

STUDIES ON THE CHEMISTRY AND PHYSIOLOGY OF SALMON GROWTH  
HORMONE

by

Graham Forsyth Wagner  
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Graham Forsyth Wagner 1984  
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Approval

Name: Graham Forsyth Wagner  
Degree: Doctor of Philosophy  
Title of Thesis: Studies on the Chemistry and Physiology of Salmon Growth Hormones

Examining Committee:

Dr. G.H. Geen, Chairman

Dr. B.A. McKeown, Senior Supervisor

Dr. P.C. Oloffs

Dr. M.J. Smith

Dr. K.K. Nair, Public Examiner

Dr. L.M. Srivastava, Public Examiner

Dr. E.M. Donaldson, External Examiner

Date approved

Aug 23/84

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**Studies on the Chemistry and Physiology of Salmon Growth Hormones**

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Author:           

(signature)

Graham F. Wagner

(name)

September 10, 1984

(date)

## ABSTRACT

The first stage of the thesis involved the purification of salmon growth hormone (GH), using a protocol of gel filtration, affinity and ion-exchange chromatography. In this manner, GH was isolated to homogeneity from both chum (Oncorhynchus keta) and coho (O. kisutch) salmon. Chum and coho GH have similar molecular weights of 23,500 and 22,500 daltons respectively as estimated by sodium dodecylsulphate electrophoresis. On the basis of amino acid composition, the salmon GHs have all of the salient characteristics of a vertebrate growth hormone. When tested in the conventional mammalian GH bioassay, the rat tibia test, chum GH had significant bioactivity whereas coho GH did not. When tested in rainbow trout (Salmo gairdneri), both chum and coho GH significantly elevated the rate of growth in two separate experiments. Chum and coho GH were also compared immunologically using an antiserum raised in rabbits against chum GH. Both GHs were found to be immunologically identical. The antiserum also specifically stained the GH cells of four species of pacific salmon.

The second stage of the thesis involved the development of a radioimmunoassay (RIA) for salmon GH. Using the antiserum prepared for the immunological studies, a standard curve was constructed for measuring plasma GH in the range of 2.5-160 ng/ml. This RIA was found to be valid for plasma GH measurements in the genera Salmo and Oncorhynchus. The RIA was then used to monitor plasma GH levels in rainbow trout under a variety of

experimental conditions. Handling stress had no effect upon plasma GH levels in trout. Long-term starvation resulted in plasma GH levels that were approximately nine times higher than those in a control group maintained on a constant feeding regime.

Plasma GH levels in rainbow trout were observed to change on a weekly basis in a manner similar to the change in growth rate, but with a lag phase of one week. The correlation coefficient calculated for these two parameters was not significant. However, when the plasma GH data were phase-shifted forward by one week, the correlation coefficient then calculated was statistically significant. It appears therefore, that a rise in plasma GH precedes the actual onset of an accelerated growth phase and may be a determining factor of the cyclical pattern of growth.

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I would like to dedicate this thesis to my son, Lyle Forsyth Wagner.

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## A. Introduction

Somatotropin, or growth hormone (GH) is a polypeptide that is synthesized, stored and released by a distinct cell type in the vertebrate pituitary gland. The hormone consists of a single chain of 191 amino acids, interconnected by two disulphide bridges and depending upon the species, has a molecular weight between 20-23,000 daltons (Farmer and Papkoff, 1979). The name of the hormone is derived from its specific role in controlling somatic growth and development from infancy until adulthood through a variety of mechanisms that include increased DNA, RNA and protein synthesis in bone, muscle, skin, connective tissue and the visceral organs. Growth hormone also exerts positive effects on the retention of bone salts and plasma electrolytes and stimulates fat metabolism in order to conserve carbohydrate and protein reserves (Frantz, 1976). While the actions of growth hormone are extremely diverse and continue throughout the lifetime of the individual organism, its precise mechanism of action in promoting these cellular processes is poorly understood (Ahren et al., 1976).

The earliest evidence for a growth promoting substance in the pituitary gland is that of Evans and Long (1921) who reported accelerated body growth in rats injected with bovine pituitary extract. In a series of experiments employing hypophysectomized tadpoles (Rana boylei), it was subsequently

demonstrated that this growth-promoting activity was confined to the anterior portion of the pituitary gland (Smith and Smith, 1922, 1923). However two decades followed before the first purification of growth hormone from bovine pituitary glands was reported by Evans and Li (1944). Since that time, GHs have been isolated from a wide range of mammalian species, culminating with the complete sequencing of human GH by Li et al., in 1969.

Concurrent with much of the early work on mammalian GHs, Regnier (1938) observed that hypophysectomy of the swordtail (Xiphophorus helleri) also resulted in a complete cessation in body growth. This observation was confirmed later in the killifish (Fundulus heteroclitus) by Pickford (1953a) who also demonstrated that administration of ovine GH caused a resumption of growth (Pickford, 1953b). The first isolations of GH from teleost fish were conducted by Wilhelmi (1955) using pollock (Pollachius virens) and hake (Urophycis tenuis) pituitary glands. Although the methods for assessment of purity employed by Wilhelmi may be considered crude by today's standards, he determined by paper electrophoresis that hake GH contained fewer contaminants than pollock GH. Both GH preparations were found to have molecular weights (22-26,000 daltons) similar to the mammalian GHs and each caused a resumption in body growth of hypophysectomized killifish (Pickford, 1954). In contrast, neither preparation had demonstrable bioactivity in the hypophysectomized rat weight gain test or in the rat tibia test (Wilhelmi, 1955), the standard bioassay for mammalian GHs

(Greenspan et al., 1949). These latter observations in the rat were interpreted by Wilhelmi (1955) as being due to the phylogenetic uniqueness or specificity of the teleost GH molecule.

More recent investigations on two teleost species have yielded GHs which have been well characterized and bioassayed; tilapia (Sartherodon mossambica) GH which was isolated by Farmer et al., (1976) and carp (Cyprinus carpio) GH isolated by Cook et al., (1983). Structurally, both GHs are single chain polypeptides with molecular weights of 21,200 and 22,500 daltons respectively. In terms of amino acid composition, each satisfies the criteria which have been established by one author for the vertebrate GH molecule; a low histidine and methionine content, a high content of leucine and glutamic acid, a single tryptophan residue and two disulphide bridges (Wilhelmi, 1974). Both GHs possessed growth hormone activity (increased body length and weight) in homologous (tilapia GH) and near homologous (carp GH) fish species (Clarke et al., 1979; Cook et al., 1983). In addition to the teleosts, GH has also been isolated from the sturgeon (Acipenser guldensstadtii (Farmer et al., 1980)) and the blue shark (Prionace glauca (Lewis et al., 1972)).

The first attempt to demonstrate the presence of GH in the salmon pituitary gland was conducted by Jampolski and Hoar in 1954. Despite the high dosages employed, they were unable to stimulate weight gain in the rat or goldfish (Carrasius auratus) with an alkaline extract prepared from chinook salmon

(Oncorhynchus tshawytscha) pituitaries. Pituitary extracts from the coho salmon (O. kisutch) have proven to be equally as ineffective in the rat tibia test (Hayashida and Lagios, 1968). More recent investigations however, have provided better evidence for the presence of a salmon GH. An immunoreactive GH-like substance was first detected in the serum and pituitary gland of the sockeye salmon (O. nerka) by radioimmunoassay and by immunocytochemistry, using an antiserum to ovine GH (McKeown and van Overbeeke, 1971, 1972). It has subsequently been shown that salmon pituitary extracts (chinook) are indeed capable of promoting fish growth (Higgs et al., 1978) and that the chum salmon (O. keta) pituitary contains a specific fraction which has somatotropic activity when injected into hypophysectomized rainbow trout (Salmo gairdneri) (Komourdjian and Idler, 1979). However, despite all of the efforts that have been made to purify teleost GHs, the physiology of these hormone preparations, other than increasing body growth, have been virtually unexplored. In fact, the development of the first teleost GH radioimmunoassay (carp) has only just been published (Cook et al., 1983) despite the fact that purified teleost GH has been available for nine years.

The purpose of this investigation was therefore to isolate, characterize and bioassay the GH molecule from two species of Pacific salmon and to develop a radioimmunoassay for physiological studies in salmon and other closely related species. In this way, some insights will hopefully be gained



regarding the role of this hormone in the growth and development of the salmonidae.

## B. Materials and Methods

### Purification of Salmon Growth Hormones

Pituitary glands from both chum (Oncorhynchus keta) and coho (O. kisutch) salmon were collected between September and November with the cooperation of British Columbia Packers, Steveston, B.C. The purification protocol used was similar to that of Idler et al., (1978) with modifications.

The first modification involved the use of a single G-75 column for the first gel filtration step. Idler et al., (1978) used two columns connected in series in the original procedure. A second modification involved the choice of pH and buffer salt for the ionic exchange procedure. Whereas the original protocol employed ammonium bicarbonate adjusted to pH 9.0, my procedure employed Tris-acetate, adjusted to pH 8.1. The final modification was the inclusion of a desalting step following the ion exchange procedure. This was necessitated as the Tris-acetate used in the ion exchange is non-volatile.

