THE UNIVERSITY OF OKLAHOMA
GRADUATE COLLEGE

# THE THYROID RESPONSE TO PURIFIED MAMMALIAN THYROTROPIN AND PARTIALLY PURIFIED FISH THYROTROPIC FACTORS IN GOLDFISH (CARASSIUS AURATUS) AND KILLIFISH (FUNDULUS HETEROCLITUS)

A DISSERTATION
SUBMITTED TO THE GRADUATE FACULTY
in partial fulfillment of the requirements for the
degree of
DOCTOR OF PHILOSOPHY

BY
F. BLAKE GRANT
Norman, Oklahoma
1967

THE THYROID RESPONSE TO PURIFIED MAMMALIAN THYROTROPIN AND PARTIALLY PURIFIED FISH THYROTROPIC FACTORS IN GOLDFISH (*CARASSIUS AURATUS*) AND KILLIFISH (*FUNDULUS HETEROCLITUS*)

APPROVED BY

Howard L. Clemens

Richard A. Goff

Leon S. Lieuallen

Kenneth S. Mellish

DISSERTATION COMMITTEE

## ACKNOWLEDGMENT

I wish to express my gratitude to Professor Howard P. Clemens who directed my graduate research. Also, I am indebted to Professor Grace E. Pickford, Yale University, who allowed me to use her research facilities at the Bingham Laboratory.

My dissertation committee, Professors Howard P. Clemens, Leon S. Ciereszko, Richard A. Goff, and Kenneth S. Mills, have contributed helpful comments in improving the scientific expression, philosophy, and merit of this manuscript for which I am grateful.

I wish to acknowledge Dr. Y. A. Fontaine, Paris, France, for supplying the heterothyrotropic preparation and Mrs. James W. Atz for her microscopical examination of the thyroid sections. I wish to thank technicians Mr. Evertt Shackleford and Mrs. Mary Cowmeadow, and fellow graduate student, Mr. Waynon Johnson, who freely gave their assistence.

I wish to express my appreciation finally to my wife, Nancy, who helped prepare the manuscript and who offered understanding and encouragement throughout.

These studies were undertaken as partial fulfillment of the requirements for the Doctorate Degree of Philosophy at the University of Oklahoma.

The studies on goldfish were supported by Grant A-3445 of the National Institutes of Health. The experiment on the heterothyrotropic principle in collaboration with Dr. Pickford was supported by Grant GB-0132 of the National Science Foundation.

TABLE OF CONTENTS

LIST OF TABLES

## LIST OF ILLUSTRATIONS

# THE THYROID RESPONSE TO PURIFIED MAMMALIAN THYROTROPIN AND PARTIALLY PURIFIED FISH THYROTROPIC FACTORS IN GOLDFISH (CARASSIUS AURATUS) AND KILLIFISH (FUNDULUS HETEROCLITUS)

## ABSTRACT

Twenty-four hour serum iodine conversion ratios, expressed as protein bound iodine-131 (PBI-131) and butanol extractable iodine-131 (BEI-131) percentages were determined serially for 108 hours at 12-hour intervals following intraperitoneal pituitary extract (carp) injection in female goldfish. The PBI-131 percentages of extract-injected fish were consistently lower than those of fish injected with physiological saline. The margin of difference increased with time. Concurrently, the BEI-131 percentages were higher in extract-injected fish than the controls. Again, the difference increased with time. Collateral effects of the injection material and a diurnal rhythm were evident in serum PBI-131 and BEI-131 parameters.

A log-dose response between extract injection and the 24-hour conversion ratio was studied using the PBI-131 percentage as a response index, 220 hours after extract treatment. Similarly, a log-dose response was evaluated with BEI-131 percentages 36 hours after I-131 injection and 60 hours after pituitary extract injection.

The protein-radioiodine complex at 45 hours and at 9.5, 23.5, and 36 hours was characterized by Sephadex G-25 and G-100, respectively. Results obtained on the G-25 column effluent samples precipitated with TCA suggested three protein-I-131 gradients in the macromolecular range. The largest fraction of total protein in the G-100 effluent was only

weakly radioactive; almost all of the radioiodine was associated with the lower molecular weight material.

Thyroid radioiodine uptake at 72 hours was higher in freshwater-adapted killifish than in salt water-adapted killifish. However, their environmental and serum iodide-127 concentration were lower. The total thyroid iodine contents were comparable.

Acute injections of pituitary materials into intact killifish were effective in stimulating thyroid activity when initial thyroid activity was relatively low (disclosed by relatively low uptakes of controls that did not receive pituitary extract injections). On the other hand, thyroids of intact animals whose non-treated controls had high radioiodine uptake could not be stimulated by an acute pituitary extract injection.

Three carp pituitary extract injections at 48-hour intervals were effective in stimulating thyroid tracer (I-131 or I-125) uptake in 55-day post-hypophysectomial killifish. The seventy-two-hour radioiodine uptake was determined preceding, 12 days after, and 55 days after hypophysectomy in killifish.

Gel filtration with Sephadex G-100 at pH 7.0 and pH 9.0 gave partially purified thyroid stimulating preparations that were assayed by the 72-hour tracer uptake method on hypophysectomized killifish. Acrylamide electrophoresis (disc) was effective in fractioning active gonadotropic preparations but not thyroid stimulating preparations. The behavior of these hypophysial factors with these isolation methods are discussed.

The mammalian heterothyrotropic factor (HTF), thyrotro-

pin (TSH)-USP, and highly purified bovine thyrotropin (B-TSH) were compared by bioassay using 48-hour *in vivo* tracer uptakes, 96-hour *in vitro* tracer uptakes, 96-hour serum radiothyroxine levels, and mean thyroid cell heights. The four methods gave comparable estimates of thyrotropic potency for corresponding preparations, but estimations based on the thyroid cell heights were less equivocal due to less variability within injection groups. The preparations were rated on a weight basis as 1.0 μg HTF = 0.07 μg purified bovine TSH = 1.35 μg TSH-USP.

# INTRODUCTION

Studies on the purification and chemical characterization of mammalian thyrotropins have been intense in the past decade. The application of newer isolation techniques i.e. gel electrophoresis, gel filtration, counter-current distribution, and ion exchange chromatography in these studies are reviewed by Pierce and Carsten, 1960, 1963; and Bates and Condliffe, 1960, 1966. Studies on the biological effects and biological assays for mammalian thyrotropin (TSH) preparations on mammals have been equally prolific. The most recent treatise on this subject is given by Bates and Condliffe (1966) and inclusive series of reports on current studies in this area have been published (Whitilock, ed; 1960), (Werner, ed; 1963), and (Cassano and Andreoli, eds.; 1966). The more notable bioassay methods from various laboratories are listed and discussed in a recent survey by Bakke (1965). By comparison, the biochemical and physiological studies on the thyrotropic factors of fish and other lower vertebrates are indeed meager even though the existence of a pituitary-thyroid axis has been demonstrated in these classes. The literature on the interrelationships of the thyroid gland and the pituitary gland of non-mammalian vertebrates has been frequently reviewed (Olivereau, 1954, 1955; Pickford and Atz, 1957; Dent and Dodd, 1961; Dodd, et al., 1963; and Leloup and Fontaine, 1960).

Almost equally neglected as the studies on fish TSH has been the standardization of a bioassay utilizing fish as the test animal and as the hormone donor. The few quantitative data on the fish's thyroid response to TSH have exclusively dealt with either mammalian preparations of varying degrees

of purity or crude non-mammalian pituitary extracts. These results must often be interpreted with reservations. It is difficult to assess the effects of injecting large amounts of foreign protein into a small test animal and of injecting crude extracts containing a variety of contaminants, hormonal or otherwise, that may cause unknown interactions. Stress effects are also especially important considerations in thyroid research. The implications of biochemical evolution cannot be ignored either (Geschwind, 1959). It is likely that mammalian thyrotropins differ from those of fish in having different temperature optima and exert their effects in association with different enzyme systems. For example, Dent and Dodd (1961) showed that a threshold response in newly hatched dogfish (12° C) required at least fifty times as much ox TSH as that required for such a response in man (37° C). Either of the above interpretations could apply.

Examples of such incongruencies on thyrotropin assays as mentioned above are rife in the literature on non-mammalian thyroid research. Clearly, an attempt to standardize an assay for fish thyrotropin that provides phylogenetic nearness of the recipient to the donor species is desirable.

The methods for assessing thyroid activity in fish almost exclusively employ a radioiodine isotope and measure thyroid uptake rates or uptake percentages of the injected dose by direct counting (Gorbman and Berg, 1955; Fromm and Reineke, 1956; Fontaine and Fontaine, 1956) or by autoradiography (Olivereau, 1955); thyroid excretion rates (Swift, 1955; Riggs, 1952) or the amount of radioactive hormone released by the gland (Berg, Gorbman, and Kobayashi, 1959; Chavin, 1965; Hickman, 1962).

Methods that employ probing the thyroid area or methods that measure the radioactivity of the isolated gland (thyroid area) cannot be readily accomplished on goldfish because in this species the gland is diffuse, about two-thirds of the follicles reside in the head kidney (Chavin, 1956a, 1956b). Therefore, an assay method with this species would preferably be carried out on serum or otherwise employ the laborious and lengthy histological methods (Pickford, 1954).

The present study was directed toward following some of the parameters of serum iodine from intact goldfish that indicate the thyroid's response to exogenous pituitary extract. It attempts to relate a carp pituitary extract injection to a thyroid response and then, relate an extract dosage range to a biological response range.

Further studies using a partially purified fish thyrotropic factor were carried out on intact and hypophysectomized killifish. In other experiments on killifish several parameters of the thyroid response to purified mammalian TSH were tested simultaneously. With these data an evaluation of the most valid and readily measured index of thyroid activity could be made. Also, further observations were made on the known variables in iodine metabolism that could affect the test animal's response to exogenous TSH and affect the interpretation of the assay results.

## METHODS

### Part I

### Experiments on goldfish

All fish were taken from the University of Oklahoma Fisheries Research Center holding ponds at the indicated times of year. Prior to their acclimation in the experimental environment, the conditions described during radioiodine treatment, the fish were kept in indoor holding-tanks and allowed to adjust to general aquarium conditions (room temperature, indoor lighting, aeration, "building noises", and regular feeding) for seven to ten days.

A. Total serum I-131 and protein bound I-131 following a single radioiodine injection.

This pilot study was designed to determine the time required for maximum serum iodide conversion to protein bound iodine and the time for optimum serum iodine levels for determining these conversion ratios. Female goldfish (61 $\pm$ 6 gm, April) were injected intraperitoneally through the fleshy tissue around the urinogenital openings with 0.064 microcuries of radioiodine per gm body weight (NaI-131 with a trace amount of carrier NaI-127) and kept in temperature-controlled (19 $\pm$ 0.5° C), standing water. The fish were acclimated in the system five days prior to injection in an attempt to allow iodine equilibration. Blood samples were drawn with clean, dry syringes from the right or left efferent branchial artery (1.0 to 2 ml/fish) at sampling periods of 8, 17, 25, 33, 41.5, 45, and 49.5 hours after radioiodine injections. The whole blood was allowed to clot in centrifuge tubes, the clots were centrifuged at 4000 rpm for 10 minutes, and the sera were decanted and frozen in vials for

subsequent analysis. Clotting was complete after five- ten minutes at room temperature. This method for collecting serum at the noted sampling times was used throughout the studies on goldfish. The sera from three fish sampled at 45 hours were pooled and a 2.0 ml portion of this was passed through a Sephadex G-25 column (1x18 cm, 19-29 cm hydrostatic head, $\mu$ = 0.16, pH = 7.0, phosphate buffer) at room temperature. The column effluent, consisting of 1.0 ml samples that were collected throughout the protein range, and 0.35 ml aliquots of serum from each of the above serum samples were added to 2.0 ml of 20% (wt/vol) trichloroacetic acid (TCA) and centrifuged until clear, (Hickman, 1961; Eales, 1963). The supernatants were decanted and the precipitates washed twice with 0.5 ml of 5.0% TCA. The washes and supernatants of each individual sample were combined, and the volumes adjusted with water to 4.0 ml for scintillation counting. The precipitates were reconstituted in 2.0 ml of 0.01 N NaOH and made to 4.0 ml. The column samples following the protein range of effluent were prepared for counting by taking 1.0 ml from each and adjusting the volume to 4.0 ml with water. All the samples contained in uniform vials were counted in a NaI crystal, well detector (Tracerlab Kelekett Mark II). Counts were made for 5000 counts per minute (cpm) or 10 minutes, whichever was the shorter.

B. Effect of pituitary extract on the two-day conversion ratio in serum I-131.

This experiment was designed to determine the effect of a single injection of pituitary extract (the aqueous supernatant of one extraction of homogenized fresh frozen carp glands with pH 7.0, $\mu$ = 0.16 phosphate buffer) on the per-

centage[▽] of serum PBI-131. Seventy-two female goldfish (74 ± 18 gm, April) were marked with color-coded beads secured with a loop of thread through the dorsal fin area of the fish for nine experimental and nine control groups of four and acclimated as above for five days. Half the fish (experimental groups) received pituitary extract (100 μg fresh gland equivalent/gm body weight) and all fish received radioiodine (0.05 uc/gm with trace amount of NaI-127) 24 hours prior to each individual sampling period. The injection carrier was Ringer's solution (10 μl/gm). Along with the above groups, six non-marked fish were included as non-I-131 injected controls to indicate the amount of radioactivity the fish would take up from the I-131 accumulation in the water over the experimental period. Blood was drawn as above at nine successive periods (intervals of 12 hours each) following the injection of pituitary extract. Serum samples were kept frozen until precipitated with TCA as above, (Man, 1962).

