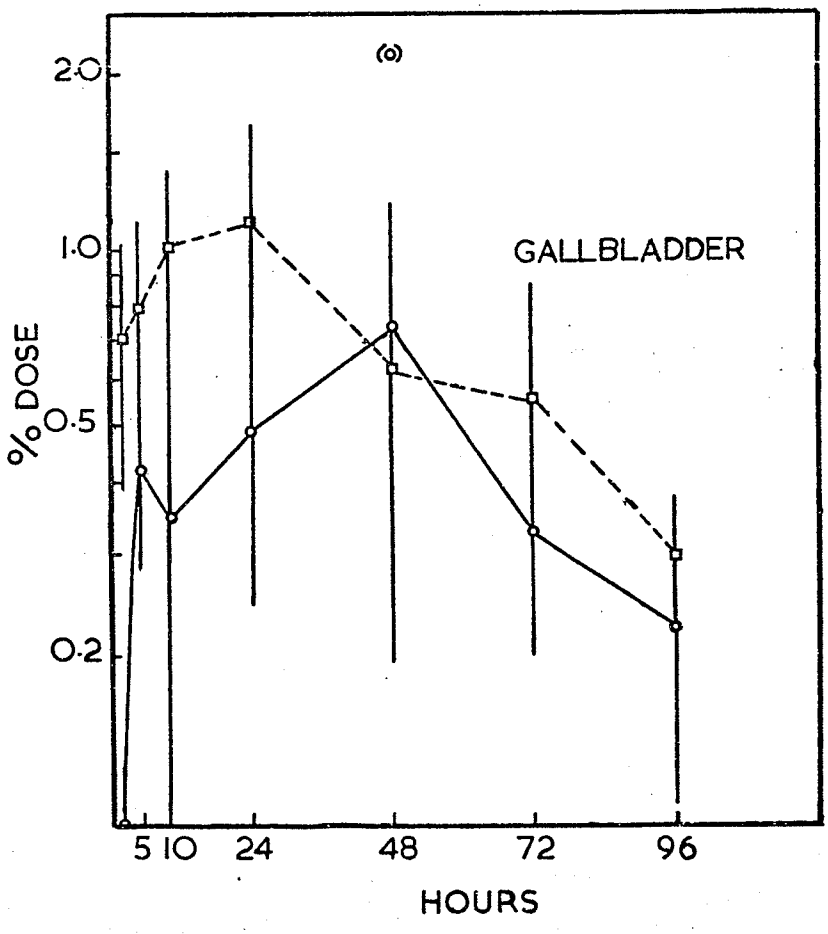
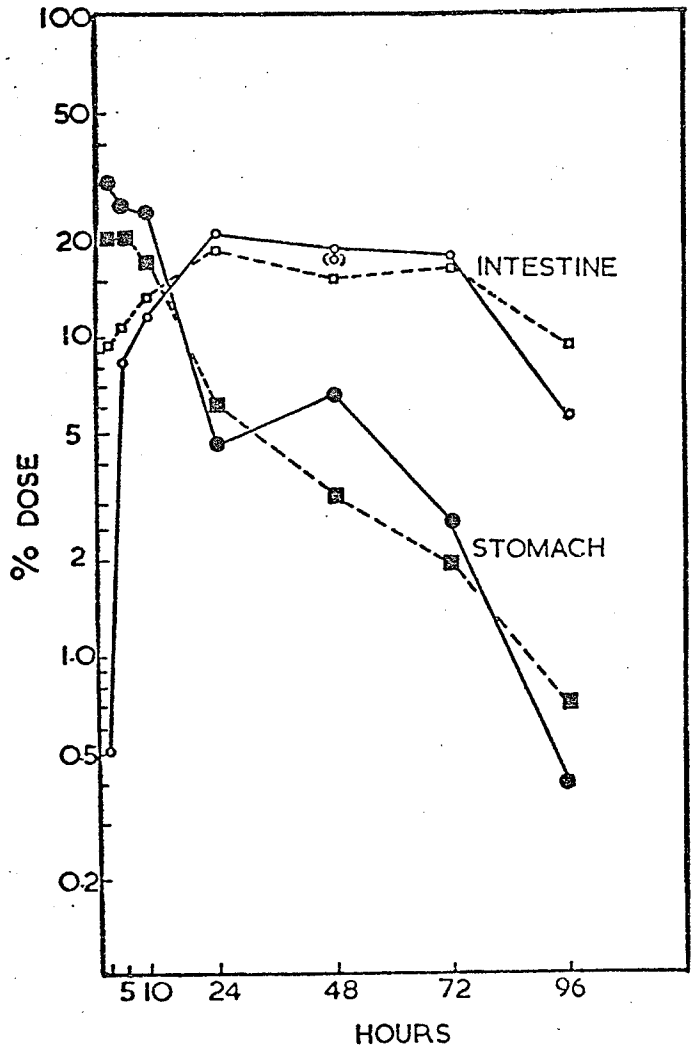
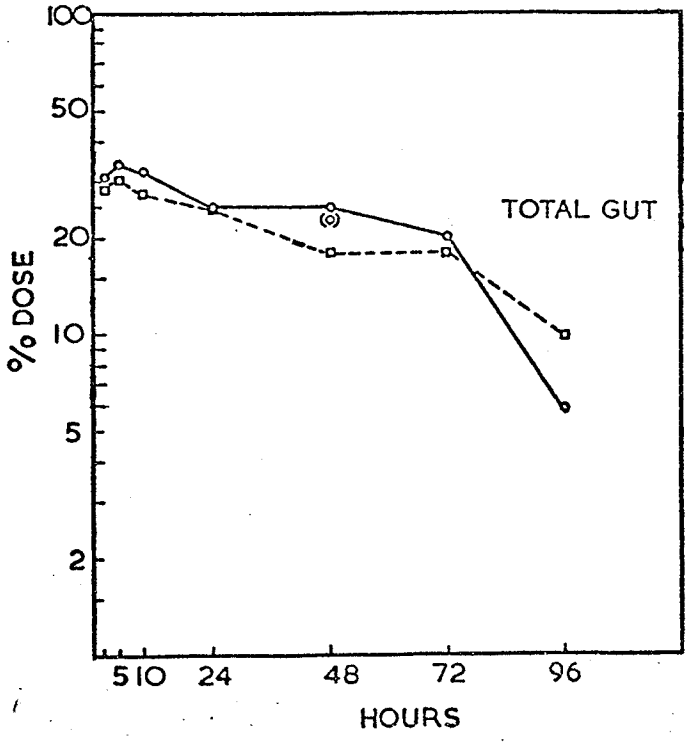

2.0
1.0
0.5
0.2
% DOSE
GALLBLADDER
5 10
24
48
72
96
HOURS

FIGURE 9. Percentage of radioactivity in stomach, (● = T_4*, ■ = T_4*-gl) intestine (o = T_4*, □ = T_4*-gl) and total gut (o = T_4*, □ = T_4*-gl) of brook trout at various times after T_4* (——) or T_4*-gl (- - -) stomach-injection. Each point represents a mean ($\pm$ 2 standard errors) for 5 - 8 fish. The point in parenthesis for each gut region represents mean if one aberrant fish injected with T_4* is included. Means $\pm$ 2 standard errors in Appendix V.


100
50
20
10
5
2
1.0
0.5
0.2
% DOSE
INTESTINE
STOMACH
5 10 24 48 72 96
HOURS


100
50
20
10
5
2
% DOSE
TOTAL GUT
5 10 24 48 72 96
HOURS

Total gut patterns for the T_4 and T_4 -gl groups were similar, with 2 hour levels at about 30 percent followed by a gradual decrease to 72 hours, and a steep decrease from 72 to 96 hours. These data emphasize the long period necessary for appreciable intestinal excretion of radioactivity, and also the low initial levels of gut radioactivity.

At 24 hours most bile radioactivity from T_4 fish corresponded to thyronines on paper chromatograms, with little glucuronide conjugate (Fig. 10), while at 48 hours the reverse was true. For T_4 -gl fish, the bile at 24 and 48 hours revealed mostly glucuronide, with less thyronines.

TLC of bile from T_4 fish (Fig. 11) at 48 hours revealed mostly T_4 with less glucuronide, while TLC of bile from T_4 -gl fish at 24 hours revealed a radioactive pattern similar to the paper chromatography of this sample.

The results of stomach injection of bile iodothyronines indicated that absorption of radioactivity from the gastrointestinal tracts of the fish of both groups was low as revealed by low plasma levels, especially of TCA-precipitable (protein bound) radioactivity. The TCA soluble fraction (unbound) of plasma radioactivity showed higher levels than the bound fractions, and the unbound levels were consistently higher for T_4 fish than T_4 -gl fish. Biliary excretion of

FIGURE 10. Paper radiochromatograms (BAW) of bile at 24 (1) and 48 (2) hours after T_4* (T_4), or T_4*-gl (GL) injection into the stomach of brook trout. The position of authentic materials are shown by arrows (T = thyronines, GL = glucuronide).