Routinely, 35 g of frozen pituitaries were homogenized in 70 ml of ice-cold 0.003 M Tris-acetate buffer, containing either 0.001 M phenylmethylsulphonylfluoride or 1000 kallikrein inhibitor units/ml of Trasylol (Boehringer-Mannheim) as protease inhibitors. The homogenate was adjusted to pH 8.5 with 1 N NaOH

and stirred for 3 hours at 4°C. The homogenate was centrifuged at 50,000 x g for 30 minutes and the supernatant was decanted and saved. A second homogenization was done on the pellet followed by centrifugation and decantation as before.

The supernatants were combined (150 ml approximately) and applied to a 10 x 92 cm, Sephadex G-75 column equilibrated with homogenization buffer. The column was calibrated with molecular weight standards (45,000 and 25,000 daltons) and the void volume was determined with blue dextran (Pharmacia).

The 18,500-31,000 molecular weight fractions were saved (1500 ml) and passed through a column of Concanavalin-A-Sepharose (ConA) to remove the glycoproteins, principally gonadotropic and thyrotropic hormones. The sample was adjusted to the pH and ionic strength of the ConA column before application (0.001 M MnCl<sub>2</sub>, 0.001 M CaCl<sub>2</sub>, 0.001 M MgCl<sub>2</sub>, 0.5 M NaCl, 0.04 M Tris HCl, pH 7.7). The material eluting from the column was saved and the column was flushed with 200 ml of ConA buffer. The first 100 ml of the flush were saved and added to the eluate, resulting in a final volume of approximately 1600 ml.

To concentrate the protein, the eluate was adjusted to 80% saturation with ammonium sulphate, stirred overnight at 4°C and centrifuged at 50,000 x g for one hour. The supernatant was discarded and the pellet was dialyzed for four hours in 0.003 M Tris-acetate, pH 8.1. Prior to dialysis, the tubing was boiled for one hour in 0.5 M EDTA and rinsed several times with

distilled water. This procedure served to denature contaminating proteases and chelate any divalent cations which may have been still present in the tubing.

The dialyzate was then centrifuged at 50,000 x g for thirty minutes and the supernatant (30 ml) was applied to a calibrated, 5 x 92 cm, Sephadex G-75 column, equilibrated with 0.003 M Tris-acetate, pH 8.1.

The 18,500-31,000 molecular weight fractions were saved (150 ml) and applied directly to a 1.6 x 50 cm column of DEAE Bio-gel (Bio-Rad) equilibrated with the same buffer. The column was flushed with several bed volumes of buffer before applying a gradient of 300 ml 0.003 M and 300 ml 0.06 M Tris-acetate, pH 8.1. All fractions (5.1 ml) were analyzed by absorption spectrophotometry (280 nm) to locate the proteins which had eluted from the column.

Conductivity measurements were also done on each fraction using a meter (Radiometer) calibrated with a wide range (0-0.1 M) of Tris-acetate standards, in order to correlate the elution profile of the column with the gradient. The protein peaks identified by spectrophotometry were analyzed by polyacrylamide disc gel electrophoresis at pH 8.3 (Ornstein, 1964) to identify those fractions which contained the presumptive growth hormone (GH). Under these electrophoretic conditions, most tetrapod GHs (Nicoll and Licht, 1971) and all fish GHs (Farmer et al., 1976, 1980; Cook et al., 1983) have a characteristic pattern, consisting of three closely spaced bands, that is easily

recognized.

The presumptive GHs from both chum and coho salmon eluted in the same manner from the ionic exchange column, as two distinct peaks between 0.025 and 0.045 M Tris-acetate. These peaks were designated Peak I (PI) and Peak II (PII) according to their order of elution from the column. Under the above specified electrophoretic conditions, PI consisted of a single band with an Rf of 0.35, while PII consisted of two bands with Rf's of 0.41 and 0.45. Invariably, some degree of cross-contamination occurred between the two peaks and they were consequently pooled, lyophilized and desalted on a 3 x 85 cm Sephadex G-100 column, equilibrated with 0.015 M ammonium acetate, pH 8.1. This final gel filtration also removed contaminating proteins of lower molecular weight which eluted at the trailing edge of PII.

In one instance however, chum salmon PI and PII were distinctly separated with no apparent cross-contamination, as verified by electrophoresis. In this case, the peaks were individually lyophilized and desalted as described earlier. Following the desalting step, the fractions containing GH were pooled and lyophilized, yielding a fluffy, white powder.

The final electrophoretic characteristics of both chum and coho salmon GH (PI and PII combined) were determined in disc gels at pH 8.3 (Ornstein, 1964) and in acid-urea gels at pH 4.5 (Davis et al., 1972). Their respective molecular weights were estimated by sodium dodecylsulphate (SDS) electrophoresis

(Laemmli, 1970).

In order to compare chum PI and PII immunologically, antibodies were raised against PII in New Zealand white rabbits. Animals were injected intradermally (Vaitukaitus et al., 1972) at approximately thirty sites with 25 ug of PII, once every month for a period of four months. The chum PII used for the immunization consisted of two bands, with Rf's of 0.41 and 0.45, under alkaline (pH 8.3) electrophoresis. They were bled from an ear vein ten days after each injection. The hormone was emulsified in Freund's Complete Adjuvant for the first injection and Incomplete Adjuvant (Difco) thereafter. The development of antibody titer was monitored by immunocytochemistry and by binding studies with iodinated chum GH (see later materials and methods). Chum PI and PII were then compared for immunological relatedness, with antisera from the fourth bleeding of rabbit #2 (R2-4), by Ouchterlony's (1968) double gel diffusion technique.

Table I

Procedural Steps for the Isolation of Salmon Growth Hormone

1. Homogenize pituitaries, centrifuge the homogenate and save the supernatant.
2. Gel filter the supernatant on Sephadex G-75. Save the 18,500-31,000 dalton eluent.
3. Remove glycoproteins by passing the eluent through a column of Concanavalin-A Sepharose.
4. Concentrate the Concanavalin-A eluent with 80% ammonium sulphate, centrifuge and then dialyze the precipitate.
5. Gel filter dialyzate on Sephadex G-75 and save the 18,500-31,000 dalton eluent.
6. Apply eluent to a column of DEAE Bio-gel equilibrated with 0.003 M Tris acetate, pH 8.1.
7. Elute growth hormone with a 0.003-0.060 M gradient of Tris acetate, pH 8.1. Lyophilize fractions between 0.025-0.045 M Tris acetate.
8. Desalt growth hormone on a column of Sephadex G-100.
9. Lyophilize fractions containing growth hormone.

## Bioassay of Salmon Growth Hormones in Rainbow Trout

In the first trout bioassay, chum salmon PI and PII were tested (double-blind) in intact, juvenile rainbow trout, Salmo gairdneri ( $5.34 \pm 1.3$  g,  $x \pm S.D.$ ,  $n=40$ ), in a manner similar to Komourdjian and Idler (1979), to demonstrate their bioactivity and comparative growth-promoting abilities. The fish were obtained from the Sun Valley Trout Farm, Mission, B.C. Four groups of ten fish were acclimated in separate 20 L glass aquaria, supplied with flow-through dechlorinated tap water ( $12^{\circ}\text{C}$ ) and maintained on a 12D:12L photoperiod. Following a two week acclimation period, the fish were injected intraperitoneally, twice weekly with either chum PI, PII or ovine GH (1 I.U./mg, N.I.H.-GH-S10) at a dosage of 1  $\mu\text{g/g}$  body weight (BWT) for 24 days. The fourth group, serving as a control, received equivalent dosages of bovine serum albumin (BSA). The fish were weighed prior to each injection and the dosages were adjusted accordingly. Oregon moist pellets (Moore-Clark) were provided to satiation twice daily.

As the mean initial weights of the controls and the group receiving chum PII were significantly different from one another ( $P < 0.005$ , Student's t-test), the total weight gains of each group were compared instead using the Student's T-test. The fish were not individually tagged in this experiment. Therefore, the standard deviations derived from the mean initial and final



weights for each group were combined, to obtain a pooled standard deviation (Zar, 1974) according to the formula;

$$\text{Pooled S.D.} = \sqrt{\frac{(\text{S.D. Initial})^2(n-1) + (\text{S.D. Final})^2(n-1)}{2n-2}}$$

Groups were considered to be significantly different if  $P < 0.05$ . Growth was also compared by calculating regression lines for the cumulative percent weight gains of each group and alternately, the cumulative absolute weight gains of each group over the course of the experiment. The slopes in each case were then compared by one-way analysis of variance and the Student-Newman-Keuls multiple range test. Slopes were considered to be significantly different if  $p < 0.05$ .