The non-I-131 injected fish were sampled two fish each time at the third, sixth, and ninth 12 hour intervals. Again serum aliquots (0.5 ml) were treated with TCA and counted as before.

The sera remaining after TCA precipitation were pooled within each group and a 1.0 ml aliquot of each pooled group

---

[▽] The serum PBI-131 percentage is the ratio (expressed as a percent) of the radioactivity associated with the serum proteins to the total serum radioactivity; similarly, the BEI-131 percentage refers to the ratio of the radioactivity in the butanol extract of serum to the total radioactivity extracted from serum. These definitions apply throughout the following experiments.

was extracted once with 5.0 ml of n-butanol (saturated with distilled water) at room temperature.

In a serum extraction with these conditions, free and protein bound thyroxine, triiodothyronine, and iodinated amino acids, as well as free iodide partition between the aqueous and butanol phases. Three such extractions are necessary to remove all forms of iodine quantitatively (Taurog and Chaikoff, 1948). For the sake of simplicity, and since comparative results between fish receiving hormonal injections and their controls were desired, only one extraction was made. The butanol was later acidified to facilitate the thyroxine-protein complex's dissociation (Chaney, 1958). A sufficient volume of anhydrous butanol was used on acidified killifish sera to dissolue all of the serum water and eliminate any partitioning between butanol and water of serum.

Four ml of the n-butanol phase were extracted with an equal volume of 4 N NaOH-5% $Na_2CO_3$ solution and 3.5 ml of the resulting alkaline and butanol phases were counted separately with consistent geometry as before. The para-hydroxyl groups of triiodothyronine and thyroxine do not dissociate at the pH of the aqueous, alkaline solvent and they remain (94-96%) in the organic phase; whereas, monoiodo- and diiodothyronine, iodinated tyrosine, and iodide are extracted from the organic phase. The butanol fraction thus contains the serum radiothyroxine and radiotriiodothyronine and the ratio of the radioactivity in the butanol extract to the radioactivity in the alkaline and butanol extracts represents the radiothyroxine conversion ratio.

Background counts taken every hour throughout counting periods indicated no detectable machine drift nor instability.

C. Log-dose response relationship between crude pituitary extract and serum PBI-131 percentages.

Five groups of goldfish (female, 74 ± 22 gm, March) were injected as above with 1000, 300, 100, 30, and 10 ug fresh gland equivalent/gm body weight, respectively; a sixth group was injected with 1.0 microunit of USP (Mussett and Perry, 1955) standard thyrotropin/gm; and a seventh group received a carrier injection only. Seventy-two hours after the injections, each fish received 0.2 μc I-131/gm, intraperitoneally in 0.6% NaCl-trace NaI-127 solution (10 μl/gm). Iodine-131 conversion ratios were determined on individual serum samples collected 48 hours later by the TCA precipitation method previously described.

D. Log-dose response relationship between crude pituitary extract and BEI-131 percentages (radiothyroxine conversion ratios) in serum.

Female goldfish (72 ± 16 gm, August) were treated with 0.2 μc carrier free NaI-131/gm and pituitary extract (10, 50, 100, 200, and 1000 μg/gm) to establish a log-dose to response relationship between pituitary extract and BEI-131 (60 hours after extract injection; 36 hours after I-131 injection). The sera (0.5 ml aliquots) were extracted once with n-butanol (2.0 ml) saturated with 1.0 N HCl and the butanol phase was extracted once with 2 N NaOH-5% $Na_2CO_3$ (2.0 ml) and again with 4 N NaOH-5% $Na_2CO_3$ (2.0ml). Counts were made on each of the three resulting extracts (1.8 ml aliquot).

E. Serum I-131 distribution in Sephadex G-100 gel filtration.

Control sera (pooled from four fish) collected 9, 23.5, and 36 hours after I-131 injection, were passed separately

through a Sephadex G-100 column (1x18 cm, 19-29 cm hydrostatic head, $\mu$ = 0.16, pH = 7.0, phosphate buffer) at room temperature. Effluent samples were analysed for total protein by the Folin-Ciocalteu method (modification described by Bailey, 1962) and total iodine radioactivity.

## Part II

### Experiments on killifish

Fish were kept in temperature regulated rooms (20 ± 0.5° C) and on an artificial eight-hour day length. They were fed daily on Aronson's mix (about 15 μg iodine/100 gm body weight daily) supplemented weekly with frozen brine shrimp or daphnia. The fish were taken from a tributary to New Haven harbor and given routine treatment for external body parasites including manually picking Ergasilus sp. off the gills. After a week of acclimation, routine feeding was begun. After hypophysectomy (method of Pickford, 1954), identifying fin clips were made and the fish were fasted five days to allow healing of the roof of the mouth. Prior to handling the fish were anesthetized in tricainemethanesulphonate (0.4 mg/ml). All injections were intraperitoneal (10 μl/gm).

A. Thyroid activity assessment

Thyroid uptake of radioiodine was measured either on dissected thyroids (thyroid areas) or on live fish with a scintillation probe and expressed as a percentage of the injection dose (0.02-0.5 μc NaI-125 or NaI-131, carrier free, in 10.0 μl physiological saline per gram body weight).

1. Probe counting Various arrangements for probing killifish thyroids were tried to determine the most accurate measurement of thyroid radioactivity. The most suitable system consisted of a mechanical stage that permitted positioning an anesthetized fish held in a Lucite carrier on its back so that its whole thyroid area$^{\nabla}$ was exposed being about

$^{\nabla}$ This area was determined by scanning the lower jaw when

3-4 mm below a 0.625 cm-bore collimator and about 28-29 mm below a shielded NaI scintillation detector (Baird Atomic model 812-S). Counts for 2-4 minutes were sufficient (4000 cpm or greater) for fish injected with 0.5 μc I-131/gm; the gamma energy of I-125 was too low for accurate probing. Initially, radioactivity of the abdomen (including the background radiation) was subtracted from the thyroid measurement, but since the thyroid activity was exceedingly high relative to that of the abdomen (less than 40 cpm), this correction was not necessary. The use of a spectrometer for counting pulses only in the photoelectric energy range made background ($10 \pm 4$ cpm) corrections unnecessary.

It was possible with the probe to follow the net loss of thyroid radioactivity on individual fish from day to day. On this basis, the optimal period for measuring uptake differences in control and stimulated fish was selected.

2. Well counting For determining the radioactivity of thyroids in the well-scintillation detector the thyroids were dissected out at autopsy, placed in vials containing Bouin's fixative, and counted after one change of fixative about 24 hours later. Good count rates were obtainable with radioiodine dosages as low as 0.02 μc/gm or even less if the thyroids were highly stimulated. Usually, the 0.5 μc/gm dose

---

the fish was placed on its back and its head raised so that the surface of the lower jaw was perpendicular to the collimator. This arrangement was sufficiently reproducible. The peak of activity was found to be restricted to 1-3 mm along the medial axis of the lower jaw corresponding to the area just above the point where the afferent gill arteries branch off from the ventral aorta.

was too high for optimal counting rates in the well detector, in which case, the radioactivity was allowed to decay to the optimal level before counting. Counts above about $300 \times 10^3$ caused hysteresis in the detector's phototube; high counts also made a correction for dead time, the refractory period following an impulse, necessary.

3. Butanol extraction The butanol extraction method for radiothyroxine differed from Mougey and Mason's (1962) modification of Taurog and Chaikoff's (1948) procedure only in its application to smaller serum volumes (20-150 µl) and in the estimation of radioiodine rather than the estimation of iodine by its catalytic action on ceric ion reduction.

The butanol extraction procedure was applied in a pilot study on intact fish injected with 0.5 µc I-131/gm to determine the sampling time for the maximal rate of serum radioiodine conversion to radiothyroxine along with thyroid radioiodine fixation as determined by probing the thyroid area.

4. Thyroid cell heights Mean thyroid cell heights, expressed in microns, were determined by the method used by Pickford (1954). One hundred follicles from groups of five sections (7 µ thick) taken every 50th transverse section (alternating groups of five sections, every other 20 through 25 sections, were stained with hematoxylin-eosin and azan-Heidenhain's "tri-chrome," azocarmine, counterstained with aniline blue and orange-G) across the whole gland of each fish were used in estimating cell heights. The glands were fixed in Bouin's fixative and stained with hematoxylin-eosin for general microscopic examination and with azan to disclose resting, active, and mixed follicles.

B. Iodine-127 estimation

Determinations of the stable isotope of iodine were made on the aquarium water (Long Island Sound sea water, about 25‰), sera and thyroids from fish adapted to freshwater, and sera and thyroids from fish kept in sea water. Pooled sera (50-150 µl) and individual thyroids were put in one-ml, thick-walled Pyrex test tubes with 50 µl of 4N $Na_2CO_3$, dried in an oven at 90-98° C, covered with Pyrex covers, and ashed in a muffle furnace at 600 ± 25° C for two hours. The ash was dissolved in a standard volume (200 µl for serum; 1.0 ml for thyroids) of 2N HCl and the iodine estimated by its catalytic action on ceric ion reduction by arsenite. Rogina and Dubravcic's (1953) modification of Chaney's (1938) procedure was followed after the organic material was ashed. The same procedure, excluding ashing, was followed for an appropriate quantity of aquarium water. Iodine determinations were not made in every experiment; they were done initially with thyroid radioiodine uptakes of salt water and freshwater fish for an evaluation of how the serum iodide pool, the water iodine concentration[▽], and dietary iodine related to uptake percentage.

C. Pituitary injection preparations

Crude pituitary preparations either in the form of a

---

▽ Except for two experiments, subsequently noted, the saltwater fish were kept in standing water which was changed at frequent enough intervals (7-10 days) to prevent iodine deficiency. Moreover, iodine was supplemented in the diet. A preliminary experiment on fish in 50% sea water and 50% freshwater showed radioiodine uptakes comparable to those of fish in 100% sea water (Long Island Sound).

brei of frozen whole pituitaries[▽] (carp), the supernatant after centrifugation diluted with an equal volume of distilled water (carp), or the supernatant of an equal volume, single extract of brei (carp and buffalofish) with 0.1N NaCl-phosphate, pH 7.0 buffer, served as the starting materials for several subsequent separation procedures.

Initially, a carp pituitary starting material, used effectively in the isolation procedures of the gonadal hydration principle (Clemens and Grant, unpublished), was employed, but excessive loss of thyrotropic activity made its use unfeasible. The procedure involved extracting freshly thawed glands with pH 4.25, $\mu$ = 0.1 pyridine-acetate, centrifuging, desalting on a Sephadex G-25 gel column, and lyophilizing. The step that caused loss of thyrotropic activity was found to be lyophilizing with the volatile buffer. Also, exceedingly small traces of pyridine actually inhibited radioiodine uptake. By reconstituting this material in distilled water and re-lyophilizing it to completely remove the trace of pyridine, some thyrotropic activity was detectable.

The neutral brei-extract (carp) was centrifuged at 60,000xg, 5.0° C., for 20 minutes. The resulting dense, turbid protein solution was analyzed for total protein by the Folin-Ciocalteu method and for total protein (Ponceau S stain) and glycoprotein (periodic acid-Schiff's reagent) following electrophoresis on a Beckman Microzone system.

---

[▽] Fresh frozen carp (Cyprinus carpio) and buffalofish (Ictiobus, sp.) glands were obtained through Dr. Howard P. Clemens from Stoller Fisheries, Spirit Lake, Iowa.

1. Gel electrophoresis Further purification of the starting preparation was carried out on several variations of gel electrophoresis (carp) and gel filtration (buffalofish). One series of injection fractions was prepared by eluting slices of a large-tube disc electrophoretic run. The electrophoretic run was comparable to the analytical system described by Ornstein and Davis (1964) in every way except in tube size (22 mm), running time (five hours at 15 ma), protein load (44.4 mg of total protein-carp pituitary brei extract-as determined by microzone electrophoresis), and sample application (applied over the spacer gel in 8.0 ml of semi-fluid homogenized spacer gel polymer mixed with the protein sample). The gel showed refractile and colored protein zones at the end of the run that corresponded to stained zones of an analytical disc electrophoretic run of the starting material. Immediately after the run, the gel cylinder was sliced transversely into nine approximately equal sections from the leading edge of the tracking dye to the small pore gel-spacer gel interface. The slices were weighed to the nearest milligram and the weights were related accurately to gel length. Slices in sequence from one through nine corresponding to the direction of migration were pooled, crushed, allowed to elute 14 hours, in 10 ml of distilled water with constant agitation, centrifuged through polyethylene filtering discs, concentrated by pervaporation (about 5 hours) at near freezing temperature, made to 6.0 ml with distilled water, alloted to daily injection quota, and frozen. The injection fraction 1, 2, 3, 4, and 5 corresponded to slices 7 and 8, 5 and 6, 3 and 4, 1 and 2, and the spacer gel, respectively. An estimate of the protein con-

centration of each fraction by the microzone electrophoretic method was attempted, but the amounts, less than 700 μg/ml in the two most concentrated fractions, 3 and 4, were too small for accurate quantitation. Larger protein loads, 148 mg and 370 mg of starting-material total protein were tried, but the system did not operate successfully due to clogging at the top of the small pore gel.