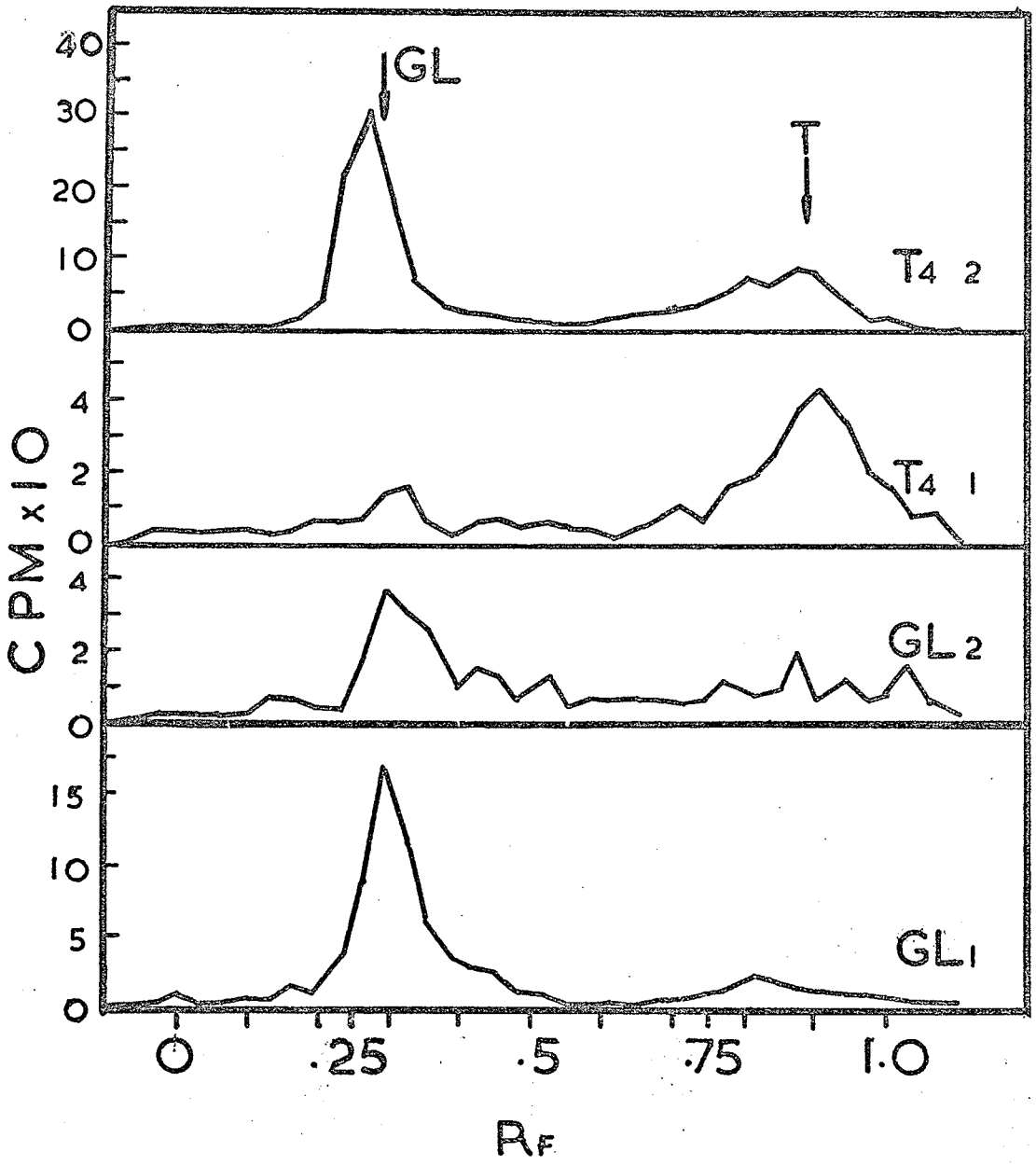
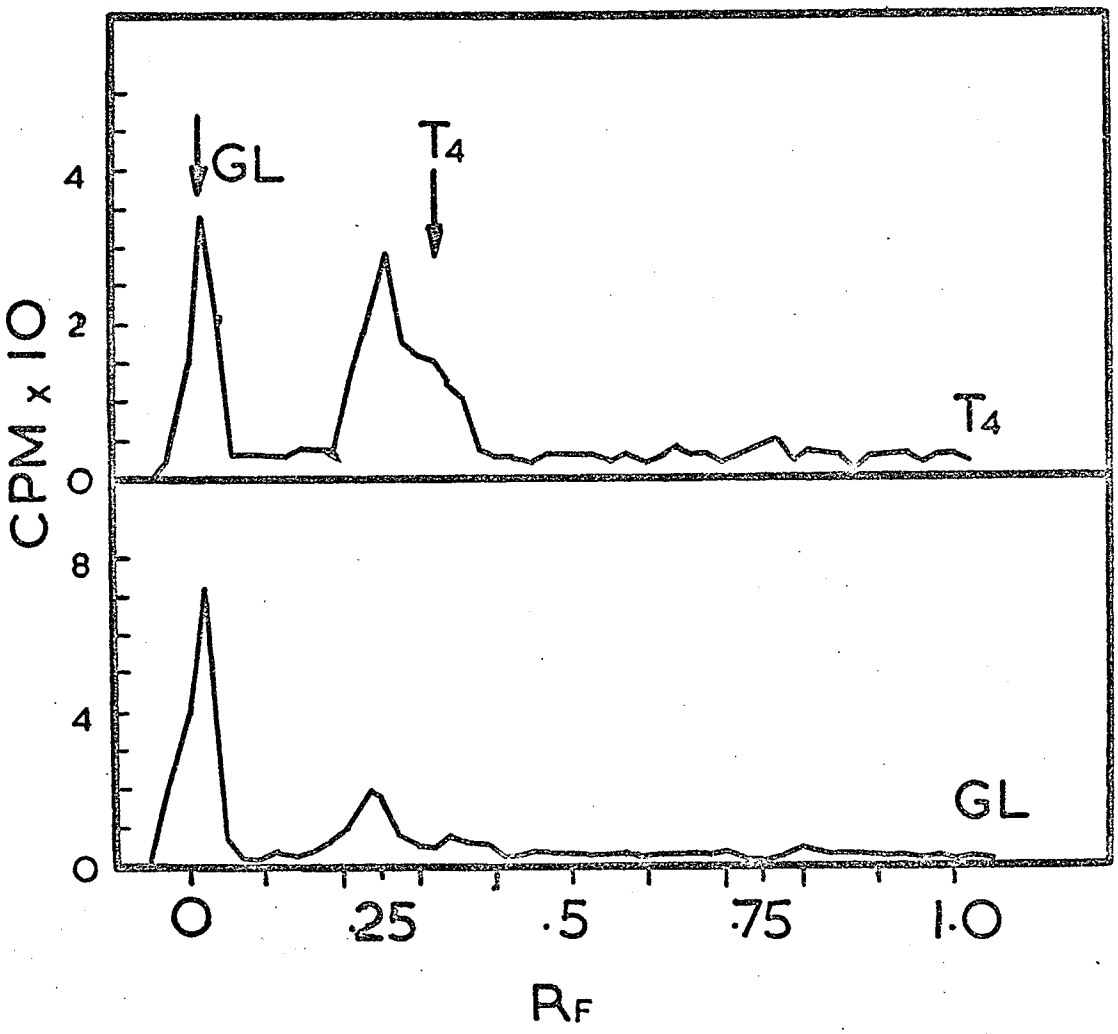

40
30
20
10
0
IGL
T
T4 2
4
2
0
T4 1
4
2
0
GL 2
15
10
5
0
GL1
CPM x 10
0
.25
.5
.75
1.0
RF

FIGURE 11. Thin-layer radiochromatograms (BEA) of bile 24 hours after T_4*-gl (GL), or 48 hours after T_4* (T_4) injection into the stomach of brook trout. The position of authentic materials are shown by arrows (T_4 = thyroxine, GL = glucuronide).


GL
T4
T4
GL
CPM x 10
4
2
0
8
4
0
0
.25
.5
.75
1.0
RF

absorbed radioactivity was significant in both groups, although the T_4 -gl gallbladder levels were slightly higher. The time of maximum biliary excretion was earlier (24 hours) for T_4 -gl than for T_4 (48 hours) fish. The low initial gut levels (at 2 hours) of radioactivity in all fish indicate that substantial leakage may have occurred that was not accounted for. The total gastrointestinal tract required about 72 hours to excrete significant amounts of radioactivity.

Uptake of Radioiodide Following Stomach Injection

In order to ascertain the effect of exogenous radioiodide in the T_4* preparations on absorption of radioactivity by stomach-injected fish, an abbreviated study of stomach injection of $Na^{125}I$ was performed on trout. Radioiodide (1 - 2 μCi) was injected into 32 trout (26 - 78 g) using distilled water as the vehicle with 0.1 ml as the injection volume per fish. The fish were held at 12 C and eight were killed and bled at each of 1, 4, 10 and 24 hour p.i. Percent dose was determined for bound and unbound plasma fractions, and also for some tissues and organs. No correction was made for initial leakage.

Fig. 6 shows plasma unbound radioactivity levels for fish stomach-injected with radioiodide. Almost all the radioactivity was in the unbound fraction, although the levels

were low. Maximum uptake (0.034 percent per ml) occurred at 10 hours after which the levels declined.