Chum and coho salmon GH (PI and PII combined) were then compared for growth-promoting ability in trout, in two further double-blind experiments (experiments two and three). Rainbow trout were obtained as before and acclimated to the experimental conditions for at least two weeks prior to each study. Eight groups of fish ( $n=10-11$ ) were confined in separate 30 L fibreglass tanks supplied with dechlorinated tap water and maintained on the natural photoperiods of July (Expt. 3) and August-September (Expt. 2). The water temperature fluctuated between  $10-12^{\circ}\text{C}$  in both experiments. The fish were fed Moore-Clark fry feed three times daily at a ration level of 15%/BWT/week. In this case, each fish was individually marked prior to the beginning of these two experiments by fin clipping. Six of the groups were then injected intraperitoneally with

0.25, 0.5 or 1.0 ug/g BWT of chum or coho GH, bi-weekly for a total of three weeks, while two groups served as the injected (vehicle only) and non-injected controls. The hormone preparations were dissolved in 0.15 M NaCl adjusted to pH 8.5 with 1 N NaOH. These preparations were also adjusted with respect to their concentrations such that each fish received the equivalent of 10 ul of vehicle/g BWT (including the injected controls). The fish in these experiments were re-measured at the end of each week (as opposed to twice weekly in experiment one) and the hormone dosages and rations were then readjusted accordingly.

In experiment two, the fish ( $6.57 \pm 0.39$  g,  $\bar{x} \pm S.D.$ ) were re-measured with respect to weight after each week and the length was re-determined at the end of the experiment. Instantaneous linear growth rates (Brown, 1945) were then calculated on this basis for length and weight. At the end of the experiment, the body water content was determined for each fish by drying the carcasses to a constant weight at 110°C.

In experiment three, the fish ( $4.14 \pm 0.31$  g) were re-measured with respect to both weight and length after each week and growth rates were calculated in the same manner. The formula for calculating instantaneous linear growth rate was  $100(\ln WT - \ln Wt)/(T-t)$ , where WT and Wt are the body weights at times t (initial) and T (final) respectively, resulting in a growth rate calculation on a %/day basis. For the calculation of growth rate on the basis of length, the same formula was used.

Alternately, the slopes of the cumulative percent weight gains (not length) of each group over three weeks were determined for both experiments two and three.

Following experiment three, the growth rates (weight only) were monitored weekly in all fish, now untreated, for a period of four months on a natural photoperiod (Sept-Dec) and the same ration level.

The instantaneous linear growth data from experiments two and three were analyzed by one-way analysis of variance (GH treatment) and Duncan's multiple range test, as well as by two-way analysis of variance (GH treatment, week) following transformation of the data (  $GROWTH + 0.5$  ). The data from the four month growth study following experiment three were analyzed by one-way Analysis of Variance to estimate the degree of interaction between growth rate and week. The slopes of the cumulative percent weight gains of the various groups over three weeks in experiments two and three were compared by analysis of variance and Duncan's multiple range test. Groups were considered to be significantly different if  $p < 0.05$ .

#### Bioassay of Salmon Growth Hormones in the Rat Tibia Test

Chum and coho salmon GH were each compared in the conventional mammalian GH bioassay, the rat tibia test (Greenspan et al., 1949). Male, Sprague-Dawley rats, which had been hypophysectomized at 28 days of age, were obtained from

Charles River Ltd., Mass., U.S.A. The rats were used ten days post-operatively.

In one experiment, four groups of five rats received daily intraperitoneal injections of either; 70 ug chum GH, 70 ug ovine GH (1 I.U./mg, N.I.H.-GH-S10), the equivalent of 5 mg of a sockeye (*O. nerka*) salmon pituitary extract or 0.9% NaCl.

The sockeye extract was prepared by homogenizing 0.5 g of pituitaries in 10 ml of ice-cold 0.9% NaCl. The homogenate was then adjusted to pH 8.5 with 1 N NaOH, stirred for 3 hr at 4°C and centrifuged at 20,000 x g for 30 minutes. Rats were injected daily with 100 ul of the supernatant, representing the extract from 5 mg of pituitary glands. As the GH content in fresh pituitaries comprised 5% of the wet weight of the gland by radioimmunoassay, this 5 mg of extract would then contain approximately 250 ug of growth hormone.

In a second experiment, four groups of rats received daily intraperitoneal injections of; 40 or 80 ug of coho GH, 80 ug of ovine GH or 0.9% NaCl.

The hormones were dissolved in 0.9% NaCl adjusted to pH 8.5 with 1 N NaOH. In both experiments, the rats were injected for four days and sacrificed the following day.

Both tibiae were removed from each rat, sagittally sectioned with a razor blade and preserved in 10% formalin. The tibiae were then rinsed in distilled water for 30 minutes, soaked in acetone for 1 hr, rinsed again in distilled water for 30 minutes and soaked in 2% silver nitrate for 3 minutes. Following a brief

rinse in distilled water, the tibiae were exposed to an incandescent light until the calcified portions turned dark brown. The tibiae were then rinsed in 10% sodium thiosulphate, followed by distilled water and stored in 80% ethanol. Following this staining procedure, all areas of the tibial epiphysis are dark brown except for the cartilage plate, which remains white. The width of the cartilage plate was determined using a Zeiss photomicroscope, calibrated with an eyepiece micrometer. Ten measurements were recorded for each cartilage plate and the widths obtained for the left and right tibiae were averaged. Groups were compared by a Student's T-test and considered to be significantly different if  $p < 0.05$ .

#### Amino Acid Analysis of Salmon Growth Hormones

For the amino acid analysis of coho GH, 200 ug of material were hydrolyzed in 200 ul of 6M HCl for 22 hours under nitrogen at 110°C. Three separate analyses were conducted on 10-20 ug aliquots of this single hydrolyzate using a Dionex Amino Acid/Peptide Analyzer, measuring at 570 nm, except for proline (440 nm).

For chum GH, three separate hydrolyses were done on 50 ug aliquots of the same preparation, for 22 hours at 110°C. Five analyses were conducted on these hydrolyzates. Following each individual analysis, the computed ratios of each amino acid were converted to absolute values on the basis of 191 residues/

molecule, the chain length of human GH. The values obtained from each analysis were then averaged to obtain the final amino acid composition for the two salmon GHs. No correction was made for hydrolytic destruction.

Immunocytochemical and Immunodiffusion Studies with an Antiserum to Chum Salmon Growth Hormone

To test the specificity of the antiserum raised against chum salmon GH for the GH cells in the salmon pituitary, immunocytochemical studies were done. The development of antisera has been described earlier (Purification of Salmon GHs).

Pituitaries from yearling coho salmon were obtained from Capilano Hatchery, North Vancouver, B.C. Sockeye salmon pituitaries were collected from upstream migrants of the "Chilko race". Chum and pink salmon (*O. gorbuscha*) pituitaries were collected from upstream migrants of the "Chilliwack race". Tissues were fixed in either Bouin's or Bouin-Hollande solution, dehydrated, embedded in paraplast (56°C) and sectioned at 5  $\mu$ m. Growth hormone cells were localized using the peroxidase-antiperoxidase staining technique (Sternberger, 1974). Prior to staining, all sections were dewaxed in toluene, hydrated and equilibrated in diluent buffer (0.05 M Tris-HCl, pH 7.6, containing 0.15 M NaCl). All sections were then pre-treated with undiluted normal goat serum for 10 min. at room temperature

to reduce nonspecific background staining. Rabbit anti-chum GH serum (1/500 in diluent buffer) was then applied and incubated overnight at 4°C. Goat anti-rabbit gamma globulin was then applied (1/10 dil. for 10 min. at 22°C) followed by rabbit peroxidase anti-peroxidase serum (Miles Yeda Ltd.) at a 1/50 dil. for 10 min. at 22°C. After each antiserum application, the slides were washed for at least 15 min. in diluent buffer or 0.15 M saline. The sites of peroxidase activity were then visualized by transferring the slides to 0.01% hydrogen peroxide containing 0.025% (W/V) 3,3-diaminobenzidine (Graham and Karnovsky, 1966). The slides were then washed thoroughly in distilled water, dehydrated and mounted.

Several control procedures were carried out to determine the specificity of the reaction. These included the application of non-immune rabbit serum (NRS) as the primary antiserum and pre-saturation of the antiserum with chum GH. In the case of coho, adjacent sections were stained with hematoxylin and eosin to confirm that the immunoreactive cells corresponded to the acidophils of the caudal pars distalis.

Pituitary homogenates of all species (including chinook, O. tshawytscha, in this case) were tested for cross-reactivity with the antiserum using the Ouchterlony (1968) double gel diffusion technique. Pituitaries (0.5 g) were homogenized in 1 ml of ice-cold 0.2 M Tris-HCl (pH 8.3) and then centrifuged at 1000 x g for 15 minutes. The supernatants were then tested against neat antiserum for cross-reactivity and were also compared with one

another and with purified chum GH for immunological similarities.