2. Gel filtration I Another series of injection fractions were prepared by flowing 5.0 ml of the buffalofish pituitary starting material (prefiltered on a short G-25 Sephadex column) through a Sephadex G-100 column (1.6 x 72 cm, 12 cm hydrostatic head, μ = 0.005, pH 7.0 phosphate buffer, 8 ± 1° C water jacket), calibrated with Dextran blue and riboflavin. The effluent, 24 ml/hour was collected manually from a 4.0 ml metering siphon, and a 10 μl aliquot of each effluent sample was analyzed for total protein by the Folin-Ciocalteu method. Three milliliter aliquots from successive samples corresponding to 59.0 through 79.0, 80.0 through 107.0, and 108.0 through 147.0 ml of effluent and covering the entire protein resolution range were pooled, lyophilized, weighed to the nearest 0.1 mg, reconstituted in 5.0 ml of physiological saline each, distributed in daily injection quota, and frozen to give fractions I, II, and III, respectively.

3. Gel filtration II A third series of injection fractions were prepared by flowing 5.0 ml of buffalofish pituitary starting material (prefiltered on a short G-25 column) through a temperature-regulated (5.0 ± 0.1° C), G-100 column (1.6 x 72 cm, 12 cm hydrostatic head, μ = 0.005, pH =9.0, TRIS-HCl buffer). This column was calibrated in the way as the one described above and had similar bed characteristics

but a slower flow rate, 16 ml/hour. The effluent was metered by a 4.0 ml siphon to give samples 1-27, beginning at the end of the void volume (51.0 ml) and ending at a volume equivalent to the collective aqueous volume inside the gel beads (159.0 ml).[▽] Ten microliters from each sample were taken for Folin-Ciocalteu total protein determinations, and 2.0 ml of each sample were pooled for injection according to the following schedule: 1-5, fraction I; 6-10, fraction II; 11-14, fraction III; 15-19, fraction IV; and 20-27, fraction V. The fractions were lyophilized and weighed as before but reconstituted in physiological saline to give concentrations of 3.5, 3.5, 3.6, 3.5, and 2.7 mg/ml for fractions I, II, III, IV, and V, respectively and frozen in daily injection quota.

D. Mammalian thyroid stimulating preparations[▽▽]

The following mammalian preparations were prepared for daily injection quota by dissolving the lyophilized protein in a buffered (pH 7.0) gelatine solution (100 ml of 0.5 M NaCl, 17.5 ml of 0.2 M $NaH_2PO_4$, 30.5 ml of 0.2 M $Na_2HPO_4$, 0.5 gm gelatine, adjusted to 500 ml with distilled $H_2O$).

(1) Three dosage levels of bovine heterothyrotropic factor, HTF 7-51-C (Y. A. Fontaine) 100 μg/ml, 10 μg/ml, and 1.0 μg/ml. This preparation, assayed on starving trout, was equivalent to 8.2 TSH-USP units/mg; on mice, it was equivalent to 0.07 TSH-USP units/mg.

---

[▽] This range of effluent, determined by the Dextran blue and riboflavin calibration, corresponds to the protein resolution range.

[▽▽] These materials were previously assayed by, and provided by, Y. A. Fontaine (Fontaine and Le Belle, 1965; Fontaine and Burzawa-Gerard, 1967).

(2) Two dosage levels of purified bovine thyrotropin, B-TSH 8-18-A (Y. A. Fontaine) 100 mU/ml and 5.0 mU/ml. The activity of this preparation assayed on mice, was equivalent to 14.2 TSH-USP units/mg.

(3) A single level of reference standard USP-TSH, 10.0 mU/ml. The activity of this preparation was arbitrarily taken as 0.074 units/mg on mice and trout.

E. Schedule of experiments

1. Hypophysectomy and thyroid regression Thyroid radioiodine uptakes of females from the same catch (July)- six intact, five 13-day, six 55-day, and seven 55-day post operative hypophysectomy injected with 1:1 carp pituitary brei-distilled water preparation-were determined. This contained the equivalent of about 500 mg of fresh-frozen pituitaries/ml. Hematocrits, gonad and body weights, and general liver colors were noted at autopsy. The extract injected fish received three injections (5 mg/gm) at 48-hour intervals; radioiodine followed the third injection by 24 hours. Thyroid radioiodine uptakes were measured 72 hours after the tracer injection.

2. Acute pituitary extract injection into intact fish with low endogenous thyroid activity Thyroid uptakes of intact males (3 July) injected once with physiological saline, 3.0 mg/gm lyophilized pyridine-acetate extract of carp pituitaries (described above), or the 1:1 brei supernatant- distilled water preparation, were determined with the probe. The tracer was injected 24 hours after the pituitary preparation and the thyroids were probed everyday after for 9 days (controls) and for 10 days (pituitary preparation).

After probing on day 9, the control fish were again

injected with 5 mg/gm of the pyridine-acetate extract after it had been re-lyophilized (described above) to remove the trace of pyridine, and another tracer dose was given on day 10, 24 hours later. Only a very small amount of the original I-131 injected (1% without correcting for decay) remained in the thyroid glands of these fish when they were given the second tracer dose. Thyroid uptakes were determined on days 1, 2, 4, 6, and 8 after the second tracer dose was given.

3. Acute pituitary extract injection into intact fish with high endogenous thyroid activity Thyroid radioiodine uptakes of intact males (8 Aug.) in freshwater injected once with physiological saline or 0.15, 0.5, 1.5, 5.0, and 15.0 mg/ml of the re-lyophilized pyridine-acetate extract (described above) were determined with the probe. The tracer was injected 24 hours after the pituitary preparation and the thyroids were probed on 1, 2, 3, 4, and 5 days after the tracer was given.

A similar experiment was run on intact males (3 Oct.) in salt water. The 0.15 mg/ml dose was excluded, and the fish were probed on days 2 and 3 after tracer injection.

4. Chronic injections of gel electrophoretic fractions into hypophysectomized killifish Males (groups of five), six weeks post-hypophysectomy, were given 10 injections, each injection scheduled every other day. There was a group for each fraction, 1 through 5, and one group for physiological saline (six fish). Twenty-four hours after the tenth injection, a tracer dose was given followed by autopsy 72 hours later. Along with thyroid dissections for subsequent uptake measurements, the testes and body weights, nuptial colora-

tion, hematocrits, and general autopsy data were recorded.

5. Chronic injections of gel filtration (I) fractions into hypophysectomized killifish Female killifish, five weeks post-hypophysectomy, were grouped six fish each, corresponding to respective injection materials, fractions I, II, III, starting material, or physiological saline (seven fish), and each received its respective injection material five times, once every other day. The fifth was followed 24 hours by a tracer injection which was then followed 72 hours by autopsy and thyroid uptake measurements in the well detector.

6. Chronic injections of gel filtration (II) fractions into hypophysectomized killifish Male killifish, four weeks post-hypophysectomy, separately grouped six fish each in sea water-recirculated aquaria corresponding to respective injection categories I, II, III, IV, V, and physiological saline, were injected six times, one time every other day. The sixth injection was followed 24 hours by a tracer iodine injection and the tracer was followed 72 hours by autopsy. Along with thyroid dissections for subsequent uptake determinations, the testes and body weights, nuptial coloration, hematocrits, and general autopsy data were recorded. The heads of suspected fish were preserved, decalcified, and examined microscopically for pituitary remnants.

7. Chronic injections of mammalian thyroid stimulating preparations into hypophysectomized killifish Screened[▽]

---

▽ The fish in this experiment were hypophysectomized by Dr. Grace E. Pickford. A pituitary remnant in an operated fish is indicated after about six weeks by a significant increase in standard length or any trace of nuptial coloration.

male killifish, 9.5 weeks post-hypophysectomy, separately grouped five fish each in sea water recirculated tanks corresponding to respective injection categories, (1) 100 μg-, (2) 10 μg-, and (3) 1.0 μg HTF 7-51-C/ml; (4) 100 mU-, and (5) 5.0 mU B-TSH 8-18-A/ml; (6) 10 mU USP-TSH/1.0 ml (four fish); and (7) buffered gelatine carrier, were injected thrice weekly for a month. Twenty four hours after the 12th injection, radioiodine was given and 48 hours after radioiodine injections the fish were probed. Twenty-four hours after tracer injections and 24 hours after probing, the 13th and 14th hormone injections respectively, were given. Records of standard length, liver weight and color, body and testes weights, hematocrits and other autopsy data were recorded at autopsy, 96 hours after radioiodine injections. The thyroids were dissected out for uptake determinations and subsequent histological procedures. Individual serum samples were analyzed for radiothyroxine according to methods mentioned above.

# RESULTS

## Part I

## Experiments on goldfish

### A. Total serum I-131 and protein bound I-131 following a single radioiodine injection.

The total serum I-131 peaked abruptly within eight hours after the radioiodine was injected and fell at a uniform rate until about the 25th hour where the rate of decrease changed until 50 hours after injection. The serum PBI-131 rose and dropped abruptly during the first 20 hours and then began to rise at a uniform rate to 40 hours after injection before it began to decrease again. These relationships are shown graphically in Fig. 1.

The serum from fish sampled at 45 hours after injection was resolved into two major fractions by Sephadex G-25, (Fig. 2). The second fraction, containing compounds having molecular weights less than 1000, contained almost all of the radioactivity. The protein gradient represented by the total counts (precipitate and supernatant) showed at least two fractions or species of compounds having radioactivity, while the protein gradient represented by the supernatant counts showed the possibility of a third species of macromolecules having radioactivity. TCA precipitation caused differential dissociation of the protein-iodine complexes: in the five successive samples that constituted the protein effluent, the precipitate contained 68.5%, 71.0%, 62.0%, 75.5%, and 56.0% of the radioactivity in each milliliter sample. Further heterogeneity of the overall protein effluent was indicated by the original two ml sample volume spreading beyond the amount characteristic for the column.

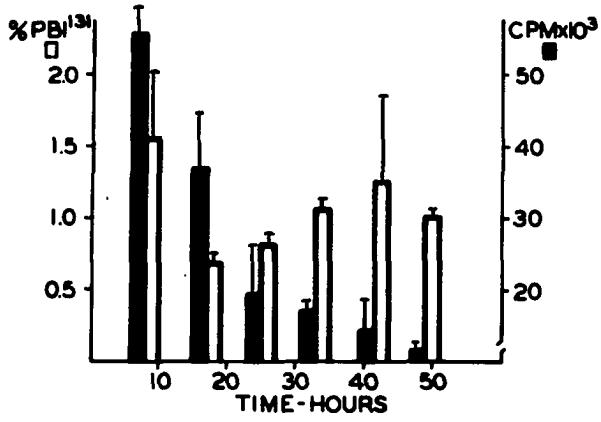


Figure 1. Total serum radioactivity (solid bars) and corresponding fraction of radioactivity associated with serum proteins (open bars). Vertical lines = S. E., N = 4.

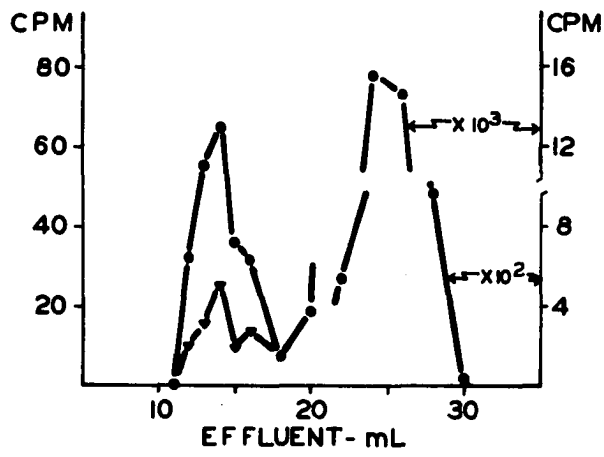


Figure 2. Sephadex G-25 separation of serum radioactivity 45 hours after tracer injection: total effluent radioactivity (●-●); TCA supernatant radioactivity (▼-▼).

B. Effect of pituitary extract on the two-day conversion ratio in serum I-131.

The two-day conversion ratios of both control and experimental fish were unpredictably variable for the first five sampling periods. Both groups had high conversion ratios the first sampling period, dropped considerably the second, rose slightly the third, and dropped to the lowest level of the sampling sequence the fourth period. During the interval between the 2nd and 3rd twelve-hour periods, all the remaining fish that had recieved pituitary extract ovulated completely. An overall decreasing trend superimposed on a circadian rhythm is suggested from the initial high at 12 hours to 48 hours after extract injection. Between the 4th and 5th periods, the PBI-131 of both groups rose significantly. Following the fifth period, both groups began to show a predictable trend, a gradual but definite PBI-131 increase (Figure 3).

The effects of pituitary extract were indicated by two significant differences between the experimental and control fish: the conversion ratios of the experimental fish were consistently lower for all sampling periods, and all experimental fish that had not been sampled ovulated after the second period. A more pronounced difference in conversion ratios existed between controls and experimental fish between 60 hours and 108hours than between 12 hours and 48 hours after extract injection.

The BEI-131 percentages (radiothyroxine conversion ratios) of the pituitary injected fish showed a general increase with time, but a sharp rise and fall occurred just after ovulation. The BEI-131 percentages of the con-

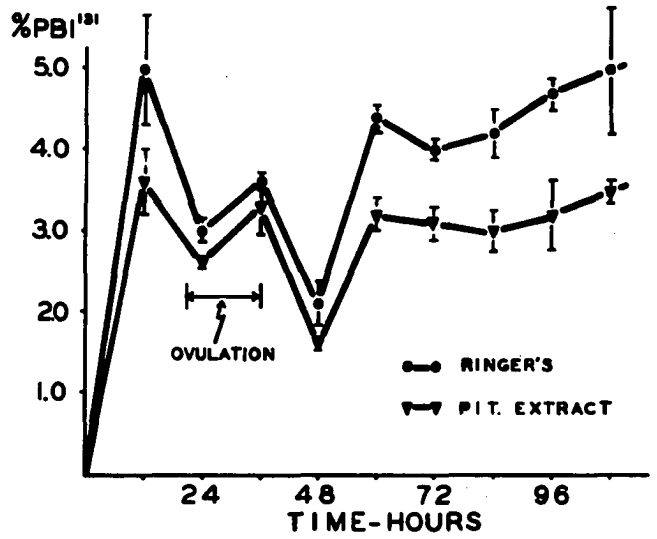


Figure 3. Serial 24-hour conversion ratios of female goldfish after injection with pituitary extract or carrier. The vertical lines = one S. E. above and below the means (N= 4).