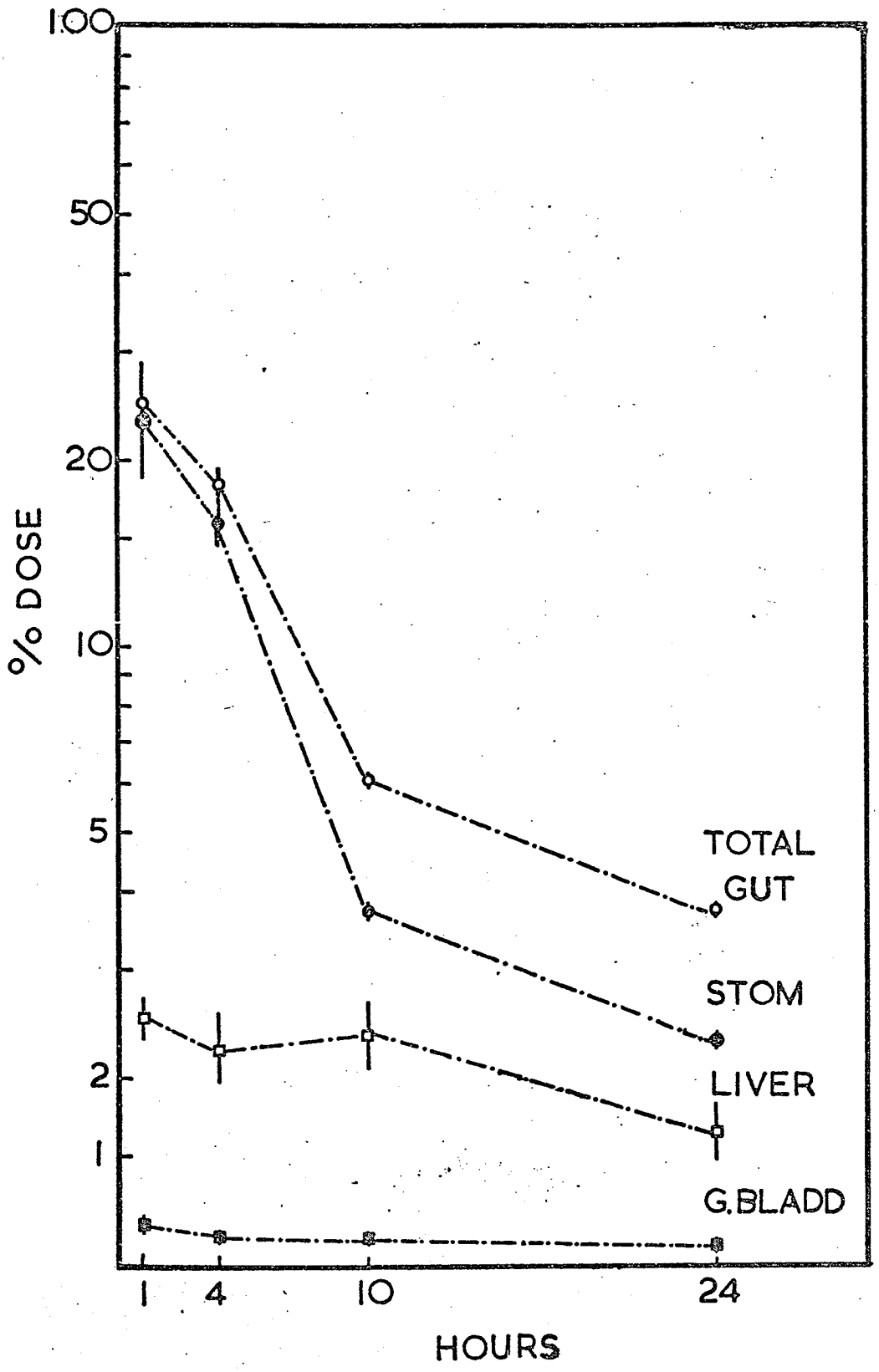
About 2.5 percent of the dose was rapidly (by 1 hour) taken up by the livers of the fish (Fig. 12), and this did not decrease until 24 hours. The gallbladders also showed maximum uptake (about 0.5 percent per ml) at 1 hour, but the levels were 4 to 5 times less than the livers.

Both the stomach and the total gut levels (Fig. 12) decreased sharply from 1 hour (25 percent) to 24 hours (4 to 6 percent). Since plasma levels and liver-bile uptake did not indicate a correspondingly large increase, it is likely that the gut decrease was largely due to leakage and not absorption. Very little radioactivity reached the intestines.

Determination of Binding of Biliary Iodothyronines to Plasma Proteins By TCA Precipitation

Plasma binding of constituents of endogenous radioactive bile (T_4 67 percent: T_4 -gl 33 percent by TLC) was compared with that of T_4* (97 percent T_4 by TLC) added to cold bile. This was done to determine if T_4 -gl would be precipitated with plasma proteins. The endogenous material showed 39.5 percent (38.8 to 40.3) bound and 60.5 percent (59.7 to 61.2) unbound, while T_4* was 71.8 percent (71.2 to

FIGURE 12. Percentage of radioactivity in total gut (o), stomach (●), liver (□) and gallbladder (■) of brook trout at various times after radioiodide-stomach injection. Each point represents a mean ($\pm$ 2 standard errors) for 8 fish.


100
50
20
10
5
2
1
% DOSE
TOTAL GUT
STOM
LIVER
G.BLADD
1
4
10
24
HOURS

72.3) bound and 28.2 percent (27 to 28.8) unbound. The T_4* binding agrees with the findings of Falkner (1971) by this TCA precipitation method. The results suggest that a radioactive component in endogenous bile from T_4* - injected fish tends to bind very little to plasma proteins in this fish. TLC of the TCA supernatants of the samples showed that all of the radioactivity was T_4 for the T_4* in bile, while for endogenous bile samples 51.3 percent (49 to 52) existed as T_4 -gl with 48.8 percent (48 to 51) as T_4. T_4 -gl appears to have less tendency than T_4 (at least by TCA analysis), to bind to plasma proteins of trout.

Uptake of Bile Iodothyronines Following Transintestinal Injection

As an attempt was made to compare intestinal absorption of naturally produced bile radioiodothyronines and T_4* in a few large brook trout. Five fish A, B, C, D and E (301 to 543 g), acclimated 13 C and starved for over 2 weeks, were each operated on and injected transintestinally with either endogenously-produced radioactive trout bile (A, B and C) or T_4* in whole cold trout bile (D and E). The percentage of radioactivity of the dose in the bound and unbound fractions of plasma taken from the caudal artery serially at 2, 5, 10, 18 and 24 hours. The fish were killed at 24 hours and the

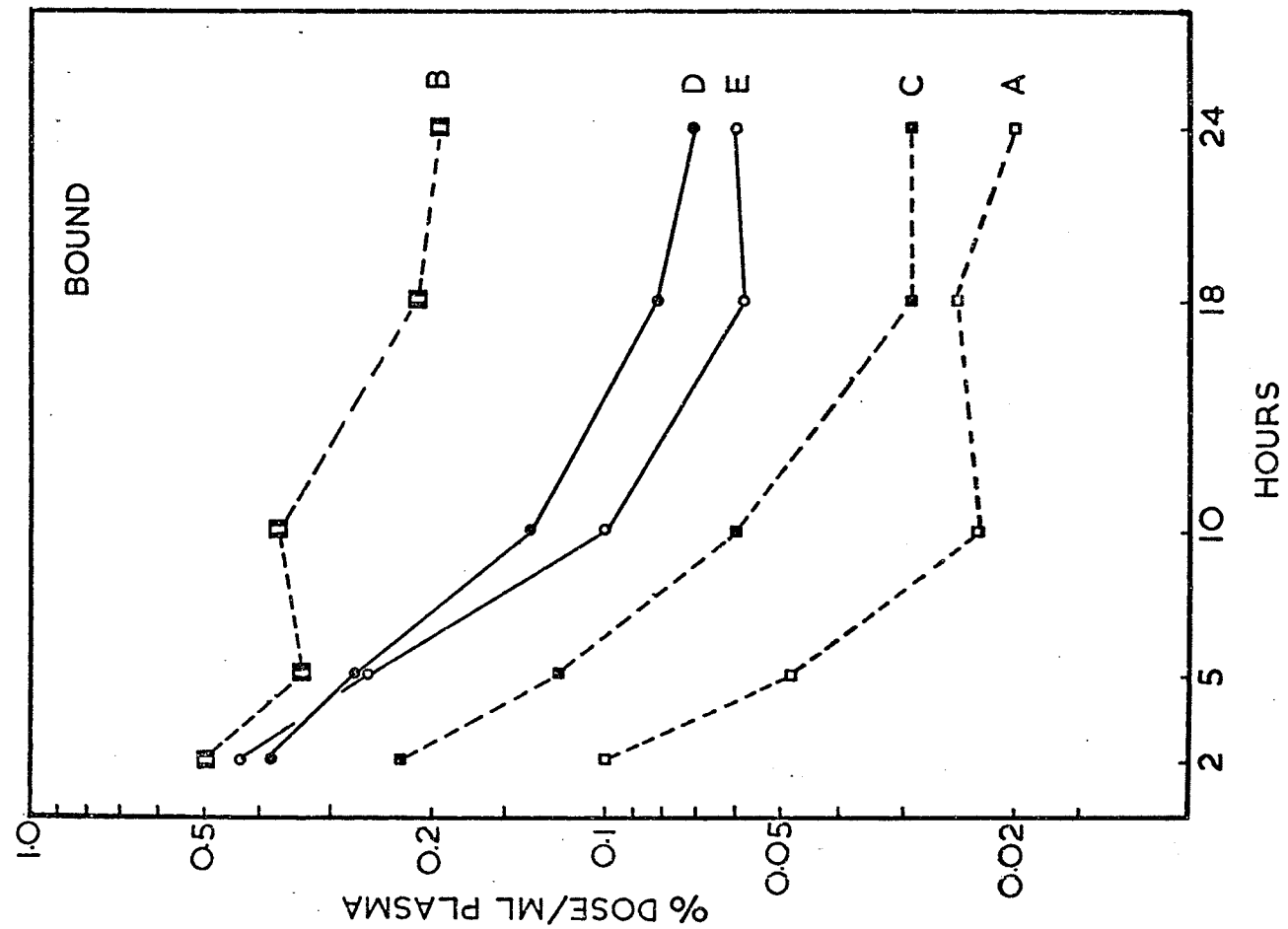
percentage of the dose in several organs and tissues determined. From these an estimation of absorption was calculated for each fish. Some TLC (Bakerflex BEA) was performed on bile, plasma (of 2 fish) and various tissue extracts.