#### Development of a Radioimmunoassay for Salmon Growth Hormone

The antiserum used for the development of the radioimmunoassay (RIA) was generated as described earlier. The development of antiserum titer was monitored by its ability to bind with  $^{125}\text{I}$ -chum salmon GH. For the iodination of salmon GH (Thorell and Johansson, 1971), one mCi  $\text{Na}^{125}\text{I}$  (2 ul), 10 ug of lactoperoxidase (80 units/mg, Sigma) in 10 ul of 0.5 M phosphate buffer, pH 7.6 and 10 ul of 0.003% hydrogen peroxide were added to 10 ug of chum GH in 30 ul of 0.5 M phosphate buffer, pH 7.6. The reaction was carried out in a 12 x 75 mm borosilicate test tube sealed with parafilm. Following 5 minutes of constant agitation, the incorporation of  $^{125}\text{I}$  into the GH molecule was determined by precipitating an aliquot of the reaction mixture in a test tube with 10% trichloroacetic acid. After centrifugation, the radioactivity in the tube was determined using a Beckman DP-5000 gamma counter. The supernatant was then decanted and the tube was re-counted. The activity in the pellet was expressed as a percentage of the total activity in the tube to obtain the percent incorporation. Further aliquots of hydrogen peroxide were added until 50-60% incorporation was achieved, resulting in a label with a specific activity of 50-60 uCi/ug of GH. Any further additions of hydrogen peroxide after



this level of incorporation had been achieved resulted in no further increase in the specific activity.

The iodination mixture was then diluted with 100  $\mu$ l of 10% sucrose and applied to a 1 x 7 cm, Sephadex G-50 column, equilibrated with 0.015 M ammonium acetate, pH 8.1, containing 0.15 M NaCl and eluted with the same buffer. Ten drop fractions of the eluate were collected in borosilicate test tubes containing 1 ml of 0.05 M phosphate-buffered saline with 1% BSA, pH 7.6. Twenty microliter aliquots of each fraction were counted to obtain the column profiles of  $^{125}\text{I}$ -GH and unreacted  $\text{Na}^{125}\text{I}$ . The five peak tubes of  $^{125}\text{I}$ -GH were made into aliquots ( $5 \times 10^6$  cpm) and stored at  $-20^\circ\text{C}$ .

Before use, the label was always repurified on a 2 x 40 cm, Sephadex G-100 column, equilibrated with 0.015 M ammonium acetate-buffered saline (pH 8.1), containing 0.1% Thimerosal (Sigma) as a preservative. Approximately sixty 1.2 ml fractions were collected and the column profile was determined by radioactivity as before, using 100  $\mu$ l aliquots instead. This repurification separated monomeric  $^{125}\text{I}$ -GH from contaminants of higher molecular weight and dissociated  $^{125}\text{I}$ . If this procedure was not followed, the non-specific binding of the label to the borosilicate assay tubes was increased. The labelled GH remained useful for up to a week after the second gel filtration.

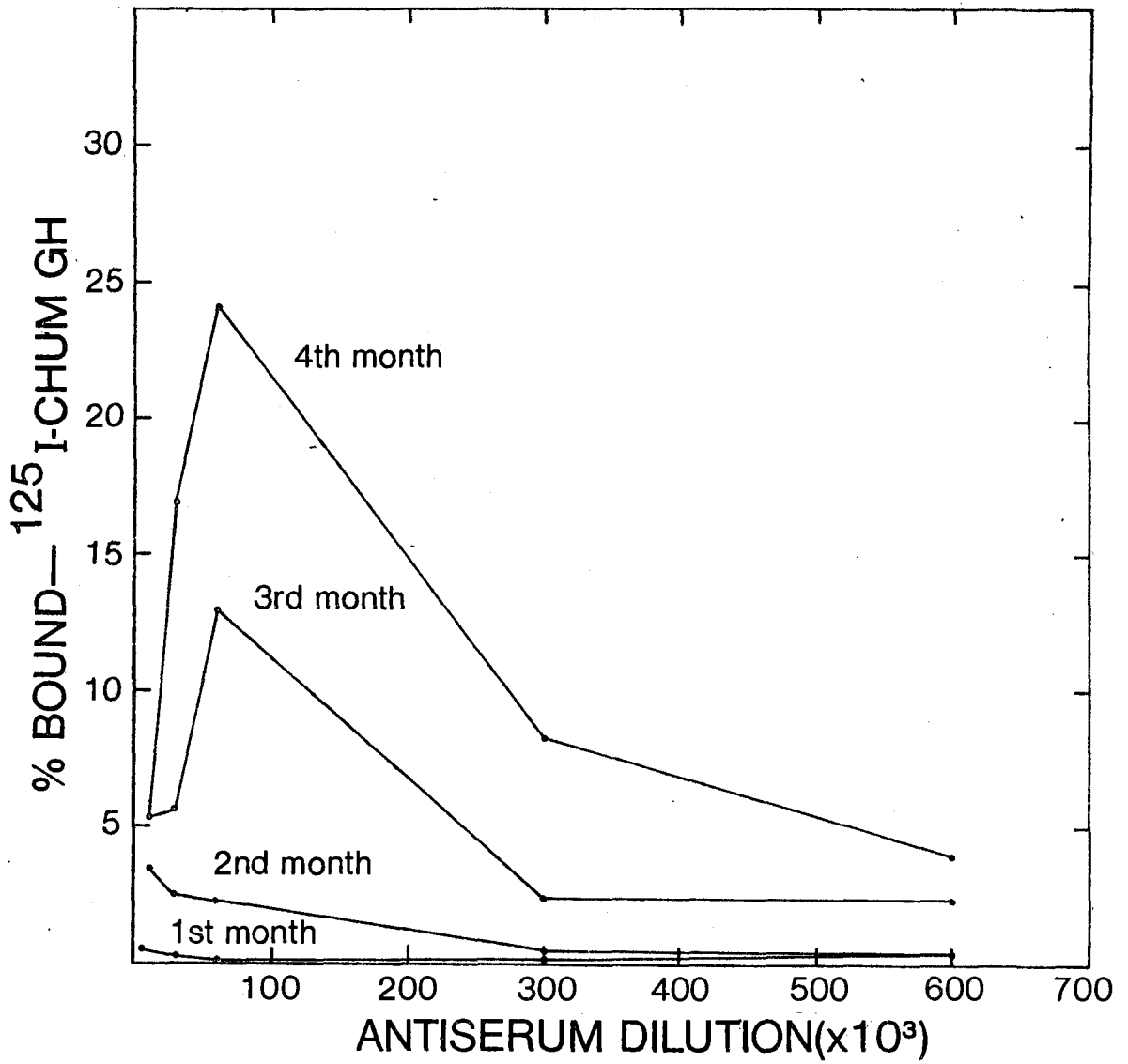
For the binding studies, a wide range of dilutions (1:1000-100,000) was prepared with the antisera from the first through the fourth bleedings of rabbit #2. The diluent buffer

used for all assay components was 0.01 M phosphate-buffered saline, pH 7.6, containing 1% BSA and 0.1% Thimerosal. One hundred microliters of the various antibody dilutions were added to 12 x 75 mm borosilicate test tubes, followed by 100 ul of 2% normal rabbit serum (NRS), 200 ul of diluent buffer and 100 ul of labelled GH (6,000 cpm). The tubes were mixed and incubated for three days at 4°C, although maximum binding of the labelled hormone is essentially complete after two days. To separate the antibody-bound labelled GH (bound) from the unbound labelled GH (free), 0.5 units of goat anti-rabbit gamma globulin (GARGG-Calbiochem) in 100 ul of diluent buffer were added to all tubes. The tubes were mixed and incubated for 24 hours at 4°C, to ensure that the immunoprecipitation between the GARGG, the NRS and the GH antiserum was complete. Prior to centrifugation for 30 minutes at 1000 x g, 2 ml of diluent buffer were added to all tubes. By increasing the supernatant volume in this manner, the non-specific binding of free label to the borosilicate tubes was reduced considerably upon decantation. The test tubes were drained for 15 minutes after decantation and blotted before counting. The non-specific binding (NSB) tubes contained 100 ul of diluent buffer in place of the antiserum. The percentage of label bound to the antibody was calculated by the following formula;

$$\% \text{ bound} = (\text{CPM in precipitate} - \text{NSB} / \text{total CPM added} - \text{NSB}) \times 100$$

All antiserum dilutions were tested in triplicate. The fourth

Figure 1. The development of antibody titer in rabbit #2 following monthly injections of 25 ug of chum salmon growth hormone. The rabbit was bled ten days following each injection and the serum titer was then determined by its ability to bind  $^{125}\text{I}$ -chum salmon growth hormone. The highest titer was obtained after four monthly injections. The serum obtained from the fourth bleeding was subsequently used for the development of the radioimmunoassay.



bleeding of rabbit #2 (R2-4) had the highest titer (Fig. 1).