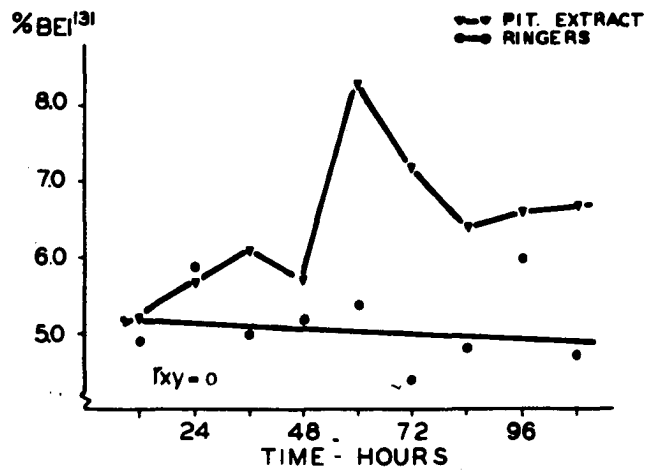


Figure 4. Serial 24-hour radiothyroxine conversion ratios (%BEI -131) following pituitary extract or carrier injection in female goldfish (serum pools of four fish).

trol fish were variable but showed no overall significant trend. The 2nd and 8th periods are high but probably have no special significance (Fig. 4). The fish that had no injection of I-131 accumulated very slight amounts of radioactivity (30-50 cpm/0.5 ml serum), even though the I-131 build-up in the water was considerable, (1394 cpm and 1813 cpm in 6.0 ml at 48 and 72 hours respectively).

C. Log-dose response relationship between crude pituitary extract and serum PBI-131 percentages.

An inverse relationship existed between the log-dose and the PBI-131 percentages, (Fig. 5). Concurrently, the relative amounts of radioiodine were generally lower in the higher PBI-131 samples: the counts were 9,250; 14,000; 14,100; 8,500; 21,750; 19,500; 16,000 and the PBI-131 percentages were 8.3; 8.6; 7.3; 8.3; 6.9; 6.8; 6.3; for the controls; the 1.0 μU TSH/gm; the 10; 30; 100; 300; and 1000 μg/gm, respectively.

D. Log-dose response relationship between crude pituitary extract and BEI-131 percentages (radiothyroxine conversion ratios) in serum.

The 60-hour BEI-131 percentages varied directly with the dosage, (Fig. 6). The BEI-131 percentages were extremely high (for all injection levels and the controls) compared to those obtained when carrier I-127 was injected. Compare BEI-131 percentages of Fig. 4 (carrier iodide) to those of Fig.6, (carrier free).

E. Serum I-131 distribution in Sephadex G-100 gel filtration.

Figure seven shows the relative protein (Folin-Ciocalteu) and total radioactivity of the Sephadex column

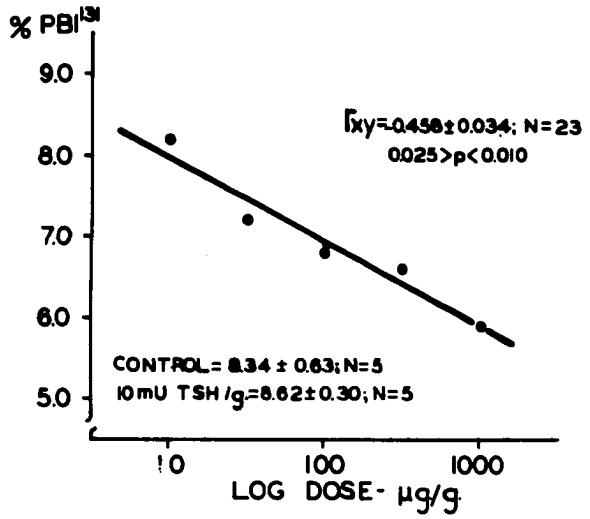


Figure 5. Twenty-four-hour serum PBI-131 conversion ratios determined 220 hours after pituitary extract injection (each point represents the mean of four or five fish).

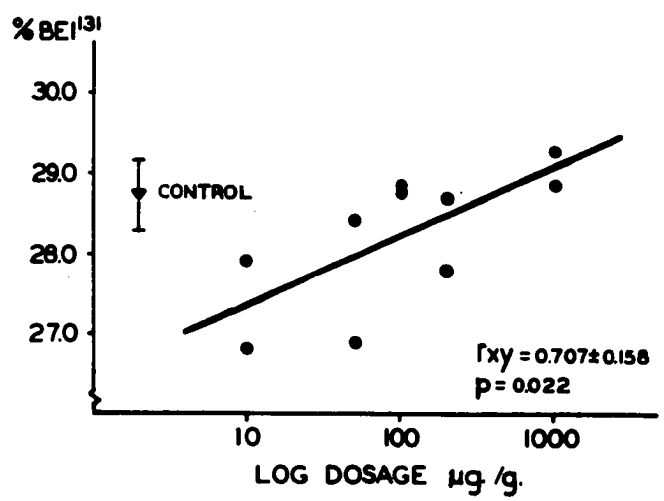


Figure 6. Thirty-six-hour radiothyroxine conversion ratios determined 60 hours after pituitary extract injection (● = serum pool of two fish; ⧗ = mean ± S. E., N = 5).

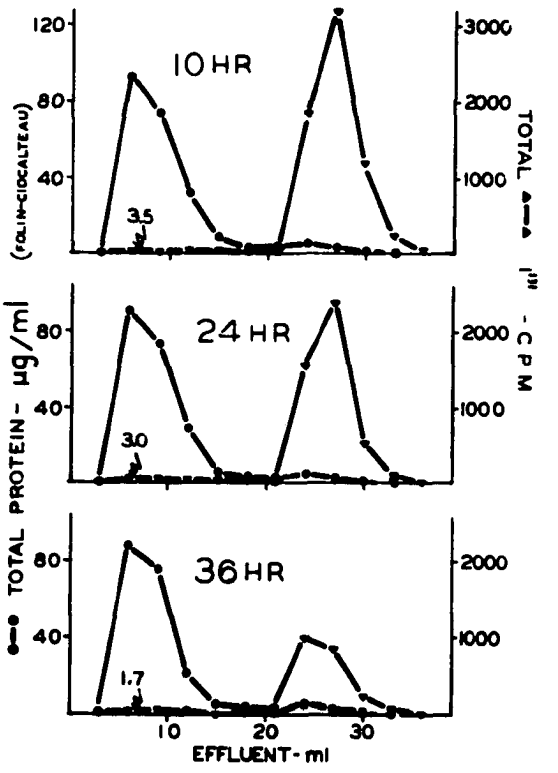


Figure 7. Sephadex G-100 separation of sera following tracer injection. (●-●) = total protein; (▼-▼) = total radioactivity. The arrows indicate the average percentages of radioactivity associated with the protein effluents.

effluents. Almost all of the serum protein appeared in the first of the two peaks, while essentially all of the radioactivity was associated with the second. No change in the radioactivity $R_f$ with time was detectable. The average percentage of radioactivity in the first peak decreased with time (3.5% at 9 hours, 3.0% at 23.5 hours, and 1.7% at 36 hours) paralleling the decrease in total serum radioactivity.

## Part II

Results of preliminary studies on killifish.

1. Thyroid radioiodine uptake of freshwater- and salt water-adapted killifish.

The radioiodine uptake of intact male killifish adapted to freshwater was higher than that of their controls in salt water. The fish in both groups were of a comparable degree of sexual maturity and had comparable thyroid iodine contents, but their serum iodine levels varied inversely with their thyroid tracer uptakes, Table I. Although the small quantities of available serum precluded relating serum iodine levels of individual fish to tracer uptakes of individual fish, an inverse relationship was suggested by the average values in these two parameters. If it were a strictly linear inverse relationship between serum iodine and tracer uptakes that resulted from the dilution of tracer iodine by the serum iodide pool, it was not apparent from these data: the salt water group's serum iodine level was about 2.5 times greater than that of the freshwater group, while the uptake percentage was about 0.75 times as much. However, a descrepancy of this magnitude may be within the limits of error in the methods since no assessment was made on an individual basis. Controlling the serum iodine level in tracer uptake studies is obviously of extreme importance if the tracer uptake is to be a valid index of thyroid activity. Serum iodine levels of both groups were in the upper part of the physiological range of freshwater species (comparing them to reported values on freshwater species with low to high iodine diets) and, were in the lower part of the physiological range of marine species; the thyroid iodine content

Table 1. Radioiodine uptake of intact (Sept.) male killifish in iodine-rich (sea water) and iodine-poor (freshwater) environments.

| Group | N | % Uptake[1] | GSI[2] | Serum iodide µg % (pool) | Thyroid $I_2$[3] µg/100 gm bwt. |
|---|---|---|---|---|---|
| Fresh-water | 5 | 20.0 ± 2.5 | 1.21 ± 0.75 | 18.3 | 4.7 ± 3.3 |
| Salt water | 5 | 14.9 ± 1.3 | 1.23 ± 0.59 | 45.6 | 4.8 ± 0.8 |

Sea water = 47.4 µg/liter (44.0-49.6); freshwater too low (less than 0.01 µg/ml) without 10-fold concentration.

1/ Seventy-two hour uptake (probe); mean ± S. E.

2/ GSI = percentage of gonad weight to total body wt./ mean ± half range.

3/ Mean ± half range.

remained near the mid-range in both (Hunn and Reineke, 1964; Hickman, 1962; La Roche, Johnson, and Woodall, 1965; Leloup and Fontaine, 1960).

2. Distribution of radioiodine.

The data from a pilot study on four intact killifish are summarized in Table 2. Although only limited confidence and significance can be placed on this pilot study on four individuals, it provided criteria for selecting an optimum time for sampling sera from fish injected with radioiodine to measure radiothyroxine production rates as a function of thyroid activity.

3. Tracer iodine uptakes in intact killifish with low endogenous thyroid activity.

Intact killifish were stimulated by a single injection of carp pituitary preparations to yield higher tracer iodine uptake than their controls (Table 3) when the endogenous thyroid activity (thyroid uptake of controls) was in the middle to lower part of its range (less then 1.0% in hypophysectomized killifish near complete regression to 20 to 30% uptake in intact fish at various times of the year). The greatest difference in thyroid uptake percentages between stimulated and control fish occurred between the third and fourth days after tracer injections; the differences in uptakes on either of these two days were more indicative of increased thyroid activity than a comparison of their respective regression coefficients. In fact, the stimulated fish's average uptake peaked a day later than that of the controls (Swift's regression coefficient, 1959, is more negative in thyroids with higher activities).

Re-lyophilization apparently removed the inhibitory

Table 2. Pilot study on killifish.

| I-131 Distribution | Days after I-131 injection 2 | 3 | 4 | 6 |
|---|---|---|---|---|
| Thyroid uptake percentage (probe) | ND | 2.7 | 4.0 | 4.7 |
| Serum radioactivity in the alkaline extract (cpm)[a] | 1469 | 1020 | 669 | 156 |
| Serum radioactivity in the butanol extract (cpm)[b] | 131 | 686 | 581 | 179 |
| Serum radioactivity in the residue (cpm)[c] | 141 | 50 | 57 | ND |
| Percent BEI-131 serum[d] | 12 | 42 | 48 | 54 |
| Percent BEI-131 cells[e] | 10 | 33 | 23 | ND |
| Radioactivity percentage of hematocrit[f] | 28 | 28 | 29 | ND |

a/ This extract contains iodide and organic iodine other than thyroxine (T-4) and triiodothyronine (T-3).
b/ This extract contains organic iodine (T-4 and T-3).
c/ The residue is the protein precipitated by the butanol extraction.
d/ BEI/BEI+Alkaline x 100.
e/ BEI/BEI+Alkaline x 100 from hematocrit.
f/ The percent of total blood radioactivity in the hematocrit.
ND = Not determined.

Summary: 1. Intact males, 0.5 µc/ gm body wt., standing water (20° C), dietary I-127 about 15 µg/100 gm body wt./day. 2. The radioiodine in the alkaline phase progressively decreased at a constant rate from day two to day six. 3. The BEI percentage (serum) reached a plateau day - 3 to day-4. 4. The amount of BEI-131 did not become significant until the third day where it peaked and began to decrease at about the same rate as the activity in the alkaline phase. 5. Only slight activity was found in the residue and it showed no evident trend. 6. The thyroid uptake percentages paralleled the BEI-131 percentages on days 3,4, and 6.