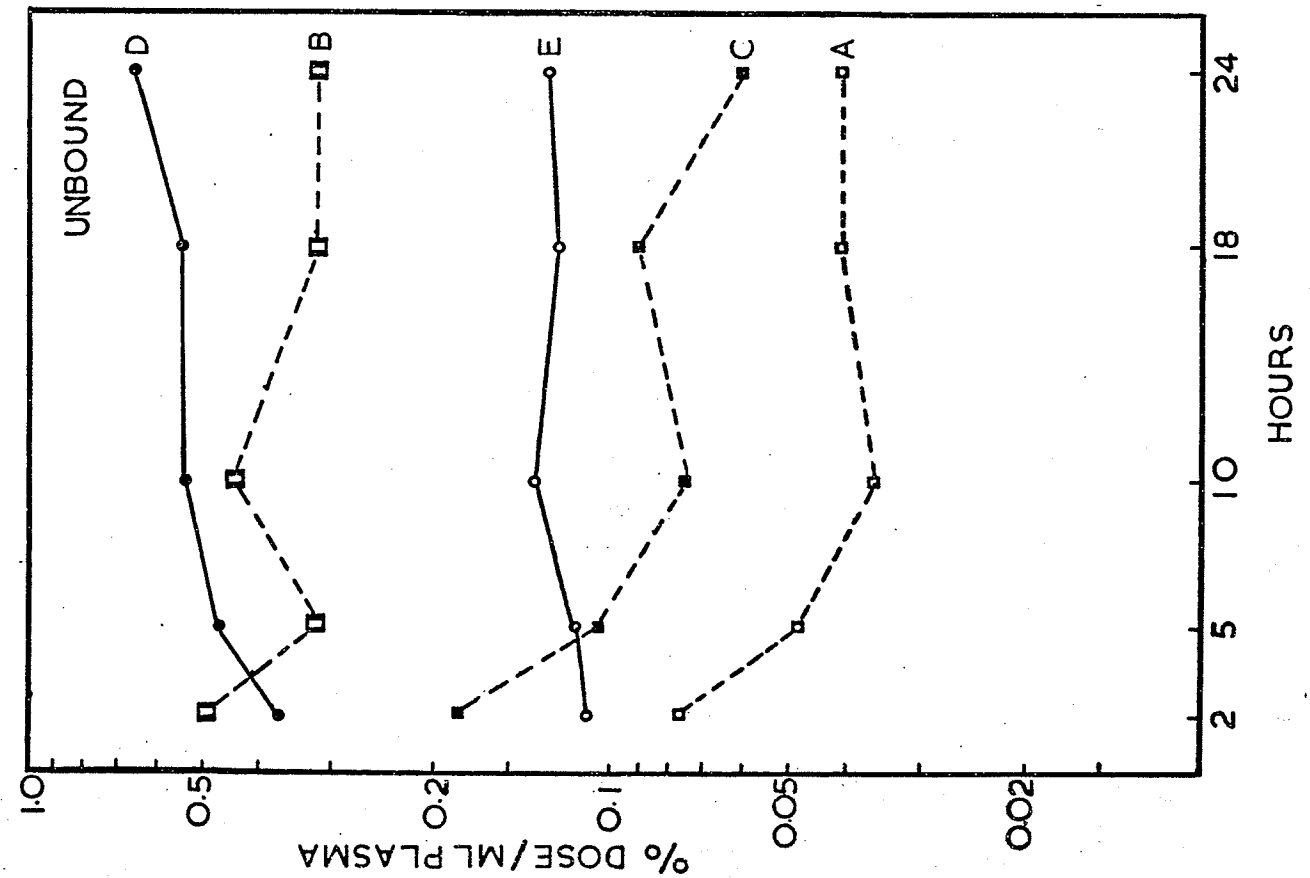
Maximum bound radioactivity levels for all fish occurred at 2 hours (Fig. 13). A, B and C showed different maximum levels (0.1, 0.22 and 0.5 percent per ml respectively), while for D and E the levels were similar (both about 0.4 percent per ml). The levels of all fish thereafter rapidly decreased to 10 hours, followed by a levelling off to 24 hours.

Maximum plasma unbound radioactivity for A, B (both about 0.18 percent per ml) and C (0.5 percent per ml) occurred at 2 hours. Thereafter these levels decreased at similar rates. However, D and E showed linear increases to 24 hours, although D showed the maximum level at 10 hours (0.14 percent per ml), whereas for E it occurred at 24 hours (0.66 percent per ml).

The 24 hour plasma samples of B and C were examined by TLC. Both showed radioactivity as glucuronide (11 to 14 percent), the majority as iodide (44 to 63 percent), and large proportions of T_4 (18 to 32 percent) with some T_3 (8 to 9 percent).

FIGURE 13. Relationship between plasma bound and unbound levels of radioactivity (percent dose per ml plasma standardized for a fish of 100 g) at various times after transintestinal injection of brook trout with T_4*. D (●), E (◇); or radioactive bile: (- - - - -), A (□), B (▨) and C (▩). Each point represents an individual fish.


BOUND
B
D
E
C
A
1.0
0.5
0.2
0.1
0.05
0.02
% DOSE/ML PLASMA
2
5
10
18
24
HOURS


UNBOUND
D
B
E
C
A
1.0
0.5
0.2
0.1
0.05
0.02
% DOSE/ML PLASMA
2
5
10
18
24
HOURS

The livers and gallbladders of A, B and C showed high percent uptake (Table V, 13 to 23 percent) and appeared to contain the majority of absorbed radioactivity in these fish. D (4 percent) and E (2 percent) showed some liver-bile uptake, but this was lower than for A, B and C.

Of the remainder of the tissues checked, only the total gut minus the isolated gut loop, and the gonads held appreciable radioactivity. The radioactivity in the gut not included in the isolated loop, may have been due to some backflow into the stomach although this is unlikely. Fish A, B, and C showed the highest levels in this area, but the high levels in B and C may have been due to direct uptake of radioactivity which leaked at time of injection. The ovaries of B and C contained 1.2 and 2.1 percent respectively, while the testes of E showed 5.3 percent. The kidneys of most fish showed some radioactivity but all had less than 1 percent.

All fish showed more than two thirds of the injected dose in the gut, with almost all of this present in the isolated gut loop.

Absorption was estimated for each fish (Table V) by subtracting total gut radioactivity from the presumed 100 percent dose. There was appreciable variation in absorption of all fish: A (18 percent), B (33 percent), C (23 percent),

TABLE V

Percentage of radioactivity in various organs at 24 hours after transintestinal injection of radioactive trout bile (fish A, B, C) or T_4* in bile (fish D, E). An estimate of absorption (100% minus total gut) is also given. The relative proportions of T_4 -gl to T_4 (*) are given with the fish receiving that particular sample of endogenous bile.

Organ	A(49:51*)	B(28:72)	C(28:72)	D	E
Liver	1.30	8.80	3.00	0.50	0.30
Gallbladder	11.40	14.20	11.90	3.70	2.05
Total gut-minus intestinal loop	2.40	3.10	11.45	1.10	0.80
Intestinal loop	79.20	63.80	66.10	72.90	87.50
Total Gut	81.60	66.90	77.50	74.00	88.30
Ovary	———	1.20	2.10	———	———
Testis	———	———	———	———	5.30
Gill	0.05	0.11	0.04	0.02	0.10
Kidney	0.10	0.35	0.12	0.25	0.30
Thyroid	0.03	0.07	0.03	0.10	0.05
Absorption Estimate	18.40	33.10	22.50	26.00	11.70
Total Recovered	94.30	92.40	94.71	79.10	96.33

D (26 percent) and E (12 percent). Since the latter two fish received only T_4*, large individual variability is suggested.

Table VI shows the TLC results on extracts of various tissues and bile of some fish. Appendix VI represents a sample of radiochromatograms.