A second series of dilutions, in narrower increments, was then made with this antiserum and the binding studies were repeated. At a range of dilutions between 1:1000-300,000, the binding of labelled GH varied from 70-9% respectively. Standard curves were then constructed with antiserum dilutions that bound between 15 and 30% labelled GH and compared for sensitivity and slope of inhibition. A 1:60,000 dilution of R2-4, which bound 23% labelled GH in the absence of unlabelled GH, was finally chosen for the RIA.

To construct a standard curve, 100 ul of R2-4 (1:60,000) were added to assay tubes, followed by 100 ul of 2% NRS, 100 ul of the respective chum GH standards (1.25-160 ng/ml) and 100 ul of diluent buffer. The zero binding tubes received 100 ul of diluent buffer instead of a GH standard while the NSB tubes were prepared as before. The volume of all tubes, prior to the addition of the label, was 400 ul.

For the measurement of plasma GH levels in salmonids, 10-50 ul of heparinized plasma were added to assay tubes, followed by 100 ul of antiserum, 100 ul of 2% NRS and sufficient diluent buffer (190-150 ul) to achieve a volume of 400 ul. All tubes were mixed and incubated for 24 hours at 4°C.

One hundred microliters of labelled GH (6,000 cpm) were then added to all tubes, followed by a further 48 hour incubation at 4°C. The bound and free label were then separated as described earlier. The percentage of label bound at each

standard GH concentration (zero-160 ng/ml) and for unknowns was calculated as before. The values of % bound were then converted to % Bound / Zero Bound (%B/Bo) by the following formula;

$$\%B/Bo = (\% \text{ Bound} / \% \text{ Zero Bound}) \times 100.$$

The resulting %B/Bo values for the standards were then graphed on a semi-log plot against the standard GH concentrations (1.25-160 ng/ml). The GH concentrations in the plasma unknowns were extrapolated from the standard curve using their calculated values of %B/Bo. Each standard curve was set up in triplicate.

To determine the inherent variability within a single assay, ten replicate tubes containing 100 ul of known quantities of GH (5 and 20 ng/ml) were measured in a single assay and the coefficient of variation (CV) was calculated for each concentration. The inherent variability between assays was also determined. Pooled, heparinized plasma from rainbow trout was divided (50 ul) among a large number of assay tubes and stored at -20°C. Three tubes were then included in each assay and the CV was determined on the basis of four separate RIA's.

To determine the specificity of the RIA for plasma GH from the genera Salmo and Oncorhynchus, parallelism studies were conducted. Pooled plasma from rainbow trout and coho salmon was serially diluted in assay buffer to obtain a wide range of final plasma concentrations; 1.5-100% in the case of trout and 3.1-50% in the case of coho salmon. One hundred microliters of these dilutions were assayed in triplicate with a normal standard curve. All %B/Bo values were then converted to logits

[ $\log(B/B_0/1-B/B_0)$ ] and the data was graphed on a log-logit plot. The slopes were calculated for the standard curve and the plasma dilutions and compared statistically by a Student's T-test (Zar, 1974). As the transformations resulted in negative logits for some values of y, one logit unit was added to all y values prior to the calculation of slopes. The slopes were considered to be significantly different if  $p < 0.05$ .

A parallelism study was also conducted with a coho pituitary extract. Coho pituitaries (0.5 g) were homogenized in 10 ml of ice-cold 0.01 M phosphate-buffered saline, pH 7.6, and the homogenate was centrifuged at 2000 x g for 30 minutes. The resulting supernatant was serially diluted (1:32,000-512,000) and 100 ul aliquots were assayed in triplicate with a standard curve and the coho plasma dilutions as described above.

Recovery studies, in which known quantities of chum GH were added to trout plasma, were also conducted. Trout plasma volumes of 10, 20 and 30 ul were spiked with 100 ul of a 2.5 ng/ml solution of GH and measured in a single assay (in triplicate) with tubes containing plasma alone (10, 20 and 30 ul, not spiked).

Plasma measurements were also done with hypophysectomized killifish (Fundulus heteroclitus) to verify that the only source of immunoreactivity in fish plasma was from the pituitary gland. Killifish were obtained from John Cameron, a commercial dealer in New York City. For the hypophysectomy, a small incision was made in the roof of the mouth overlying the pituitary gland of

anaesthetized fish, using a fine scalpel. The underlying musculature and connective tissue were teased away with forceps to expose the parasphenoid bone.

The pituitary gland was then exposed with a dentist drill and removed with careful suction using a pasteur pipette. Upon recovery from anaesthesia, the fish were transferred to 1/3 seawater (10°C) and maintained on a fixed photoperiod (12L:12D). Plasma volumes of 10-40 ul were then assayed in triplicate along with plasma from intact killifish.

Plasma from coho salmon was fractionated on a Sephadex G-50 column to determine if the plasma immunoreactivity was of the same molecular weight as pituitary GH. A 1.6 x 58 cm column, equilibrated with 0.015 M ammonium acetate, was first calibrated with  $^{125}\text{I}$ -GH, to determine the elution volume of pituitary GH. Approximately  $1 \times 10^6$  cpm of  $^{125}\text{I}$ -GH in 1 ml of column buffer, containing 5% sucrose, was applied to the column and eluted with a constant pressure head. Approximately sixty 3.3 ml fractions were collected and the column profile was determined by monitoring each fraction in a gamma counter. One milliliter of heparinized coho plasma, containing 5% sucrose, was then applied to the column and eluted with column buffer with the same pressure head. Sixty 3.3 ml fractions were collected and 200 ul aliquots of each fraction were assayed in triplicate by RIA.



## The Effects of Handling Stress on Plasma Growth Hormone Levels in Rainbow Trout

Rainbow trout (Sun Valley Trout Farm, Mission) were subdivided into four groups (N=10-12) and acclimated in separate 30 L fibreglass aquaria for four weeks prior to the experiment. The fish were maintained on a natural photoperiod (Sept.) in flow-through dechlorinated tap water (10°C) and fed Moore-Clark fry feed once daily to satiation.

On the morning of the experiment (9:00 A.M.), two of the groups were netted and held out of the water for thirty seconds to induce handling stress. The fish were then returned to their respective tanks and subsequently sampled, one and seven hours following the induction of stress. A control group, which had not been disturbed, was also sampled at each time period. The fish were netted individually, the caudal peduncle was severed and blood was collected in heparinized capillary tubes. The blood was centrifuged, the hematocrits were recorded and the plasma was stored at 4°C. The sampling was alternated between the control and experimental groups to equalize any disturbance to the fish. The following day, plasma GH levels were measured in all groups, using 20 ul aliquots of plasma in triplicate, by RIA. The data were analyzed by a Student's T-test and groups were considered to be significantly different if  $p < 0.05$ .

## The Effects of Long-Term Starvation on Plasma Growth Hormone Levels in Rainbow Trout

Two groups (N=10-12) of rainbow trout were acclimated in separate 120 L fibreglass aquaria for a period of two weeks, in flow-through dechlorinated tap water (10°C) and a natural photoperiod (Sept.). The fish were fed Moore-Clark fry feed to satiation once daily. Following the acclimation period, food was withheld from one group for three weeks. Blood samples were then taken from all fish, alternating between tanks, in the manner described previously. Plasma GH levels were measured in triplicate by RIA, using 20 ul aliquots of plasma, the same day. The data were analyzed by a Student's T-test and groups were considered to be significantly different if  $p < 0.05$ .

## Weekly Changes in Plasma Growth Hormone Levels in Rainbow Trout

Two hundred and forty rainbow trout ( $2.82 \pm 0.29$  g,  $x \pm$  S.D.) were equally subdivided into sixteen 30 L fibreglass tanks, supplied with flow-through dechlorinated tap water (10-12°C) and were maintained on a natural photoperiod (June-August). The fish were fed Moore-Clark fry feed three times daily at a ration level of 18%/BWT/week. Each fish was tagged by fin clipping to permit identification. Following an acclimation period of two weeks, the fish were weighed on a weekly basis for the duration of the experiment (13 weeks). The

instantaneous linear growth rate of each fish was calculated on a weekly basis and all the growth rates were expressed as a population mean  $\pm$  standard error of the mean (S.E.M.). Following six weeks of weekly growth measurements, a blood sample was taken from one fish in each tank (N=16), on a weekly basis for a period of seven weeks. Blood sampling was conducted on the weighing day at 9:00 A.M., prior to the first feeding. The fish were not fed on the day prior to sampling and blood collection and separation were conducted as before. The plasma was stored at  $-20^{\circ}\text{C}$  until the end of the experiment. Plasma GH levels were determined in triplicate by RIA, using 20  $\mu\text{l}$  aliquots, for each of the seven weeks. The data were analyzed by one-way analysis of variance and the Student Newman Kuels multiple range test. Groups were considered to be significantly different if  $p < 0.05$ . Correlation equations and coefficients were calculated for the mean growth rates and plasma GH levels from weeks seven through thirteen, "in phase", (N=7) and by phase-shifting the plasma GH levels forward by one week, "out of phase", (N=6). The correlations were considered to be significant (two-tailed) if  $p < 0.05$ .