Table 3. Acute pituitary extract injection into intact (July) killifish with low endogenous thyroid activity.

| Day[1] | Uptake percentage[2] (Mean ± S. E.) | | | |
|---|---|---|---|---|
| | Carp brei (5mg/gm) | Control (saline) | Pyridine-acetate (0.3 mg/gm) | Re-lyophilized P-A (5 mg/gm) |
| 1 | 17.1 ± 3.6* | 8.3 ± 0.16 | 5.6 ± 0.66[3] | 20.1 ± 2.2** |
| 2 | 17.6 ± 3.4 * | 7.2 ± 0.16 | 4.7 ± 0.10 | 17.9 ± 2.5** |
| 3 | 16.9 ± 3.6 * | 6.3 ± 0.50 | 3.9 ± 0.10 | ND |
| 4 | 16.2 ± 3.6 * | 5.2 ± 0.55 | ND | 15.1 ± 3.0 ** |
| 5 | ND | 4.8 ± 0.69 | ND | ND |
| 6 | 13.6 ± 7.6 * | 4.3 ± 0.80 | ND | 12.9 ± 3.1 ** |
| 7 | ND | 4.0 ± 0.82 | ND | ND |
| 8 | 12.1 ± 3.4 | ND | ND | 12.0 ± 3.1 |
| 9 | ND | 3.2 ± 0.71 | ND | ND |
| 10 | 11.4 ± 3.2 | (day-1, 4th column) | ND | 10.7 ± 3.1 |

1/ Day after tracer iodine injection.

2/ N = 4 in each group.

3/ N = 2; two fish had uptakes too low to get an accurate estimation. In all fish tabulated, counts were greater than 1000/minute: the two excluded had less than 50 cpm.

*/ Significantly higher than controls.

**/ Significantly higher than initial uptake percentage.

substance in the pyridine-acetate extract. Since the first lyophilized product which actually caused lower tracer uptakes smelled of pyridine and since the re-lyophilized material which did stimulate tracer uptake did not have an odor of pyridine, it was suggested that it in some manner interfered with thyroid function.

4. Tracer uptakes in intact killifish with high endogenous thyroid activity.

There was no evidence of stimulation in tracer uptake measurements of intact killifish (Table 4) with high endogenous thyroid activities (control uptakes).

5. Hypophysectomy and regression.

The results of uptake studies on female killifish after hypophysectomy are summarized in Table 5. Post spawning females (July) had a low average GSI (gonosomatic index, the percent of gonad weight to body weight) indicative of the endogenous gonadotropic blood level. Following hypophysectomy, the GSI did not change appreciably until the fish were treated ($p < 0.01$) with a crude pituitary preparation. An indirect gonadotropic effect was also detected by a decrease ($p < 0.05$) in the hematocrit (Slicher, 1961).

Tracer uptakes following hypophysectomy reflected the removal of endogenous thyrotropin and subsequent regression of the thyroid. Three injections of the crude pituitary extract was highly effective in stimulating thyroid iodine uptake.

Results of definitive studies in killifish.

1. Gel electrophoretic fractions.

None of the gel electrophoretic fractions were effective in stimulating thyroid iodine uptake and fractions 1, 2, and 3 appeared to be inhibitory (Table 6). Sufficient thyro-

Table 4. Acute pituitary extract injections into intact killifish with high endogenous thyroid activity.

| Day after tracer inj. | (N) | Thyroid tracer uptake percentages (Mean ± S. E.) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | 0.15 mg/gm | 0.5 mg/gm | 1.5 mg/gm | 5.0 mg/gm | 15 mg/gm |
| 1 (8 Aug) | 4 | 29.7 ± 1.2 | 33.7 ± 2.0 | 30.8 ± 2.3 | 28.3 ± 2.3 | 32.0 ± 2.0 | 32.5 ± 0.3 |
| 2 (8 Aug) | 4 | 26.0 ± 1.7 | 28.9 ± 1.7 | 28.5 ± 1.7 | 26.0 ± 3.0 | 29.5 ± 1.6 | 29.0 ± 0.5 |
| (3 Oct) | 4 | 18.6 ± 2.6 | ND | 11.4 ± 1.5 | 21.8 ± 2.6 | 25.2 ± 5.8 | 21.1 ± 6.5 |
| 3 (8 Aug) | 4 | 25.6 ± 1.5 | 28.6 ± 2.9 | 28.1 ± 2.6 | 24.8 ± 4.0 | 28.5 ± 2.3 | 27.3 ± 1.0 |
| (3 Oct) | 4 | 17.5 ± 2.8 | ND | 10.9 ± 2.0 | 21.6 ± 3.5 | 25.0 ± 5.8 | 19.3 ± 1.1 |
| 4 (8 Aug) | 4 | 23.3 ± 1.4 | 25.7 ± 3.6 | 24.2 ± 2.6 | 21.9 ± 3.9 | 24.0 ± 2.4 | 23.3 ± 1.0 |
| 5 (8 Aug) | 4 | 20.3 ± 1.5 | 23.6 ± 3.8 | 22.2 ± 2.4 | 20.1 ± 3.7 | 22.3 ± 2.3 | 21.3 ± 0.5 |

ND = not determined.

Table 5. Hypophysectomy and regression in female killifish.

| Group | (N) | Radioiodine uptake %-age | GSI[1] | Hematocrit |
|---|---|---|---|---|
| Intacts | 6 (24 Oct.) | 15.1 ± 2.0[2] | 1.2 ± 0.15[2] | 37 ± 3[2, 3] |
| 13-day post-hypophys-ectomy | 5 (7 Nov.) | 7.1 ± 1.7 | 1.3 ± 0.05 | 40 ± 1 |
| 55-day post-hypophys-ectomy | 6 (12 Dec.) | 5.9 ± 2.0[4] | 1.2 ± 0.10 | 35 ± 4 |
| 55-day post-hypophys-ectomy and pit. extr. inj.[5] | 7 (12 Dec.) | 26.0* ± 3.4 | 3.3* ± 0.28 | 27* ± 2 |

1/ Percent of gonad weight to total body weight. Fish were taken in July (postspawning).
2/ Mean ± S. E.
3/ N = 5; one hematocrit tube broke.
4/ N = 5; one fish with a bad mouth sore excluded from mean.
5/ Three injections spaced 48 hours apart.
*/ Significantly different from the controls ($p < 0.05$).

Table 6. Chronic injections of gel electrophoresis fractions into hypophysectomized male killifish.

| Fraction | N | Radioiodine uptake %-age[2] | GSI[2] | Hematocrit[2] |
|---|---|---|---|---|
| 1 | 2 | 0.26 ± 0.013 | 0.39 ± 0.05 | 35 ± 3 |
| 2 | 2 | 0.61 ± 0.017 | 2.59 ± 0.32 | 40 ± 1 |
| 3 | 3 | 0.54 ± 0.22 | 3.16 ± 0.52 | 43 ± 7 |
| 4 | 5 | 2.76 ± 0.90 | 0.47 ± 0.053 | 35 ± 2 |
| 5 | 4 | 2.95 ± 0.86 | 0.28 ± 0.037 | 39 ± 2 |
| Control | 5 | 2.16 ± 0.96 | 0.23 ± 0.032 | 32 ± 5 |

1/ N = number of fish in each column. Groups of less than five are the results of excluding fish that had bad lesions on the body. After the second injection, an Achromobacter sp. infection broke out in all tanks. Aureomycin was given to every group, but fish injected with fractions 1, 2, and 3 did not recover as well as the others. The fish included were healthy.

2/ Mean ± S. E.

3/ Flowing sperm and full nuptial coloration.

tropic activity was contained in the starting material to be detected in the reclaimable eluant; therefore, the hormone's biological activity was either altered, moved out the top of the column, or was diluted to the extent that it was ineffective.

Gonadotropin was evident in two fractions, 2 ($R_f$ range 0.52 to 0.72) and fraction 3 ($R_f$ range 0.28 to 0.52). The latter fraction was more effective in stimulating gonad growth and maturation. Unhealthy fish with body lesions were excluded from these data making statistical treatment of the data less meaningful because of the small sample size. But the gonadotropic effect was unequivocal: all of the males had higher GSIs than the controls and most came to full nuptial coloration with flowing sperm. A slightly higher hematocrit also indirectly suggested the gonad stimulation.

2. Gel filtration I fractions

Fractions I and II of the gel filtration at pH 7.0 had comparable thyrotropic potencies while fraction III had an inhibitory effect (Table 7 and Fig. 8). According to the manner in which the effluent was pooled, all of the thyrotropic activity could have resided in the first protein peak or in more than one peak, but no activity was found in the effluent range corresponding to about 50,000 MW or less. Hemoglobin, a molecule of about 68,000 MW resided in the second major protein peak that comprised the majority of fraction II. A significant point was that the thyrotropic activity came off the column in the very high molecular weight range. A monomeric form of mammalian TSH would have all resided in fraction III. Further, for there to have

Table 7. Chronic injections of gel filtration-I fractions into hypophysectomized female killifish.

| Effluent (ml) | Calibration (O. D.) | | Sample No. | | Protein[1] (mg/ml) | Wt.[6] | Uptake %-age | GSI[5] | Hematocrit |
|---|---|---|---|---|---|---|---|---|---|
| 61.5 | 0.034 | Dextran blue | 1 | | 0.14 | | | | |
| 63.5 | 0.182 | | | | | | Mean ± S. E. (N) | | |
| 65.5 | 0.230 | | 2 | | 1.47 | | | | |
| 67.5 | 0.168 | | | | | | * | * | |
| 69.5 | 0.100 | | 3 | I | 3.44 | 49.0 | 32.4 ± | 2.6 ± | 44 ± |
| 71.5 | 0.043 | | | | | | 4.9 (6) | 0.2 (5) | 2 (6) |
| 73.5 | 0.040 | | 4 | | 4.00 | | | | |
| 75.5 | 0.030 | | | | | | | 1.5 (1 male) | |
| 77.5 | 0.020 | | 5 | | 4.08 | | | | |
| 79.5 | 0.010 | | | | | | | | |
| 81.5 | 0.00 | | 6 | | 2.95 | | | | |
| 85.5 | | | 7 | | 2.47 | | | | |
| 89.5 | | | 8 | II | 2.87 | | * | * | |
| 93.5 | | | 9 | | 3.48 | 68.6 | 29.5 ± | 11.0 ± | 39 ± |
| 97.5 | | | 10 | | 3.72 | | 5.0 (6) | 1.4 (6) | 1 (6) |
| 101.5 | | | 11 | | 3.28 | | | | |
| 105.5 | | | 12 | | 2.42 | | | | |
| 109.5 | | | 13 | | 1.64 | | | | |
| 113.5 | | | 14 | | 1.28 | | | | |
| 117.5 | | | 15 | | 1.05 | | | | |
| 121.5 | | | 16 | | 0.89 | | * | * | |
| 125.5 | | | 17 | III | 0.61 | 20.2 | 0.9 ± | 11.8 ± | 43 ± |
| 129.5 | | | 18 | | 0.47 | | 0.5 (5)[2] | 2.1 (5) | 1 (6) |
| 133.5 | | | 19 | | 0.22 | | | 6.0 (1 male) | |
| 137.5 | | | 20 | | 0.21 | | | | |
| 141.5 | | | 21 | | 0.19 | | | | |
| 145.5 | | | 22 | | 0.17 | | | | |
| 151.0 | 0.004 | | | | | | | | |
| 157.5 | 0.064 | Riboflavin | | | | | * | * | |
| 163.1 | 0.228 | | Starting material | | | | 25.3 ± | 14.1 ± | 41 ± |
| 167.3 | 0.296 | | | | | | 4.5 (6) | 2.5 (5)[3] | 1 (4)[4] |
| 172.0 | 0.190 | | | | | | | | |
| 176.8 | 0.080 | | Controls | | | | 10.4 ± | 1.3 ± | 43 ± |
| 181.8 | 0.026 | | | | | | 2.9 (7) | 0.2 (7) | 3 (7) |
| 185.4 | 0.004 | | | | | | | | |

1/ Total protein by Folin-Ciocalteu with crystalline bovine albumin as a standard. 2/ One fish excluded from mean; it had a pituitary remnant. 3/ One male in group; GSI = 6.7. 4/ Two tubes broke. 5/ Fish injected with I, II, III, and S. M. were ovulating. 6/ Lyophilized wt. corrected for buffer residue. */ Significantly different from controls ($p<0.05$).