The fish injected with T_4* (D and E) showed T_4 and iodide in all tissues checked. The bile showed T_4 but no iodide and was the only extract to show glucuronide (liver tissue was checked but low radioactivity prohibited TLC detection). R, an unknown radioactive compound migrating between glucuronide and thyronines, was detected in one bile sample. It is conceivable that R was an artefact of separation. More than 90 percent of the radioactivity in the testes of one fish was as T_4, and also more than 90 percent in the intestinal loop of both fish was as T_4. The two bile samples showed different relative T_4 -gl: T_4 proportions and T_3 was present for D.

The fish injected with radioactive bile with different T_4 -gl: T_4 proportions (A, 49:51, B and C 28:72), showed varying tissue distribution of radioactivity. Glucuronide was found in all but the kidney sample of C. R was also found in some tissues of B and C. However, whether or not

TABLE VI

Percentage distribution of radioactivity in injection material, various tissues and bile of 5 fish transintestinally-injected with either radioactive trout bile (A,B,C) or T_4* in bile (D, E), as revealed by TLC (BEA).

Fish	Extract Examined	Substances Identified				
		gl	R	T_4	T_3	Iodide
A	Injection Material	48.8	-	51.2	-	-
	Bile	70.4	-	29.6	-	-
	Intestinal Loop content	34.4	-	63.7	-	1.9
B	Injection Material	28.4	-	71.6	-	-
	Liver	24.3	70.9	-	-	4.8
	Bile	49.7	-	50.3	-	-
	Stomach Content	15.0	-	70.0	15.0	-
	Intestinal Loop content	35.6	55.5	-	8.9	-
	Kidney	12.1	38.1	26.8	10.0	13.0
	Ovary	17.6	-	63.5	-	18.9
C	Injection Material	28.4	-	71.6	-	-
	Liver	30.9	57.0	7.1	2.3	2.7
	Bile	45.7	-	50.0	4.3	-
	Stomach Content	88.7	-	11.3	-	-
	Intestinal Loop content	17.2	-	76.9	5.2	0.7
	Kidney	-	50.0	19.2	10.8	20.0
	Ovary	28.8	61.3	-	-	9.9
D	Injection Material	-	-	96.8	-	3.2
	Bile	76.1	-	11.5	12.4	-
	Intestinal Loop content	-	-	92.7	-	7.3
E	Injection Material	-	-	97.2	-	2.8
	Bile	18.7	27.0	54.3	-	-
	Intestinal Loop content	-	-	95.1	-	4.9
	Testis	-	-	93.2	-	2.7

this is a real compound is uncertain. Radioiodide was also present in some extracts and was especially prominent in kidney and ovary. B and C showed bile patterns of equal T_4 -gl: T_4, while A showed predominantly glucuronide. A and C showed proportionally less glucuronide and more T_4 in the gut loop material than was present in the injection materials. However B showed the reverse. T_4 was found in most and T_3 in several of the tissue extracts of these fish.

In summary intestinal absorption of radioiodothyronines appeared to be low (12 to 33 percent), and to have occurred early (within 2 hours). Large individual variability occurred and prohibited proper assessment of differences in absorption of the fish injected with different material.

Plasma unbound radioactivity trends for A, B and C tended to decrease after 2 hours maxima, while those of D and E showed increasing trends to mortality. The plasma bound radioactivity in all fish showed a steady decrease after 2 hours maxima.

Liver-bile uptake was definitely higher for radioactive bile-injected fish than T_4* - injected fish. Appreciable radioactivity was also found in testes or ovaries of the fish checked.

TLC of tissue and bile extracts showed few definite differences between T_4* and radioactive bile-injected fish, although glucuronide was present in most tissues in A, B and C and only in the bile of D and E. T_4 was present in most tissues of all fish with iodide in some.

DISCUSSION

Identification of Bile Iodothyronines

In brook trout biliary excretion of intraperitoneally-injected T_4* is mainly as T_4* or its glucuronide conjugate, while T_3* is excreted as T_3* or its glucuronide conjugate. Little evidence was found for the presence of sulphate esters or other T_4 degradation products. Subsequent data (Sinclair and Eales unpublished) have shown that no other major degradation products appear in this fish up to 9 days p.i. This contrasts somewhat with the findings of Osborn and Simpson (1969) for the marine plaice, where several T_4* derivatives and their sulphate or glucuronide conjugates were identified after intraperitoneal injection of T_4*. However, these authors gave no indication of the relative proportions of these other materials and they may be present in quantities too small to be detected by present methods. Lack of T_4 derivatives other than glucuronide may reflect the rapidity with which T_4 is removed from the circulation by the biliary system of this fish (Eales 1970), precluding the possibility of extensive peripheral metabolism.

While 3, 5, 3' - T_3 was the main product of β-glucuronidase hydrolysis on thin layer chromatograms of bile from T_3* - injected brook trout, lesser quantities of radioactivity occurred at slightly higher Rf's. Flock *et al* (1963) found

appreciable 3, 3' - T_2 - glucuronide in dog bile after injection of T_3*, and this substance may be present in trout. Unfortunately authentic 3, 3' - T_2 was unavailable as a standard. The presence of T_3 degradation products in bile of T_3*-injected trout, might be anticipated since T_3 is cleared from the serum more slowly than T_4 in brook trout following intraperitoneal injection at these temperatures (Eales et al 1971).

The proportion of T_4 -gl in the bile was always higher by paper chromatographic than TLC separation. Furthermore, in contrast to TLC separation (Polygram), paper separation of β - glucuronidase-treated bile revealed residual radioactivity at Rf 0.2 to 0.3. The reasons for these differences are not clear. One possibility is the presence in bile of a substance that binds free thyronines and has an Rf on paper (with BAW solvent) closely corresponding to that of thyronine-glucuronide conjugates. On the paper medium this binding persists to some extent but does not occur on thin-layer media with the solvent systems used. In view of the very high concentration gradient for unconjugated thyroxine between the bile and serum, binding of thyroxine to some bile constituent is not unexpected and has been suggested for mammals (Hillier 1971).

Gastrointestinal Uptake of Bile Iodothyronines

Stomach or transintestinal injection of bile iodothyronines indicated that while enterohepatic recycling of these substances does occur in the brook trout, it is limited.

Owing to incompletely compensated leakage of stomach-administered bile, containing either T_4* or predominantly T_4* -gl, the extent of uptake of these radioactive materials cannot be stated.

Although the data indicate that 12 to 33 percent of the iodothyronines were absorbed by 24 hours after transintestinal injection, with no obvious absorption differences between T_4* and T_4* -gl, only a few animals were used, and high absorption in one (33 percent) may have been partially due to intraperitoneal leakage. Therefore, absorption may be 20 percent or less, although further work is necessary to ascertain this. Despite limitations in techniques, the low absorption agrees with some previous work, 12 to 16 percent absorption of stable T_4 in cows (Mixner and Lennon, 1960); 15 percent or less absorption of ^{131}I-bile iodothyronines from the duodenum of the dog (Furth et al 1968); 25 percent absorption of Na ^{131}I - T_4 in the cat, (Clayton et al 1949); 20 percent absorption of crystalline T_4 in the chicken (Monroe and Turner, 1949), and 15 to 16 percent absorption of radio-

activity from ^{131}I - T_4 - endogenous bile of rats, Cottle and Veress (1965). However other species showed higher absorption; 60 to 70 percent in frogs, 58 percent in salamanders, and 25 to 60 percent in turtles (De Los Reyes and Jones, 1970); and also in the rat (Taurog et al 1953) and man (Hays, 1968). In comparison, it must be remembered that the trout were starved for a minimum of 2 weeks prior to injection, and none of the previous species mentioned were treated in a similar manner. It has been observed that during chronic starvation (Narayansingh unpublished data), the gut mass may decrease, and the accompanying tissue changes may alter gut absorption. Hays (1968) states that in man, malabsorption diseases result in poor T_4 absorption. Consequently, it is difficult to compare absorption of iodothyronines in the trout with work on other animals.

Uptake of T_4* and T_4* -gl into the blood of stomach-injected fish also indicated low absorption. For example, at 2 hours, plasma unbound radioactivity was 10 times less for T_4* and 20 times less for T_4* -gl fish, while the bound radioactivity was 100 times less for both T_4* and T_4* -gl fish, as compared to fish intraperitoneally injected with T_4* (Higgs and Eales, 1971). For fish injected transintestinally with T_4* or radioactive bile, at 2 hours the unbound plasma

fractions were 4 to 25 times less while the bound fractions were 12 to 60 times less than the plasma of T_4* - intraperitoneally-injected fish (Higgs and Eales 1971). From plasma analysis, maximum absorption in transintestinally-injected fish appeared to occur within 2 hours. This early maximum absorption agrees with Chung and Van Middlesworth (1967) for rats and Hays (1968) for man. Hays (1968) suggests that the rapid decrease in T_4* absorption with time (after 2 hours) may be due to the presence of substances (found by Chung and Van Middlesworth 1967) in the intestine, which rapidly bind the hormone and interfere with subsequent absorption. This interference may also have been promoted by the bile vehicle used. Hillier (1971) has discovered substances in the bile which bind T_4 and may block absorption.

If trout under normal conditions experienced long periods with little food, low absorption of thyroid hormones may be advantageous. Since there would be no appreciable fecal mass in the gut to restrict absorption of biliary excreted T_4 and its derivatives under these conditions, low absorption by the gut itself could be a mechanism to prevent thyroxinemia imbalance. Low absorption of the hormone, even during times of food abundance, may be advantageous to predators if T_4 were abundant in the diet (e.g. livers, gall-

bladders and thyroids of the prey).