## C. Results

### Purification of Salmon Growth Hormones

The final yield of the growth hormone (GH) from coho salmon pituitaries (7.5 mg/ 35 g) was greater than from chum salmon pituitaries (1-5 mg/ 35 g). The yield from chum pituitaries was also extremely variable, probably due to the poor condition of the glands upon arrival at the cannery.

Of the two peaks eluting from DEAE Bio-gel (Fig. 2) which comprise the presumptive salmon GH, Peak I (PI) was the more prevalent. The amount of Peak II (PII) which was obtained in the final yield varied between purifications and in one instance (coho), the fastest migrating band in alkaline gels ( $R_f=0.45$ ) was entirely absent (Fig. 4,A). Both chum (Fig. 3) and coho GH each eluted as a symmetrical peak on a Sephadex G-100 column with a  $K_{av}$  of 0.47. In alkaline (pH 8.3) gels ( see Fig. 4 for all electrophoretograms ), chum GH consisted of three bands with  $R_f$ 's of 0.35, 0.41 and 0.45 (Fig. 4,A). Coho GH consisted of two bands with  $R_f$ 's of 0.35 and 0.41 (Fig. 4,A). The fastest migrating band ( $R_f = 0.45$ ) was absent. In acid-urea gels at pH 4.5 (Fig. 4,D), chum PI and PII co-migrated as single bands. Coho GH migrated as a single band under the same conditions (Fig. 4,C). In SDS gels (Fig. 4,B), chum GH migrated as a single band

Figure 2. Elution profile of the 25,000 molecular weight fraction of chum salmon pituitaries on DEAE-Bio-gel. In this instance, the material was applied to a 1.6 x 50 cm column equilibrated with 0.003 M Tris-acetate buffer, pH 8.1, and eluted with a 0.003-0.09 M gradient (300 ml/side) of Tris-acetate, pH 8.1. The volume of each fraction collected was 5.1 milliliters. Growth hormone eluted between 0.025-0.045 M Tris-acetate as two peaks, I and II. This elution profile illustrates only the first 100 of a total of 117 fractions. In the last 17 fractions, two more protein peaks eluted, IV and V respectively. Peaks III through V have not been classified further. Fractions 22-52 were saved of peak I, while fractions 63-74 were saved of peak II.

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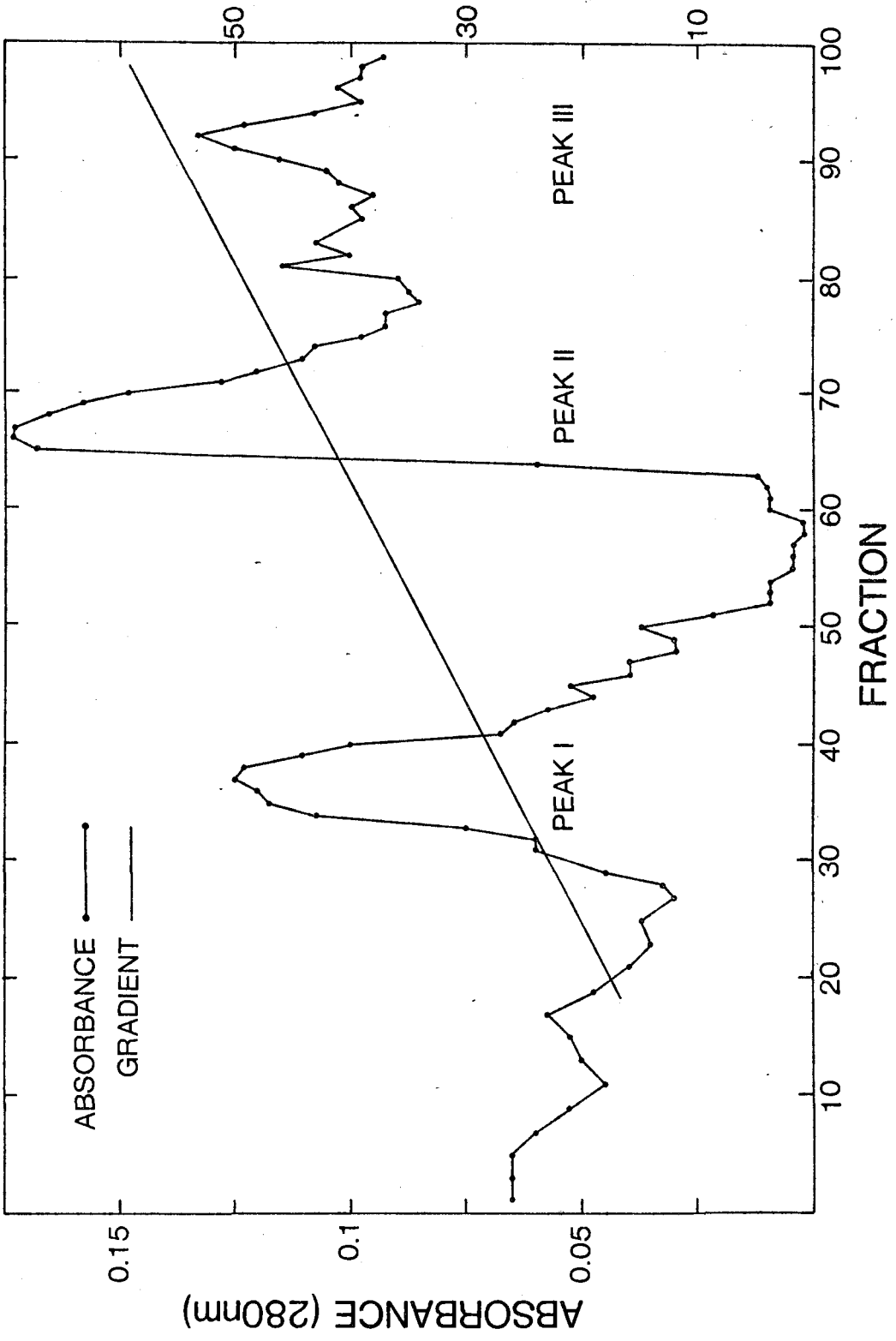


Figure 3. Elution profile of chum salmon growth hormone (Peak I and II combined) on a 3 x 85 cm, Sephadex G-100 column equilibrated with 0.015 M ammonium acetate, pH 8.1. The volume of each fraction collected was 4.6 milliliters. Growth hormone eluted as a symmetrical peak with a  $V_e/V_o$  ratio of 1.88 and a  $K_{av}$  of 0.47. Fractions 58-70 were saved.