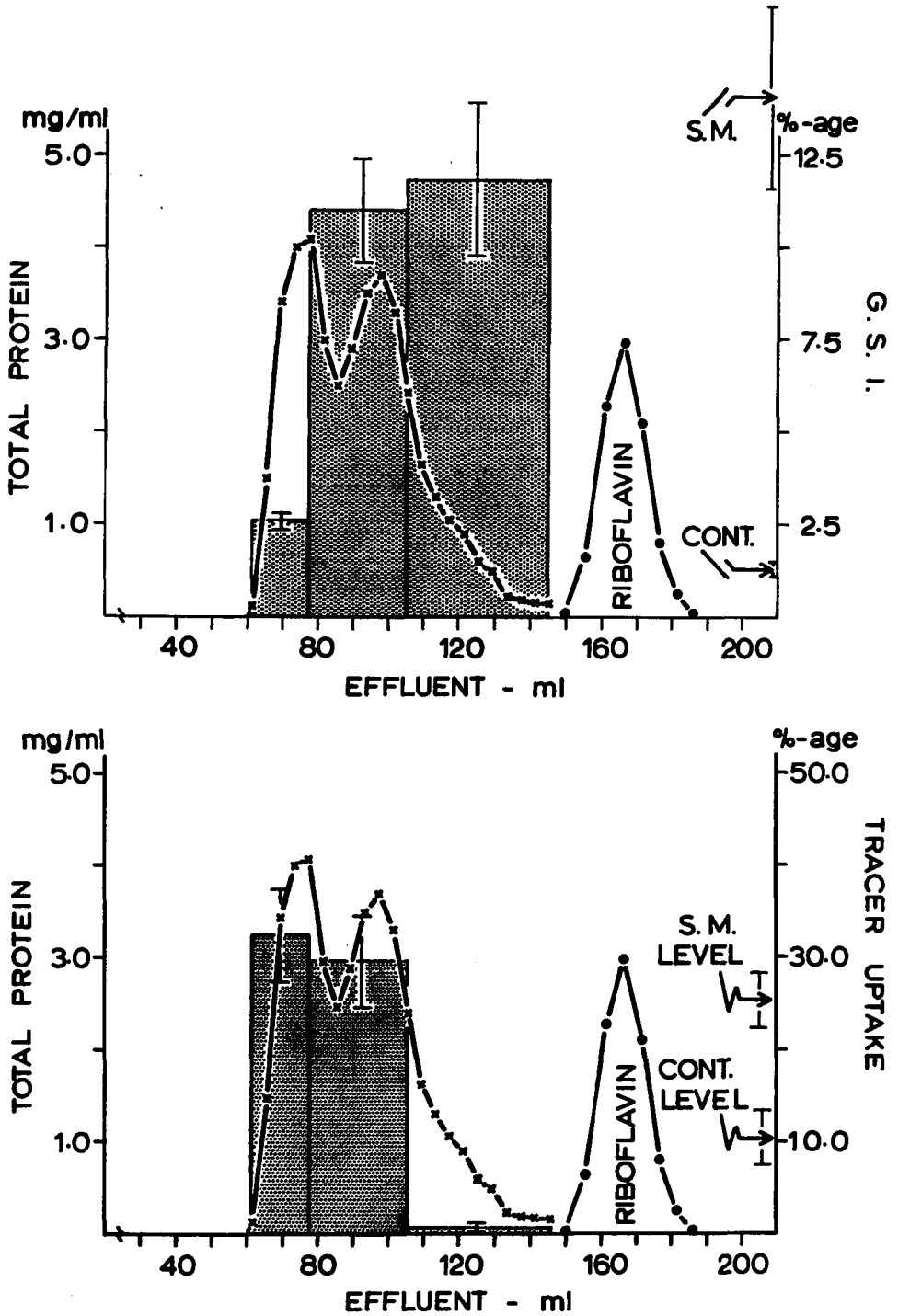


Figure 8. Sephadex G-100 gel filtration (pH 7·0, μ 0·0025). The gonadal (upper) and thyroidal (lower) response to the injected effluent is depicted with the total protein (*-*). The vertical lines represent one S. E. above and below the mean.

been a significant amount of thyrotropic activity in fraction I according to the Dextran Blue calibration, the activity would have had to have been associated with molecules of 100,000 MW or greater.

The ubiquitous gonadotropic activity at least showed a trend toward a greater concentration in the smaller molecular weight range. However, the spread it exhibited could only have occurred because of activity associated with a wide range of molecular weights. It is noteworthy that the eggs obtained from ovulating females at autopsy after fertilization by hand (sperm was obtained from the few males included in the experiment) did not develop past blastodisc formation; the eggs were atypical and only about half the size of eggs in normal spawns.

The total thyrotropic activity contained in the starting material was equal to the amount contained in fractions I and II together, assuming no activity was lost in fractionation and lyophilization, but it was not evident from the uptake data that either fraction I or II had only half or less than that contained in the starting material which was injected undiluted. Likewise, there was essentially the same gonadotropic potency in fractions II and III as in the undiluted starting material.

3. Gel filtration II fractions.

The injection data from the alkaline gel filtration fractions are summarized in Table 8 and Fig. 9. The total protein was distributed more uniformly over the protein effluent range at pH 9.0 than it was at neutrality. Further, a significant re-distribution of thyrotropic and gonadotropic principles occurred. A fraction of the thyrotropic activity

Table 8. Chronic injections of gel filtration-II fractions into hypophysectomized killifish.

| Effluent (ml) | Sample No.[3] | | Protein[5] (mg/ml) | N | Tracer uptake[1] %-age | GSI[1] | Hematocrit[1] |
|---|---|---|---|---|---|---|---|
| 52.5 | 1 | | 0.12 | | | | |
| 56.5 | 2 | | 1.06 | | | | |
| 60.5 | 3 | I[6] | 2.58 | 6 | 16.2 ± 4.0 | 0.88 ± 0.04 | 38 ± 3[2] |
| 64.5 | 4 | | 2.54 | | | | |
| 68.5 | 5 | | 2.12 | | | | |
| 72.5 | 6 | | 1.74 | | | | |
| 76.5 | 7 | | 1.62 | | | | |
| 80.5 | 8 | II | 1.28 | 6 | 13.8 ± 3.0 | 1.44 ± 0.19 | 38 ± 4 |
| 84.5 | 9 | | 1.06 | | | | |
| 88.5 | 10 | | 1.12 | | (3 with flowing sperm) | | |
| 92.5 | 11 | | 1.28 | | | | |
| 96.5 | 12 | | 1.52 | | | | |
| 100.5 | 13 | III | 1.78 | 7 | 10.4 ± 0.19 | 2.86 ± 0.25 | 42 ± 2 |
| 104.5 | 14 | | 1.84 | | | | |
| 108.5 | 15 | | 1.76 | | | | |
| 112.5 | 16 | | 1.56 | | | | |
| 116.5 | 17 | IV | 1.42 | 6 | 27.0 ± 6.6 | 7.04 ± 0.39 | 41 ± 2[4] |
| 120.5 | 18 | | 1.40 | | | | |
| 124.5 | 19 | | 1.40 | | | | |
| 128.5 | 20 | | 1.52 | | | | |
| 132.5 | 21 | | 1.72 | | | | |
| 136.5 | 22 | | 1.88 | | | | |
| 140.5 | 23 | | 1.86 | | | | |
| 144.5 | 24 | V | 1.32 | 6 | 16.6 ± 5.6 | 4.37 ± 0.24 | 36 ± 2 |
| 148.5 | 25 | | 0.50 | | | | |
| 152.5 | 26 | | 0.36 | | (1 with sperm in duct) | | |
| 156.5 | 27 | | 0.18 | | | | |
| 156.5 to 195.5=$V_i$ | | Control | | 7 | 4.4 ± 0.12 | 0.55 ± 0.07 | 37 ± 5[4] |
| $V_o$ =50.5 ml S. M. | | | | | | (not determined) | |

1/ Mean ± S. E. 2/ Rounded to the nearest integer. 3/ Calibration (comparable to G-100-I) not given. 4/ One hematocrit tube broke. 5/ Folin-Ciocalteu; crystallin bovine albumin standard. 6/ Lyophilized weights corrected for buffer residue: I-17.8 mg; II-14.7 mg; III-12.3 mg; IV-14.8 mg; V-29.1 mg. */ Significantly different from control ($p < 0.05$).

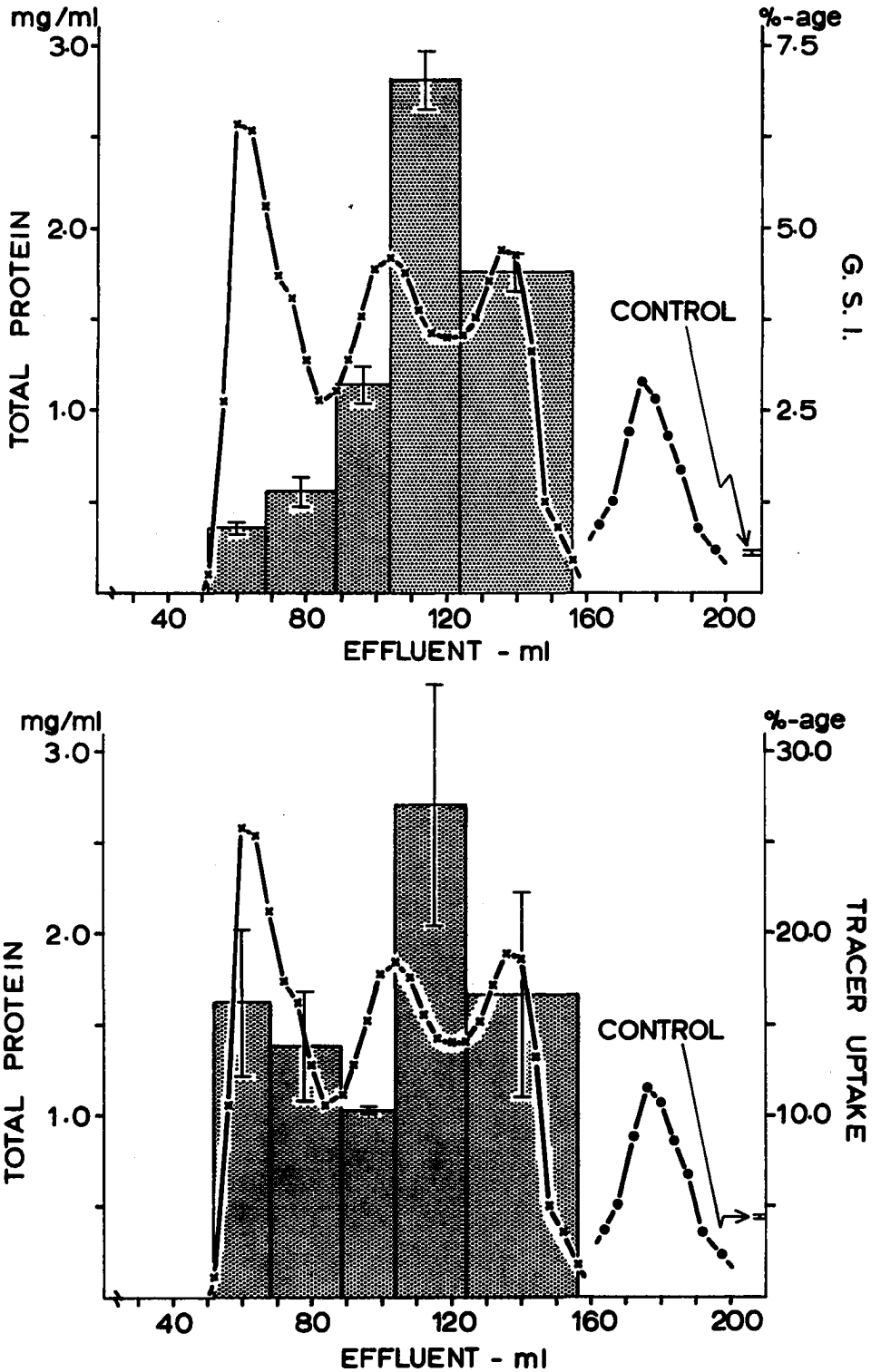


Figure 9. Sephadex G-100 gel filtration (pH 9·0, μ 0·005). The gonadal (upper) and thyroidal (lower) response to the injected effluent is depicted with the the total protein (×-×). The vertical lines represent one S. E. above and below the mean. (•-•) = riboflavin calibration peak.

was associated with molecular weights of greater than 70,000, but a greater amount of thyrotropic activity was found in fractions IV and V which corresponded to fraction III in the neutral pH run and which constituted a molecular weight range of about 50,000 to 4,000. It was impossible to evaluate how much of the lower thyrotropic potency in fraction V was attributable to its lower injection dosage, 27 μg/gm as opposed to 35 μg/gm in fraction IV, since no log-dose to response relationship was determined. The allotment for dosages of 3.5, 3.5, 3.6, 3.5, and 2.7 mg/ml in fractions I, II, III, IV, and V, respectively by lyophilized weights less buffer residue weights corresponded to 3.6, 2.7, 3.0, 3.5, and 2.4 mg/ml in fractions I, II, III, IV, and V, respectively by Folin-Ciocalteu weights.

The gonadotropic principle showed a trend toward a distribution in the lower part of the molecular weight range with a greater portion lying between the second and third total protein peaks. However significant amounts were contained in the greater than 50,000 MW range. Flowing sperm (unexpectedly) was not associated exclusively with high GSIs at autopsy. Three fish injected with fraction II had flowing sperm which was seen in only one other fish (fraction IV).

4. Mammalian thyrotropic preparations.

The data are summarized in Table 9. Within each group there was considerable individual variation, some fish were more responsive than others. However, there was close agreement between the four methods of evaluation of thyroid function: the increase in thyroid cell height was paralleled by increases in thyroid I-131 uptake and BEI-131.

Table 9. The response of hypophysectomized male killifish to the chronic administration of a heterothyrotropic factor and purified mammalian thyrotropins.

| Group | N | Thyroid cell ht. μ $\bar{X} \pm SE$ | I-131 uptake Day 2 (probe) cpm/gm wt. $\bar{X} \pm SE$ | I-131 uptake Day 4 (well) % dose $\bar{X} \pm SE$ | BEI-131 Day 4 cpm/ 10 μl serum $\bar{X} \pm SE$ |
|---|---|---|---|---|---|
| Solvent | 5 | 3.73 ± 0.26 | 67.9 ± 16.5 | 0.94 ± 0.39 | 5.8** ± 2.1 |
| HTF 1 μg/gm | 5 | 6.41* ± 0.30 | 669.1* ± 218.1 | 8.21* ± 2.98 | 299.9** ± 87.0 |
| HTF 0.1 μg/gm | 5 | 3.89 ± 0.44 | 102.0* ± 16.2 | 2.42* ± 0.59 | 7.9 ± 0.8 |
| HTF 0.01 μg/gm | 5 | 3.79 ± 0.16 | 79.1 ± 12.3 | 0.84 ± 0.24 | 7.6 ± 0.1 |
| TSH-F8-18-A 1 mU/gm | 5 | 5.12* ± 0.31 | 564.6* ± 131.3 | 9.50* ± 2.60 | 128.2* ± 41.3 |
| TSH-F8-18-A 0.05 mU/gm | 5 | 3.46 ± 0.38 | 85.8 ± 22.3 | 1.50 ± 0.58 | 6.5 ± 1.6 |
| TSH-USP 0.1 mU/gm | 4 | 5.09* ± 0.11 | 759.4* ± 322.2 | 11.90* ± 4.94 | 182.8* ± 92.5 |

* Significantly greater than the controls.