Deiodination appears to be a prominent route for peripheral metabolism of absorbed T_4 but not for absorbed T_4 -gl. The plasma unbound radioactivity levels for stomach-injected T_4* fish were much higher than those of T_4* -gl fish, even though T_4* -gl does not bind to plasma proteins. Fish transintestinally-injected with T_4* showed trends of increasing levels of unbound radioactivity over 24 hours, as opposed to the levels of fish transintestinally-injected with radioactive bile, that tended to decrease for the same period. Considering the specificity of enzymes for their substrates, the apparent lack of appreciable glucuronide deiodination is not surprising. Tata (1964) states that a low affinity of plasma proteins for a particular thyroid hormone derivative, is paralleled by a low affinity of dehalogenase for that derivative as a substrate.

The main pathway for absorbed thyroxine glucuronide appears to be biliary excretion. This was indicated by the rapid appearance of absorbed T_4* -gl in the bile of fish stomach-injected with the latter. Also, the fish transintestinally-injected with radioactive bile showed high liver-bile levels. The early (2 to 10 hour) initial decline in plasma unbound radioactivity in the latter fish may reflect

biliary excretion of T_4 -gl. Hays (1968) states that in man, glucuronide is absorbed from the gut but is rapidly excreted, probably by the kidney. While the renal route was not investigated, it is possible that considerable excretion in the brook trout is via the bile. No clear trends existed in the present study with reference to liver-bile uptake in fish transintestinally-injected with different T_4; T_4 -gl proportions in the radioactive bile. Large amounts of radioactivity were also found in the gonads of all transintestinally-injected fish, but the significance of this is not known.

TLC of several tissues of transintestinally-injected fish indicated that for fish receiving radioactive bile, some glucuronide reached tissues not included in enterohepatic circulation, while T_4* was found in almost all the same tissues. T_3* was also found in some cases and was probably the product of monodeiodination of T_4*. Several tissues showed radioiodide. TLC of plasma from two fish receiving radioactive bile indicated that glucuronide was present.

Although there were no definite differences in gut absorption of T_4* and T_4* -gl in the trout, the fact that the latter shows little tendency to be bound by plasma proteins, and is predominantly excreted in the bile, may justify calling glucuroconjugation a deactivation mechanism. Removal by the

liver of large amounts of glucuronide from the hepatic portal system before it reaches systemic circulation, may constitute a method of controlling thyroxinemia.

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APPENDIX I

To test arysulphatase activity of Mylase 'P', 0.02 (1) and 0.03 g (2) of phenolphthalein disulphate in sodium acetate buffer, were each incubated with 0.015g of Mylase 'P' at 37 ± 0.5C for 24 hours (at pH 5.0). After centrifugation the supernatants (1 and 2) along with phenolphthalein (4) and phenolphthalein disulphate (3) as standards, were chromatographed on TLC media (Bakerflex) using three solvent systems: (A) BAW 4:1:5 V/V (B) Methanol, HCl, H_2O, 70:20:10 V/V (C) Propanol, NH_4OH 2:1 V/V. All substances were detected with uV light at 250 mu. Results indicated that a rose pink color was generated in the enzyme-substrate tubes, and chromatography (A,B,C) indicated substantial breakdown of phenolphthalein disulphate to phenolphthalein in these tubes.

PH = phenolphthalein

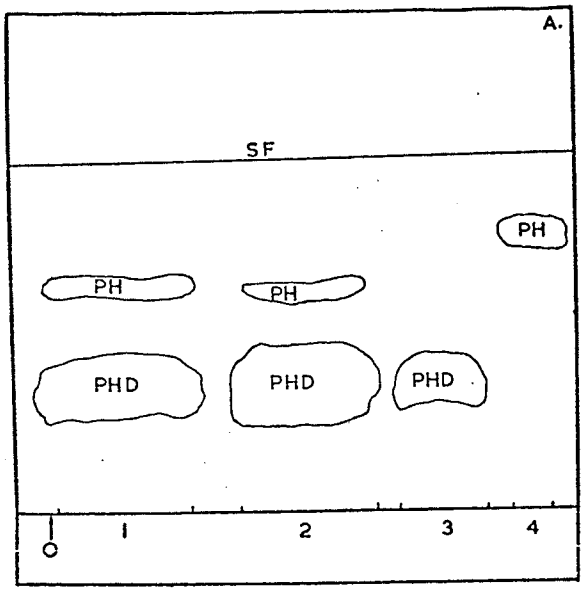
PHD = phenolphthalein disulphate

SF = solvent front

O = origin

Incubation of radioactive bile from T_3* or T_4* - injected fish with equal amounts of Mylase 'P' and saccharolactone, showed that the latter effectively inhibited hydrolytic activity of the former. Thus it is concluded that

any breakdown of the glucuronide peak by Mylase 'P' is probably due to β - glucuronidase activity in this preparation.


A.
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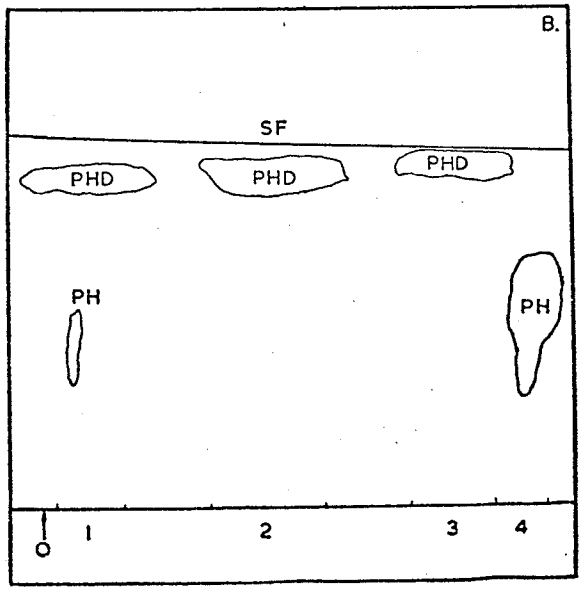
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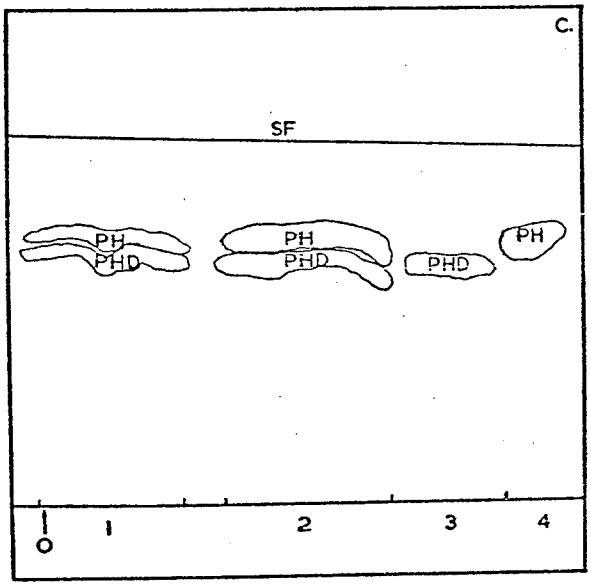
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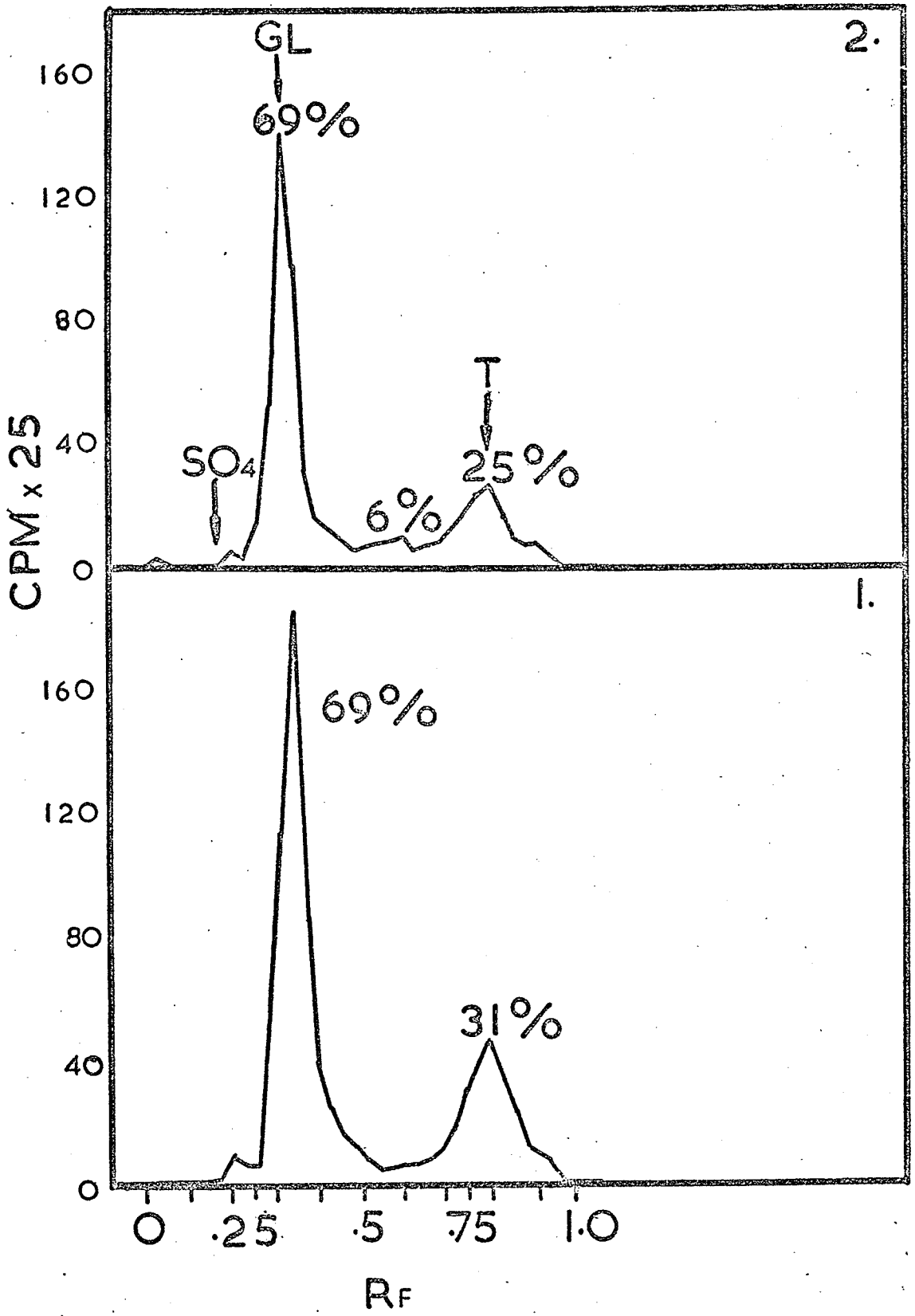
APPENDIX II