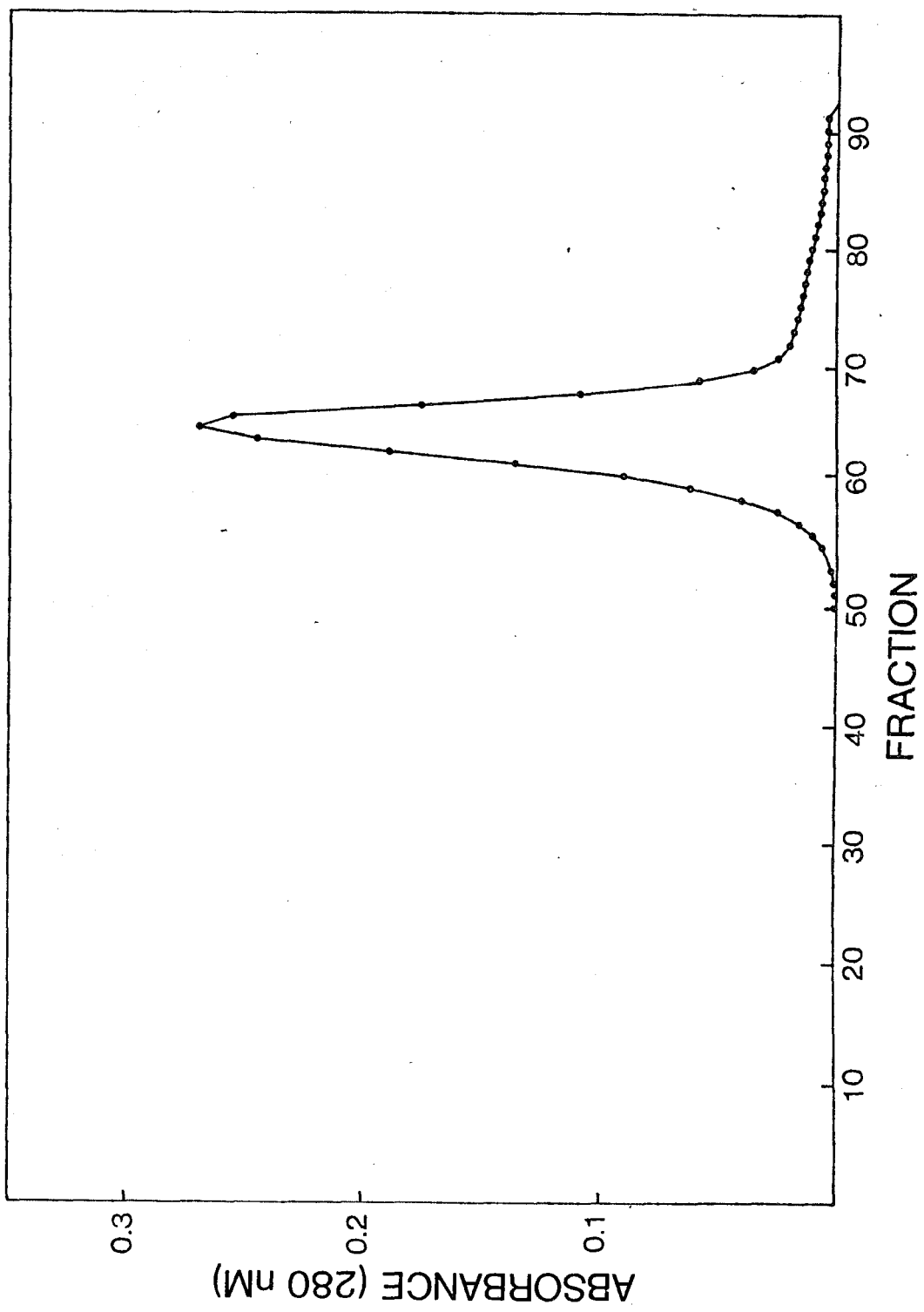




Figure 4. Electrophoretograms of chum and coho salmon growth hormone. A) Alkaline (pH 8.3) electrophoresis of coho (left) and chum (right) growth hormone. B) Sodium dodecylsulphate electrophoresis of chum (left) coho (middle) and ovine(right) growth hormone. C) Electrophoresis of coho GH in an acid-urea electrophoresis gel. D) Electrophoresis of chum PI (left) and PII (right) in an acid-urea gel along with a rostral pars distalis extract (coho) in the center well. The fastest migrating band in the extract is salmon prolactin. Approximately 40 micrograms of each GH preparation were used for each method of electrophoresis.

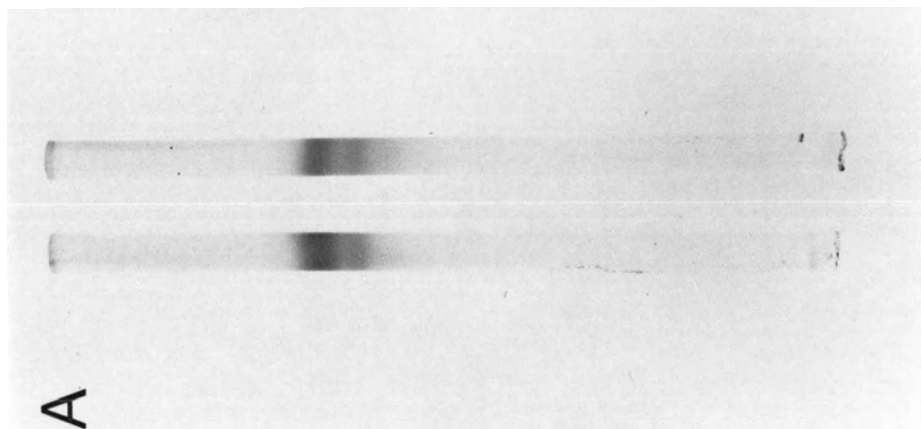
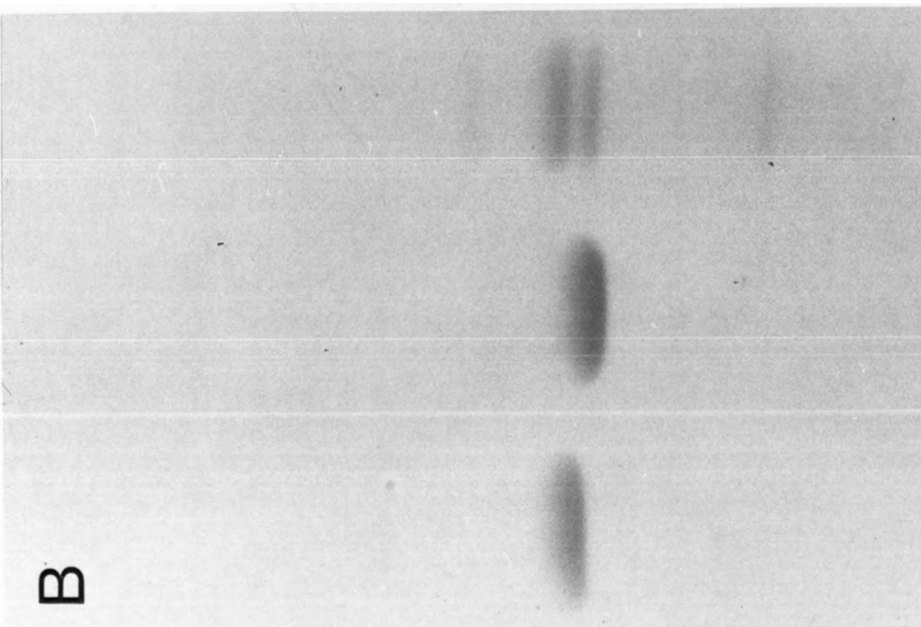
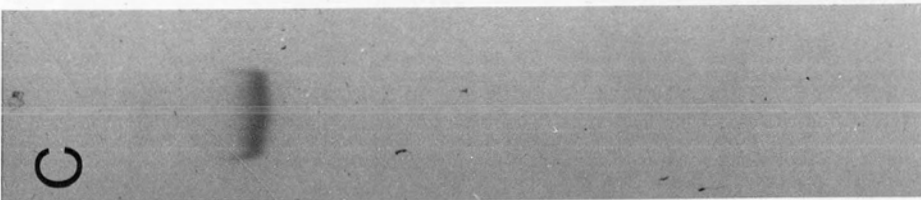
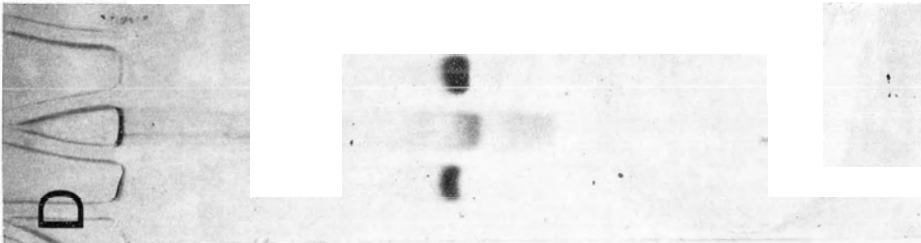
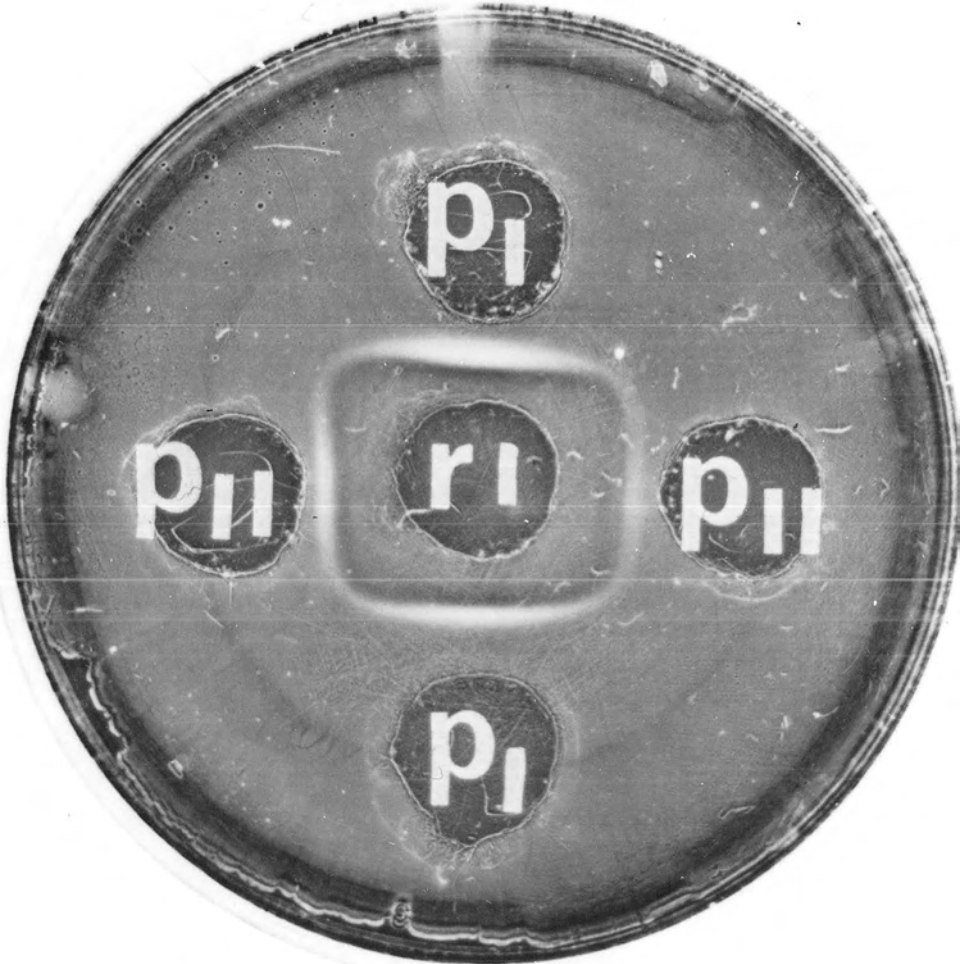


Figure 5. Immunodiffusion of chum salmon Peak I (PI) and Peak II (PII) against an antiserum raised in rabbits to chum salmon PII. There are no spurs at the junctions of the two precipitin lines, indicating immunological identity. The thickness of the precipitin lines may be due to the high concentration of the antigens or possibly to contaminants in the preparations of chum PI and PII.