** Only 4 fish.

The heterothyrotropic factor (HTF) was effective at the highest dose (x 1 μg/gm) and the response was approximately equivalent to that elicited by TSH-USP at 0.1 mU/gm. The lowest dose of HTF had no effect, but the intermediate dose (0.1 μg/gm) showed a slight but significant thyroid stimulating activity when measured by I-131 uptake (probe or well counter); thyroid cell height was not increased at this dose and the slightly higher level of BEI-131 is not significantly greater than in the controls. Purified TSH 8-18-A was active at the higher, but not at the lower dose.

The evidence suggests that 1 μg HTF (7-51-C) is approximately equivalent to 1 mU (0.07 μg) of purified TSH (8-18-A) and to 0.1 mU (1.35 μg) of TSH-USP.

## DISCUSSION

### Part I

Iodine conversion ratio, an apparently valid index of thyroid activity in humans, may be misleading when applied to fish. Arguments that strongly favor this view are based on the ways protein bound iodine is known to be affected (Hickman, 1962; Hoar and Eales, 1963; La Roche, et. al., 1965). Serum PBI determined by TCA precipitation was shown to vary directly with iodide concentrations (Hickman, 1962) and iodide contamination probably accounts for the largest source of error in the PBI conversion ratio method.[▽] This direct relationship between iodide and PBI is suggested by the high PBI-131 levels that correspond to high total serum iodine levels immediately following injection, (Fig. 1). Presumably, all serum proteins have an overall net positive charge at the low pH of TCA solution and form a strong coulombic association with anions. Iodide is present in the protein precipitate even after five washes with TCA, (La Roche, et. al., 1964). The radiothyroxine-protein complex is probably masked considerably by iodide-protein and possibly iodinated tyrosine-protein (Berg, et. al., 1959; Leloup, and Fontaine, 1960; Hickman, 1962; Chavin, 1965[▽▽]) complexes even in the second PBI-131 peak (Fig. 1) since the 40-50 hour PBI-131 percentage determined by TCA precipitation is

---

▽ Chavin extracted serum PBI-125 with Amberlite IRA-400 and got values more nearly representative of protein bound thyroxine. Subsequent thin layer chromatography separation revealed only $T_4$.

▽▽ Chavin found no iodinated tyrosines in sexually immature goldfish.

still many times higher than the PBI-131 percentage determined by gel filtration. It is likely that iodide would associate only weakly (Ingbar, et al., 1956) or not at all with serum proteins near blood pH because of their overall net negative charge. This relationship, well documented in humans, does not seem unlikely in fish since fish have the same general serum protein types. Gel filtration would have the effect of complete serum protein dialysis and would completely separate iodide from protein when they were weakly associated. On the other hand, the thyroxine-protein complex, having a high equilibrium constant at blood pH (Ingbar, 1960), should be only slightly separated by gel filtration. PBI-131 values obtained by this method may be lowered some, what but should not be inaccurate because of iodide contamination. Covalent bonds formed between iodine and protein would result in higher PBI-131 values.

In view of the effect of iodide concentration on PBI-131 determined by TCA precipitation, PBI values obviously could reflect the iodide clearance rates from the blood. In this case, fish with more stimulated thyroids would have correspondingly lower PBI-131 values. However, the difference between PBI-131 values of treated fish and their controls is not all attributable to correspondingly different iodide concentrations since a direct relationship did not exist throughout the foregoing experiments, (cf. PBI-131 and corresponding total serum radioactivity after 20 hours, Figure one; 24-hour PBI-131 values in Fig. 5 with corresponding total serum radioiodine counts). The direct relationship is not apparent after the first 20-25 hours after iodide injection.

Although admittedly not well understood at present, factors other than the above that may influence PBI conversion ratios are abnormal total serum protein concentrations, unsaturated lipids and lipoprotein concentrations, hemolysis, renal and extrarenal iodide excretion rates (Leloup and Fontaine, 1960), iodide fixation rates by tissues other than the thyroid (Leloup and Fontaine, 1960), and the recycling rates of metabolized thyroxine iodine, (Chavin, 1965).

In light of the foregoing discussion, it becomes more understandable why a truly direct relationship between treatment dosage and protein bound radiothyroxine is masked in an inverse relationship between dosage and the overall PBI-131. The overall PBI-131 percentages, the conversion ratio, indirectly indicated thyroid activity, although not as conclusively as did the butanol extractable radioiodine ratios since butanol extraction, a completely reliable method, is specific for thyroxine and triiodothyronine.

Although the amount was not assessed, stable isotope injection along with the radioactive nuclide had the effect of lowering the PBI-131 and BEI-131 percentages. A more direct line of evidence for this is presented in Part II from the results obtained on killifish. Carrier iodide apparently diluted the radioactive nuclide, lowering the radioiodide uptake percentage by the thyroid. Also, injecting varying amounts of radioiodine (0.05 - 0.2 μc/gm) appeared to cause no appreciable difference in the results of protein bound or butanol extractable iodine ratios. Only absolute amounts of measured radioactivity were affected as was expected.

Some collateral effects of injecting the non-purified pituitary extract were evident. The gravid females ovulated as was expected indicating the presence of gonadotropins in the extract. The only indirect endocrine pathway that has been shown to cause ovulation in fish is through LH stimulation of the interenals (Sundararaj and Goswami, 1966). Ovulation occurred at the expected time interval after injection (Clemens and Grant, 1964). It was not apparent what indirect effects ovulation or carrier I-127 may have had on the injected fishes' thyroid activity due to an endogenous release of TSH. An inhibitory effect of I-127 on the endogenous release of TSH should not be apparent in an acute response to exogenous TSH. This kind of feedback mechanism is believed to require a relatively longer time than the acute response to TSH injections. However, an abrupt deflection in the general rise in PBI-131 and the BEI-131 just after ovulation was evident, (Fig. 3).

It is known that ACTH, which should have been present in the extract, suppresses thyroid function, (Chavin, 1954; 1956a) in goldfish. However, ACTH's effects are more immediate and not as long lasting as those of TSH. Teolosts respond to ACTH treatment and return to normal in an extremely short time interval, a few hours in some instances, (See, TABLES 23 and 24; Pickford and Atz, 1957). This relationship is suggested by the data shown in Fig. 6. The fish responded to extract treatment, but their response originated at a lower level than the controls. This was apparently caused by the immediate effects of ACTH, a depression of thyroid activity.

# DISCUSSION

## Part II

The ultimate aim of this study was to utilize the thyrotropic response for assaying pituitary fractions from isolation precedures. Many of the collateral problems (iodine metabolism, non-thyroid biological phenomena, and problems with physical devices) that presented themselves, while basically challenging and informative, were resolved-where immediate solutions were possible-only to insure soundness of the methods and to prevent ambiguity in interpreting the results. Precedents in TSH bioassay methodology that have been workable in other species were either not applicable or not yet confirmed for the fish used in the present study. Consequently, a critique of the preliminary experimentation seems worthwhile.

Although there should be no sacrifice of real accuracy for expediency in a bioassay, the restrictions imposed by "reliable and routine," out of necessity result in a compromise. Prompted by this assumption, relatively exhaustive trials were carried out to establish a bioassay using tracer iodine uptake by intact animals having had acute hormone injections. The relatively laborious and lengthy methods of thyroid histology and hypophysectomy were not pursued initially as a compromise for "routineness" as well as for other reasons.

In all the intact fish tested, only those in one experiment showed any evidence of stimulation. This stimulation was undoubtedly only possible because the hormone recipients had relatively low endogenous thyrotropin levels. It is possible that intact fish could be used successfully

as assay animals if chronic injections were given since thyroid follicle proliferation may be a longer term response to thyrotropin than the acute effects on iodide trapping by existing follicles. However, a stimulation in intacts would still have to be interpreted with reservations: one would not know with certainty how much of the stimulation was affected by endogenous TSH which may be elevated in response to the injected materials.

Single injections of thyrotropin over a wide dosage range were ineffective (data not included in this report) in three test groups of recipients, starved intacts, fed intacts, and hypophysectomized killifish. The starved group had exceedingly low uptakes and neither of the other two groups showed any trend in tracer uptake or radiothyroxine production that correlated to log-dose. Hypophysectomized killifish showed stimulation after only three pituitary extract injections on alternate days even after 55 days of regression. Fish in complete regression (atrophy) would almost certainly take more injections and a longer time for regeneration unless extremely potent doses were given.

There are arguments in favor of not limiting assessments of thyroid activity in intact fish to any one of the established methods alone. For example, Pickford and Robertson (unpublished) found (unexpectedly) thyroid cell heights in long-term salt water-adapted killifish no different from freshwater-adapted controls. Also, Pickford and Slicher (unpublished) again found no difference in thyroid cell heights; but, thyroid uptakes and clearance ratios at the same time indicated (expectedly) higher thyroid activities in the salt water environment. Yet, data

in the present study show freshwater fish to have higher ($0.1 > p < 0.05$) uptakes but lower serum iodide levels-introducing another variable of great consequence. Other investigations, (Swift, 1960; Fontaine, Leloup, and Olivereau, 1952) suggest thyroid cell heights in intact fish are not always correlated to thyroid activity; but, in the regressed, hypophysectomized killifish treated with chronic hormonal injections in this study, they gave estimates of thyroid function comparable to serum radiothyroxine production and thyroid iodine uptakes.

With the information from these pilot experiments it was possible to formulate a basic scheme for measuring thyroid activity in definitive experiments and to understand more about what constituted an "assay animal." Several problems, however, are still unsolved and likely caused loss of sensitivity. The large variations within groups are difficult to explain and presently unavoidable. In spite of the precautions taken, there were always variations in the physiological state of randomly sampled individuals. Much of the variability could probably be overcome if one could select animals from a uniform, inbred strain. The population used for the present series of experiments showed considerable genetic variability. Individuals grossly "out of line" in their group were checked for pituitary remnants. In some cases a pituitary remnant can be obvious and can be detected by a quick examination at autopsy. However, small clusters of only a few thyrotropic cells have been shown to remain in some cases of hypophysectomy and to prevent follicular atrophy (Pickford, personal communication). Only sectioning and microscopical examination after autopsy can re-

veal this.

The pilot studies established that fish brought in from their natural habitat at different times of the year respond differently as assay organisms. Criteria used to assess thyroid activity in killifish and other species show rhythms attuned to their annual cycle (Berg, Gorbman, and Kobayashi, 1959; Swift, 1960; Fontaine, Leloup, and Olivereau, 1952; Matty, 1960). Moreover, some individuals are out of phase and large variations in the state of thyroid development are encountered. These variations appear to persist throughout regression following hypophysectomy.

Presumably, there is an optimal number and an optimal physiological state for "functional follicles" in assay fish. The optimal state probably lies between the extremes of complete post-hypophysectomial atrophy and the "natural" active state.

Gel electrophoresis, in theory at least, should be a highly effective method for obtaining a degree of purity in TSH unattainable by other protein isolation methods. Unfortunately, not enough is known about the chemical and physical properties of carp TSH to employ the correct gel electrophoretic system for its separation. The standard system employed in the present study utilized a "stacking" pH of 6.75 and a "running" pH of 9.5 which had nothing to recommend it. On the contrary, if piscine TSH has an isoionic range comparable to mammalian TSH, a cationic system would yield better results. Also, a preliminary purification step would seem necessary since the relatively large amounts of total protein necessary for charging a run with sufficient thyrotropic activity introduced the problem

of overloading and caused clogging.

Presently, it is impossible to predict whether the TSH molecule migrated as an anion or cation in the standard system employed since its isoionic point was not known. Also, in gel filtration at neutral pH it appeared to be associated with other unlike or like molecules. Biologically active proteins are known to form coulombic associations quite readily (Pierce and Carsten, 1963). It seems likely that carp TSH like mammalian TSH has a relatively high isoionic point and at a pH 7.0 or less it would have an overall net positive charge associating to a high degree with negatively charged species, especially the highly negatively charged phosphate groups of nucleic acids and nucleotides. This would account for its occurrence in the 100,000 MW effluent range in Sephadex G-100 gel filtration at neutral pH. Only further experimentation will solve these problems.

Although the gel electrophoretic system employed was ineffective for TSH, it appeared quite promising as a method for gonadotropin(s). The slices that contained a substance causing significant gonadal stimulation, a factor that promoted gonad growth and maturation, was centered closer to the origin ($R_f$ range 0.28 to 0.52) than the gonadal hydration factor ($R_f$ range 0.50 to 0.67) in carp pituitaries (Clemens and Grant, unpublished).

This hypothesis needs to be examined further with more critical slicing and more injection groups over the implicated $R_f$ range to take advantage of the high resolution properties of the isolation method.

It seems likely that two gonadotropic factors are implicated in these and other studies: one factor causing

ovulation or spermiation (hydration response) acting over a short term (LH?) and one factor stimulating gametogenesis with subsequent gonad weight increase and sex steroid production acting over a relatively long term (FSH, ICSH?). At present the problem of piscine FSH has not been resolved.

Gel filtration I

One of the most difficult problems in isolating mammalian gonadotropin and thyrotropin has been the cross contamination of one with the other. It was unexpected that gel filtration, which is not normally thought of as a "high resolution" method, should show a definite trend toward separating these two principles. Both runs in the present study showed this trend. Although there was not significant quantitative separation, the trend theoretically implies that a complete separation is possible.