Radioactive bile from fish intraperitoneally-injected with T_4* was incubated for 24 hours at 37 $\pm$ 0.5 C (a 0.25mlsample), with sodium acetate pH 5.0 buffer and 0.05 g. Mylase 'P' (2), and alone at pH 5.0 (1). The resulting supernatants were checked by paper with n-butanol equilibrated with 2N acetic acid, 1:1 (V/V). A radiochromatogram showed no substantial breakdown of major peaks at pH 5.0. The slight peak between glucuronide and thyronine peaks was probably not sulphate, since it has been shown in this solvent system that sulphate conjugates in bile migrate behind glucuronide conjugates. The positions of authentic materials are shown by arrows, and relative percentage of each peak is given.

SO_4 = known position of sulphate conjugate

GL = known position of glucuronide

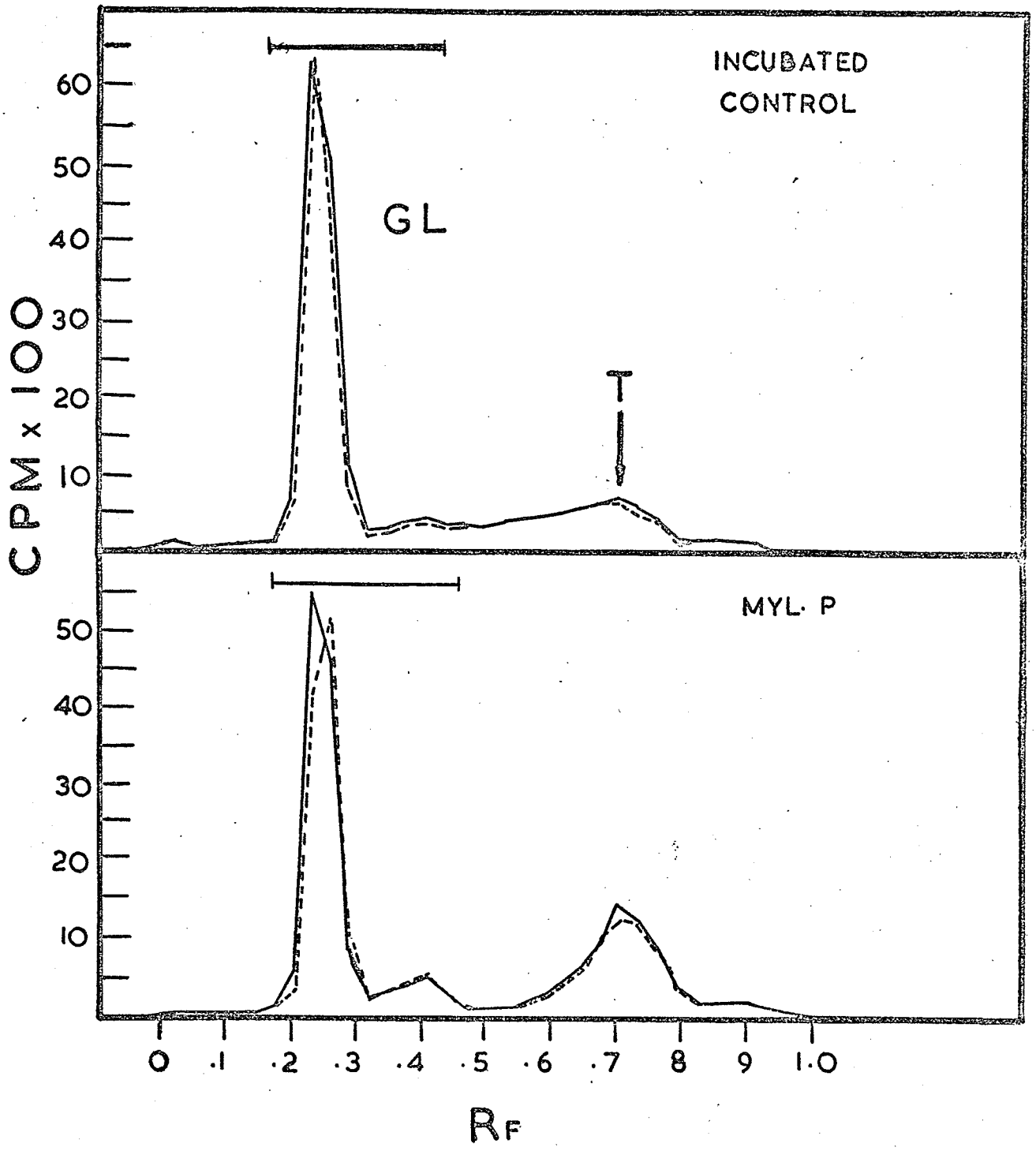
T = known position of thyronines


2.
GL
69%
160
120
80
40
0
T
SO4
25%
6%
CPM x 25
1.
69%
160
120
80
40
0
31%
0
.25
.5
.75
1.0
RF

APPENDIX III

This represents a radiochromatogram demonstrating the reproducibility of similar results of two different spottings of the same sample (_____ = initial spot, - - - - = duplicate). The position of authentic materials are shown by arrow or vertical bar GL = glucuronide.