It was not possible to determine what percentage of the charge of thyrotropin was reclaimed in the effluent nor how great a purification was achieved since the activity was not assayed quantitatively and since both fractions that contained significant potencies responded maximally relative to the starting material. In fact, the assay hints at higher potencies in both fractions than in the undiluted starting material. Likewise, fraction III contained an inhibitory substance (ACTH?) that would probably have affected the response to the starting material but was separated from fractions I and II.

The presence of two gonadotropic factors was suggested in the pituitary fractions obtained by gel filtration. Ovulation occurred despite the unripened condition of the ovaries (immature ova). This lack of coordination was obvi-

ous and suggested the presence of more than one factor acting independently on the gonads. The "normal" sequence of maturation and ovulation could have been disrupted by injecting a mixture of two factors separately responsible for each respective function.

Gel filtration II

The significant redistribution of thyrotropic activity from neutral pH to alkaline pH implies that a change occurred in the molecular dimensions associated with the activity. This change followed a logical pattern. It was predicted that a pH above the isoionic point of TSH would cause dissociations that would allow at least some of the activity to redistribute in the lower molecular weight range.

The largest portion of the thyrotropic and gonadotropic principles was not centered under any one total protein peak. If these two factors were represented in one peak that was divided, partially in fraction IV and partially in fraction V, their contribution to the total protein was slight which indicates that most of the total protein associated with these factors was "inert" in respect to these functions.

Since most of the thyrotropic principle emerged with the gonadotropin principle in the alkaline gel filtration, a preliminary run at one pH and a second run at the other should enhance their separation. One should be able to prepare these two factors with "practical" grades of purity using the two gel filtration pH systems in combination.

Mammalian thyrotropic preparations

The results reported above indicate that TSH-USP and the derived preparations act directly on the thyroid. HTF, a derived thyrotropic factor, had powerful thyroid stimu-

lating properties in the starved trout assay (M. Fontaine and Y. A. Fontaine, 1956) but little or no action on mammals. Although the trout assays were done on fish in a state of hypopituitarism and physiological hypothyroidism, they were intact and the possibility of indirect stimulation through the action of a thyrotropic releasing factor could not be excluded.

The response to the reference TSH-USP was of the expected order (Pickford, 1954). On the other hand, hypophysectomized killifish were relatively less responsive to the derived bovine TSH and HTF (about 10 times lower) than trout. In this respect the killifish's response to HTF more nearly resembled the low response of the mouse. It seems likely, therefore, that in the HTF assay involving starved trout other indirect endocrine pathways are brought into play. Fontaine has suggested that HTF has FSH-like properties (Fontaine and Burzawa-Gerard, 1967).

# BIBLIOGRAPHY


Bailey, L. 1962. Techniques in Protein Chemistry Elsevier, Amsterdam, pp. 310.

Bakke, J. L. 1965. The assay of human thyrotropin by 21 different assay laboratories in 9 different countries using 14 different methods. In, "Current Topics in Thyroid Research" (Cassano, ed.), pp. 503-512. Academic Press, New York.

Bates, R. W. and P. G. Condliffe. 1960. Studies on the chemistry and bioassay of thyrotropins from bovine pituitaries, transplantable pituitary tumors of mice, and blood plasma. In, "Recent Progress in Hormone Research" (Pincus, ed.) 16:309-352.

Bates, R. W. and P. G. Condliffe. 1966. The physiology and chemistry of thyroid stimulating hormones. In "The Pituitary Gland" (Harris and Donovan, eds.), pp. 347-410, Butterworths, London.

Berg, O. A., A. Gorbman, and H. Kobayashi. 1959. The thyroid hormones in invertebrates and lower vertebrates. In "Comparative Endocrinology" (Gorbman, ed.), pp. 302-319. Wiley, New York.

Cassano, C. and M. Andreoli (Editors). 1966. Current Topics in Thyroid Research. pp. 1-1219. Academic Press, New York.

Chaney, A. L. 1958. Protein-bound iodine. In "Advances in Clinical Chemistry" (Sobotka and Steward, eds.). 1:81-109. Academic Press, New York.

Chavin, W. 1956a. Thyroid distribution and function in the goldfish, Carassius auratus L., Ph. D. dissertation, New York University.

Chavin, W. 1956b. Thyroid follicles in the head kidney of the goldfish, Carassius auratus L., Zoologica. 41:101-104.

Chavin, W. 1965. Metabolism of iodine and thyroid hormone synthesis in the goldfish, Carassius auratus L., Gen. comp. Endoc. 5:493-503.

Clemens, H. P. and F. B. Grant. 1964. Gonadal hydration of carp (Cyprinus carpio) and goldfish (Carassius auratus) after injections of pituitary extracts. Zoologica 49(3):193-210.

Clemens, H. P. and F. B. Grant. 1966. Purification and characterization of the gonadal hydration principle of carp (Cyprinus carpio) pituitary by gel filtration and acrylamide electrophoresis, (In manuscript).

Davis, B. J. 1964. Disc electrophoresis-II. In "Gel Electrophoresis" (Fredrick, ed.), Ann. N. Y. Acad. Sci. 121(2):404-427.

Dent, J. N. and J. M. Dodd. 1961. Some effects of mammalian thyroid stimulating hormone, elasmobranch pituitary gland extracts, and temperature on thyroidal activity in newly hatched dogfish (Scylliorhinus caniculus). J. Endocrinol. 22(4):395-402.

Dodd, J. M., K. M. Ferguson, M. H. I. Dodd, and R. B. Hunter. 1963. The comparative biology of thyrotropin secretion. In "Thyrotropin" (Werner, ed.), pp. 1-28, Thomas, Springfield.

Eales, J. G. 1963. A comparative study of thyroid function in migrant juvenile salmon. Can. J. Zool. 41:811-824.

Fontaine, M, J. Leloup, and M. Oliveriau. 1952. La Fonction Thyroïdienne du jeûne saumon, Salmo salar L. (parr et smolt) et son intervention possible dans la migration d'avalaison. Arch. Sci. physiol. 6:83-104.

Fontaine, M. and Y. A. Fontaine. 1956. Détermination du pouvoir thyréotrope de l'hypophyse et du milieu intérieur de téléostéens

par mesure de la fixation de 131-I par la thyroïde de la truite arc-en-ciel (Salmo gairdneri, Rich.), J. Physiologie 48:881-892.

Fontaine, M. and J. Leloup. 1957. Sur l'existence de différences specifiques de permeabilite au radioiode des hematies de divers poissons. J. Physiol. (Paris) 49:164-169.

Fontaine, M. and J. Leloup. 1958. Sur l'existence d'une "liaison" de l'ion iodure avec certaines protéines du plasma de saumon adulte (Salmo salar, L.). Compt. Rend. Acad. Sci. (Paris) 247: 767-770.

Fontaine, Y. A. and N. Le Belle. 1965. The "heterothyrotropic factor" (HTF) from mammalian pituitaries and its eventual significance for thyroid physiology. In "Current Topics in Thyroid Research" (Cassano, ed.). pp. 425-432. Academic Press, New York.

Fontaine, Y. A. and Burzawa-Gerard. 1967. Activité heterothyréotrope et hormones gonadotropes des hypophyses de mammifères. Compt. Rend. Acad. Sci. (In press).

Fromm, P. O. and E. P. Reineke. 1956. Some aspects of thyroid physiology in rainbow trout. J. cell. comp. Physiol. 48:393-404.

Geschwind, I. 1959. Species variation in protein and polypeptide hormones. In "Comparative Endocrinology" (Gorbman, ed.), pp. 421-443. Wiley, New York.

Gorbman, A. and O. A. Berg. 1955. Thyroid function in the fishes Fundulus heteroclitus, F. majalis, and F. diaphanus. Endocrinology 56:86-92.

Hickman, C. P. 1961. The conversion ratio as a discriminatory test for thyroid activity in fish. Nature 189:1012-1013.

Hickman, C. P. 1959. The osmoregulatory role of the thyroid gland in the starry flounder, Platichthys stellatus. Can. J. Zool. 37:997-1060.

Hickman, C. P. 1962. Influence of environment on the metabolism of iodine in fish. Gen. comp. Endoc., Supplement 1, pp. 48-62.

Hoar, W. S. and J. G. Eales. 1963. Dependence of conversion ratio on the dose of radioiodine in fish. Can. J. Zool. 41:1061-1067.

Hunn, J. B. and E. P. Reineke. 1964. Influence of iodine intake on iodine distribution in trout, Proc. Soc. Exp. Biol. Med. 115(1): 91-93.

Ingbar, S. H. 1956. Concentration gradients for radioiodide in unblocked thyroid glands of rats: effect of perchlorate. Endocrinology 58:95.

Ingbar, S. H. 1960. The interaction of the thyroid hormones with the proteins of human plasma. In "Modern Concepts of Thyroid Physiology" (Whitelock, ed.), Ann. N. Y. Acad. Sci. 86:440-453.

La Roche, G., D. Carpenter, and A. Coxworth. 1964. Isolation and estimation of serum organically bound iodine (PBI) or nonanionic iodine (NAI). Univ. Calif. Rad. Lab. 11387, 152-164.

La Roche, G., C. L. Johnson, and A. N. Woodall. 1965. Thyroid function in the rainbow trout (Salmo gairdneri, Rich.) I. Biochemical and Histological evidence of radiothyroidectomy. Gen comp. Endoc. 5: 145-159.

Leloup, J. 1958. Sur la repartition des hormones thyroïdiennes (thyroxine et 3:5:3' triiodothyronine) entre les hematies et le plasma du sang de quelques poissons (Dipneuste et Téléostéens).

J. Physiol. (Paris) 30:368.

Leloup, J. and M. Fontaine. 1960. Iodine metabolism in lower vertebrates. In "Modern Concepts of Thyroid Physiology" (Whitelock, ed.), Ann. N. Y. Acad. Sci. 86:316-353.

Man, E. B. 1962. Differences in serum butanol-extractable iodines (BEIs) of children, men, and women, (note on the preservation of serum BEIs with thiouracil). J. Lab. and Clin. Med. 59:528-532.

Matty, A. J. 1960. Thyroid cycles in fish. In "Cyclical Activity in Endocrine Systems" (Barrington, ed.), No. 2 pp. 1-15. Symposia Zool. Soc. London.

Mougey, E. H. and J. W. Mason. 1962. Measurement of butanol-extractable iodide in the rhesus monkey. J. Lab. Clin. Med. 59:672-680.

Mussett, M. V. and W. L. M. Perry. 1955. The international standard for thyrotropin. Bull. Wld. Hlth. Org. 13:917-929.

Olivereau, M. 1954. Hypophyse et glande thyroide chez les poissons. Étude histophysiologique de quelques corrélations endocriniennes en particulier chez Salmo salar L. Ann. Inst. oceanogr. Monaco, 29:95-296.

Olivereau, M. 1955. Température et fonctionnement thyroidien chez les poissons. J. Physiol. 47:256-258.

Ornstein, L. 1964. Disc electrophoresis-I. In "Gel Electrophoresis" (Fredrick, ed.), Ann. N. Y. Acad. Sci. 121(2):321-249.

Pickford, G. E. 1954. The response of hypophysectomized male killifish to prolonged treatment with small doses of thyrotropin. Endocrinology 55:589-592.

Pickford, G. E. and J. W. Atz. 1957. "The Physiology of Pituitary

Gland of Fishes." New York Zool. Soc., New York. pp. 613.

Pierce, J. G., M. E. Carsten, and L. K. Wynston. 1960. Purification and chemistry of the thyroid stimulating hormone. In "Modern Concepts of Thyroid Physiology" (Whitelock, ed.), Ann. N. Y. Acad. Sci. 86(2):612-624.

Pierce, J. G. and M. E. Carsten. 1963. Preparation of highly purified thyrotropin. In "Thyrotropin" (Werner, ed.), pp. 216-231 Thomas, Springfield.

Pitt-Rivers, R. and J. R. Tata. 1959. "The Thyroid Hormones" Pergamon, New York. pp. 247.

Riggs, D. S. 1952. Quantitative aspects of iodine metabolism in man. pharm. Rev. 4:248.

Rogina, B. and M. Dubravcic. 1953. Microdetermination of iodides by arresting the catalytic reduction of ceric ions. Analyst 78:594.

Slicher, A. M. 1961. Endocrinological and hematological studies in Fundulus heteroclitus (Linn.), Bull. Bingham. oceanogr. Collect.17:1-55.

Sundararaj, B. I. and S. V. Goswami. 1966. Effect of metapiron (SU-4885) on luteinizing hormone and corticosteroid-induced ovulation and spawning in hypophysectomized catfish, Heteropneustes fossilis (Bloch) J. exp. Zool. 163(1):49-54.

Swift, D. R. 1955. Seasonal variations in the growth rate, thyroid gland activity and food reserves of brown trout (Salmo trutta, Linn.). J. exp. Biol. 32(4):751-764.

Swift, D. R. 1959. Seasonal variations in the activity of the thyroid gland of yearling brown trout (Salmo trutta Linn.). J. exp. Biol.

36:120-125.

Swift, C. R. 1960. Cyclical activity of the thyroid gland of fish in relation to environmental changes. In "Cyclical Activity in Endocrine Systems" (Barrington, ed.), No. 2 pp. 17-27. Symposia Zool. Soc. London.

Taurog, A. and Chaikoff, I. L. 1948. The nature of the circulating thyroid hormone. J. biol. Chem. 176:639-656.

Werner, S. C. (Editor). 1963. "Thyrotropin," pp. 1-392. Thomas, Springfield.

Whitelock, O. V. St. (Editor). 1960. "Modern Concepts of Thyroid Physiology," Ann. N. Y. Acad. Sci., 86:(2):311-676.