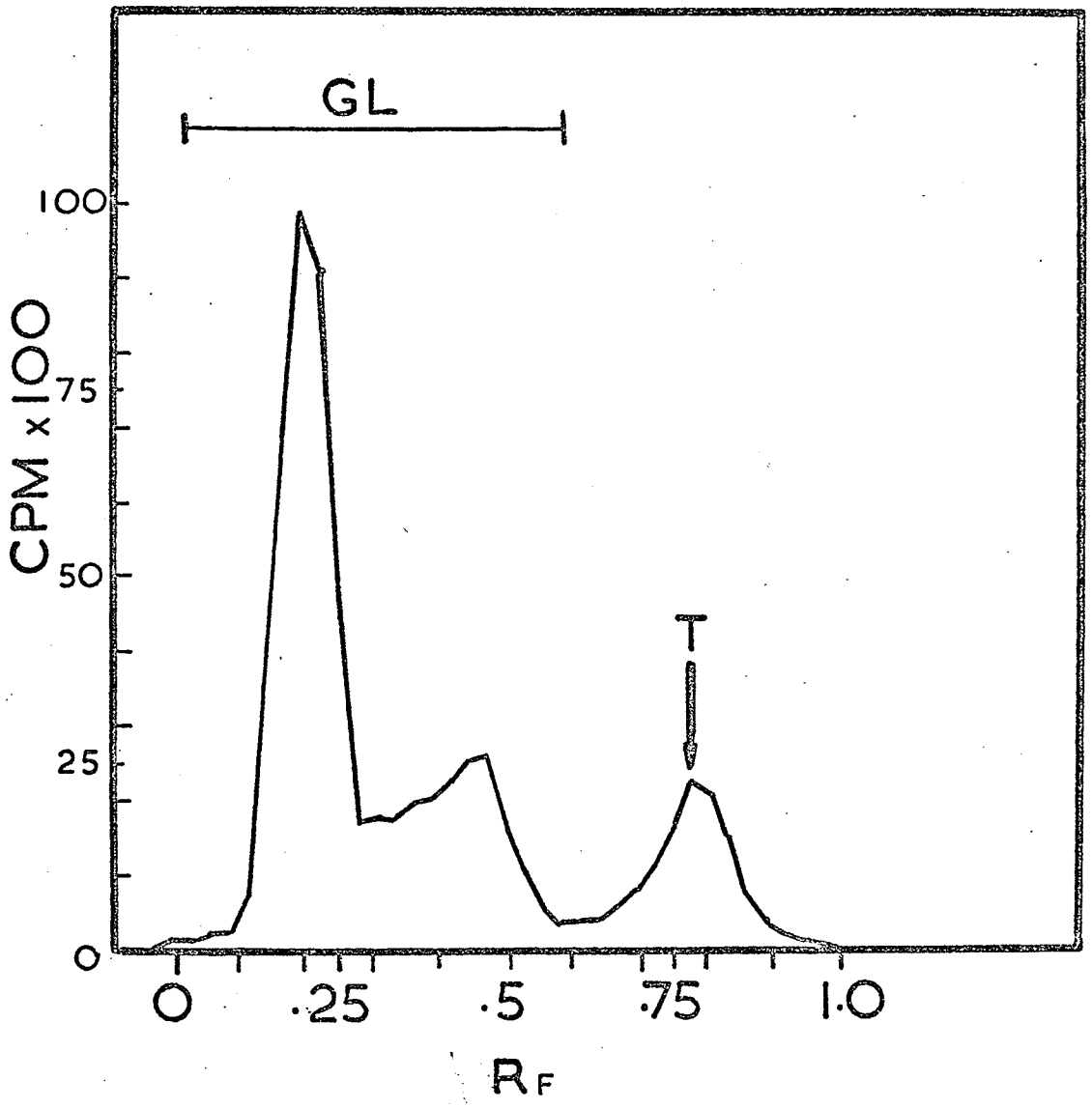
T = thyronines


INCUBATED
CONTROL
GL
T
MYL. P
CPM x 100
60
50
40
30
20
10
50
40
30
20
10
0
.1
.2
.3
.4
.5
.6
.7
8
9
1.0
RF

APPENDIX IV

Elution of iodothyronine materials migrating in the glucuronide area of the paper radiochromatograms was necessary for the collection of T_4* -glucuronide. Twenty-four hour radioactive bile was collected and chromatographed on paper (NO. 3), and a radiochromatogram determined for a middle 1-inch-wide strip (see figure). The area corresponding to glucuronide on each sheet was divided into $\frac{1}{2}$ x 3 inch pieces, which were rolled and placed in glass tubes. Distilled water was added and the tubes allowed to stand for 6 to 8 hours in darkness. Following squeezing of the strips, this method consistently eluted more than 75 percent of the radioactivity. The slightly alkaline eluate was neutralized with 0.1 N HCl and frozen on stainless steel trays. The eluate was then subjected to two lyophilization treatments in a Virtis Freeze-Mobile unit for 1 to 2 days, the first producing radioactive crystals which were then collected in 20 ml distilled water, and after re-freezing, again lyophilized. Adsorption to cellulose fibres necessitated extraction of the radioactivity with 0.1 N NaOH. After neutralization and freezing, lyophilization of the extracts was carried out in a smaller, Virtis-Automatic unit, and all crystals combined. This radioactivity from the glucuronide area of paper radiochromatograms and T_4* were each dissolved in diluted bile vehicle and adjusted to

similar levels of radioactivity. Thus a vehicle containing predominantly T_4* -gl and one containing almost entirely T_4* were obtained for stomach injection. The area between the two vertical lines on the paper radiochromatogram in the figure shown, represents the area removed as glucuronide conjugates (GL). T = thyronines.


GL
100
75
50
25
0
CPM x 100
T
0
.25
.5
.75
1.0
RF

APPENDIX V

The means ($\pm$ 2 standard error) for the stomach, intestine and total gut levels are represented here for brook trout stomach-injected with T_4* and T_4* -gl at the times sampled after injection.

Interval hr (p.i.)	Stomach		Intestine		Total Gut	
	T_4*	T_4*-gl	T_4*	T_4*-gl	T_4*	T_4*-gl
2	(30.1 ± 7.5)	(20.1 ± 5.8)	(0.5 ± 0.4)	(9.2 ± 3.8)	(30.6 ± 7.8)	(29.3 ± 7.7)
5	(25.4 ± 8.1)	(20.0 ± 8.2)	(8.4 ± 6.4)	(10.6 ± 1.8)	(33.8 ± 12.7)	(30.6 ± 9.5)
10	(20.4 ± 6.2)	(16.8 ± 4.5)	(11.5 ± 10.6)	(13.2 ± 4.1)	(31.9 ± 13.5)	(27.5 ± 6.0)
24	(4.5 ± 2.1)	(6.0 ± 2.4)	(20.5 ± 10.8)	(18.4 ± 7.0)	(25.0 ± 12.0)	(24.4 ± 8.1)
48	(6.4 ± 5.4)	(3.1 ± 1.2)	(18.3 ± 7.3)	(14.9 ± 4.5)	(24.8 ± 10.8)	(18.0 ± 4.6)
72	(2.6 ± 3.2)	(1.9 ± 1.6)	(17.3 ± 7.2)	(15.8 ± 5.9)	(19.9 ± 8.2)	(17.7 ± 5.7)
96	(7.0 ± 0.3)	(0.7 ± 0.7)	(5.5 ± 1.6)	(9.1 ± 5.9)	(5.8 ± 1.9)	(9.8 ± 6.3)

APPENDIX VI

A sample of TLC radiochromatograms of bile and a few tissue extracts from one fish (C), 24 hours after transintestinal injection with radioactive trout bile (with T_4-gl:T_4 ratio 28:72). The position of authentic materials are shown by arrows.

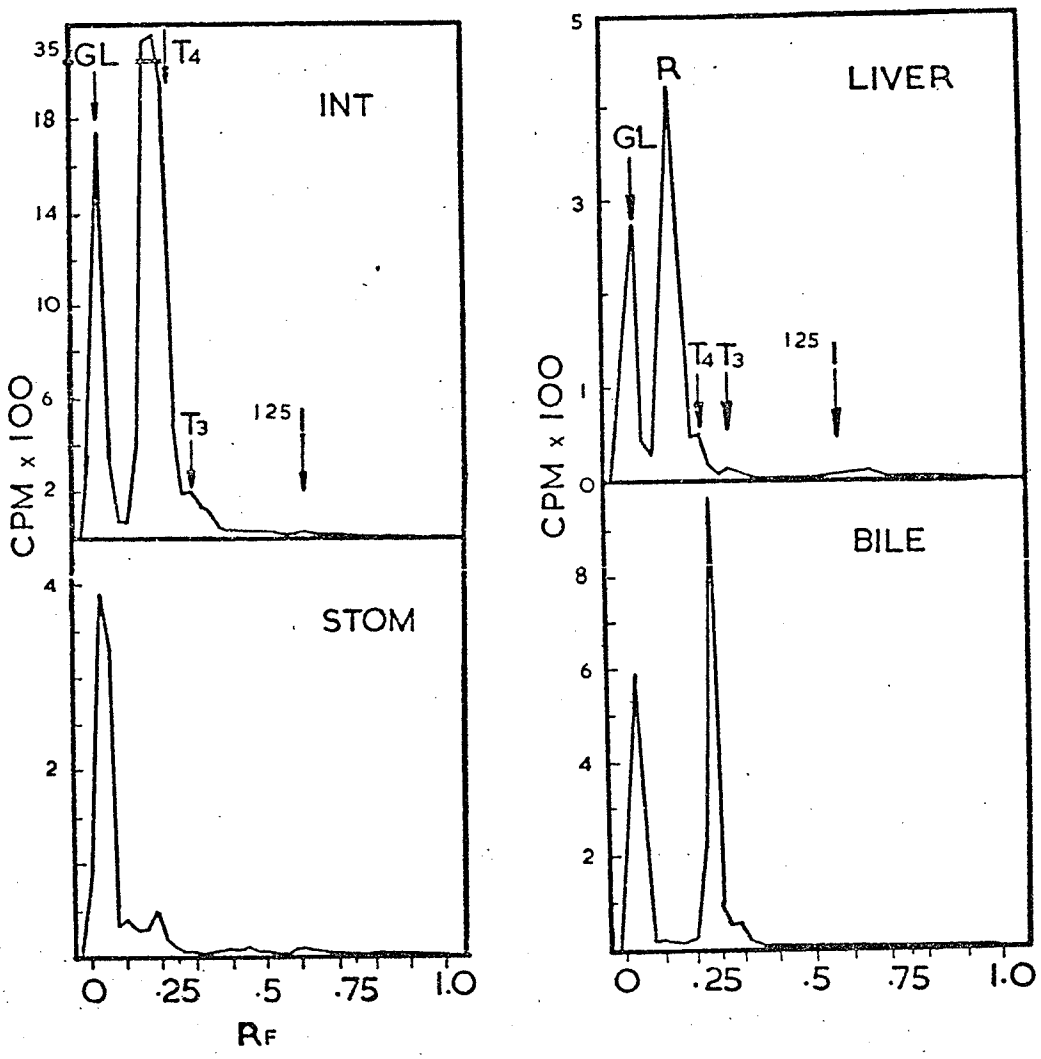
T_4 = thyroxine

T_3 = triiodothyronine

R = unknown substance

GL = glucuronides

^{125}I = iodide


GL
T4
INT
T3
125
CPM x 100
STOM
0
.25
.5
.75
1.0
RF
R
LIVER
BILE