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with a calculated molecular of 23,500 daltons. Ovine GH migrated as four bands under the same electrophoretic conditions. Coho GH migrated as a single band with a calculated molecular weight of 22,500 daltons (Fig. 4,B).

The immunodiffusion study of chum salmon PI and PII (Fig. 5) demonstrated that these proteins are immunologically identical; as indicated by the absence of spurs between the immunoprecipitin lines of the two antigens.

#### The Bioassay of Salmon Growth Hormones in Rainbow Trout

The results of experiment one, the bioassay of chum salmon PI, PII and ovine GH are shown in Table I and Figure 6. Treatment with ovine GH resulted in a total weight gain of  $5.63 \pm 0.65$  g ( $x \pm$  pooled S.D.,  $P < 0.001$ ) compared with  $3.79 \pm 0.66$  g for the BSA-injected control group. Treatment with chum PI and PII also resulted in significant weight gains of  $5.0 \pm 0.83$  g ( $P < 0.002$ ) and  $4.46 \pm 0.55$  g ( $P < 0.05$ ) respectively. When the slopes of the cumulative percent weight gains over time were compared statistically, all hormone-treated groups had slopes which were greater than the control group ( $P < 0.001$  in all cases), but not different than one another ( $P > 0.5$  in all cases). When the slopes of the cumulative absolute weight gains over time were compared statistically, only the groups treated with ovine GH and chum PI had significantly greater slopes than the controls. The slope of the group treated with Chum PII was not

Table II

Bioassay of Chum Salmon PI, PII and Ovine Growth Hormone in  
Rainbow Trout at a Single Dosage (1 ug/g body weight/biweekly)

TREATMENT	INITIAL WEIGHT	FINAL WEIGHT	WEIGHT GAIN	N	P
Control	5.91±0.39*	9.71±0.85*	3.79±0.66**	10	--
Ovine GH	5.60±0.36	11.23±0.84	5.63±0.66	10	<0.001
Chum PI	5.53±0.48	10.53±1.07	5.0 ±0.83	10	<0.002
Chum PII	4.36±0.26	8.82±0.73	4.46±0.55	10	<0.05

\* x ± Standard Deviation

\*\* x ± Pooled Standard Deviation

Figure 6. The Bioassay of Chum Salmon PI, PII and Ovine Growth Hormone in Rainbow Trout. The fish were injected bi-weekly with the above hormone preparations at a dosage of 1 ug/g/body weight, while the control group received an equivalent dosage of bovine serum albumin (BSA). When the slopes of the cumulative percent weight gains over time were compared statistically (analysis of variance followed by the Student Newman Kuels multiple range test), all hormone-treated groups had greater slopes than the control group ( $P < 0.001$  in all cases). The slopes of the hormone-treated groups were also not significantly different from one another ( $P > 0.5$  in all cases). The regression equations for the various groups are as follows; BSA( $y = 2.76X - 1.71$ ), Ovine GH( $y = 4.33X - 1.21$ ), Chum PI( $y = 4.50X + 1.11$ ), Chum PII( $y = 4.27X - 2.07$ ).

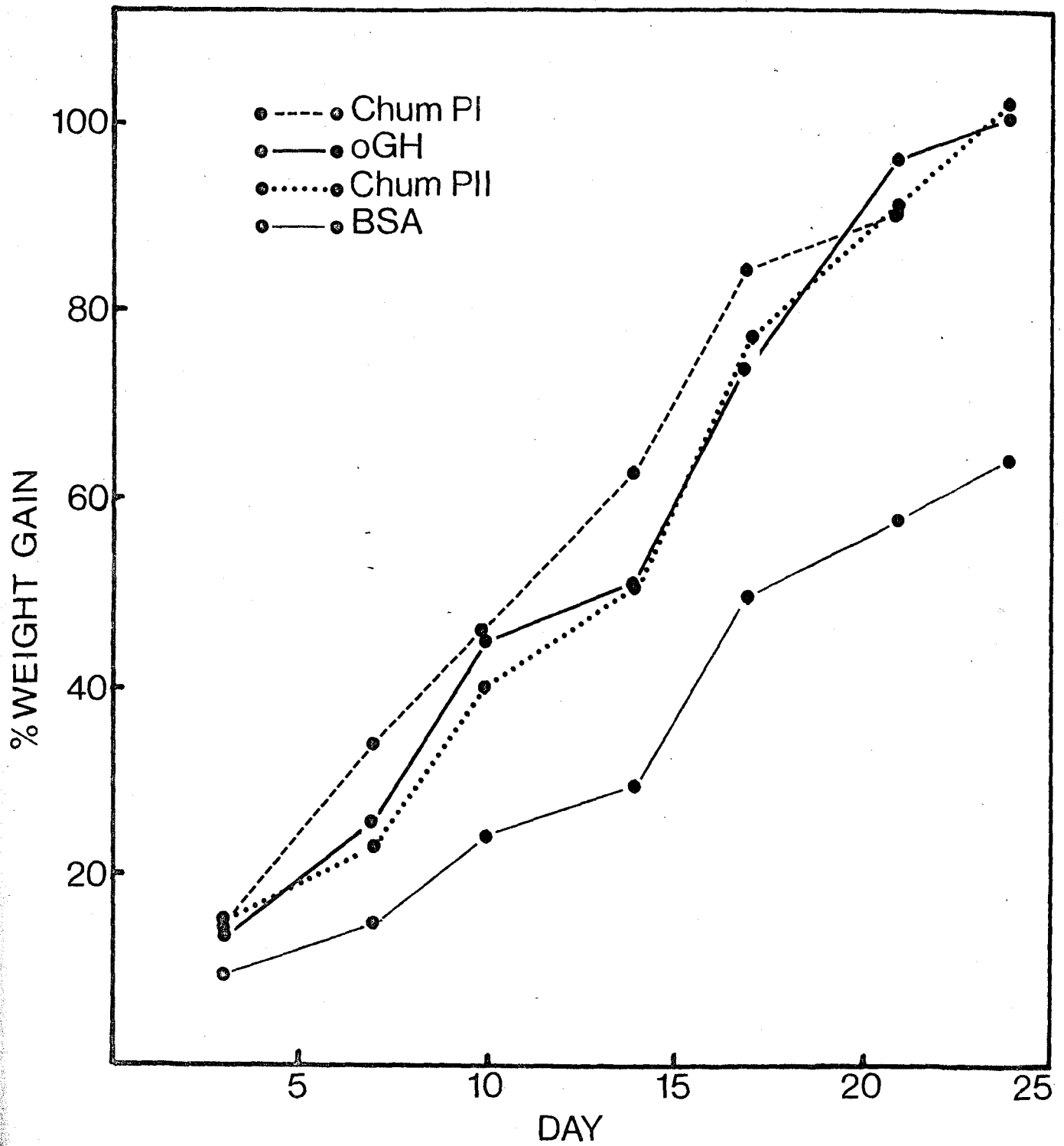




Figure 7. The bioassay of chum and coho growth hormone in rainbow trout (Experiment 2). Weekly instantaneous linear growth rates of intact rainbow trout following bi-weekly administration of chum and coho salmon GH. Each data point represented consists of the mean  $\pm$  S.E.M. of the untransformed data. Significant differences ( $P < 0.05$ ) are as follows; Week 1. Coho and chum GH (1.0 ug) higher than both control groups. Week 2. Coho GH (0.25 ug) lower than chum GH (0.25, 0.5 ug). Coho GH (0.5 ug) higher than non-injected control and coho GH (0.25 ug). Week 3. No significant differences. There was also a significant effect of week upon growth ( $P < 0.0005$ ) but no interaction between week and GH therapy ( $P = 0.12$ ). There was no effect of GH therapy upon length gains.

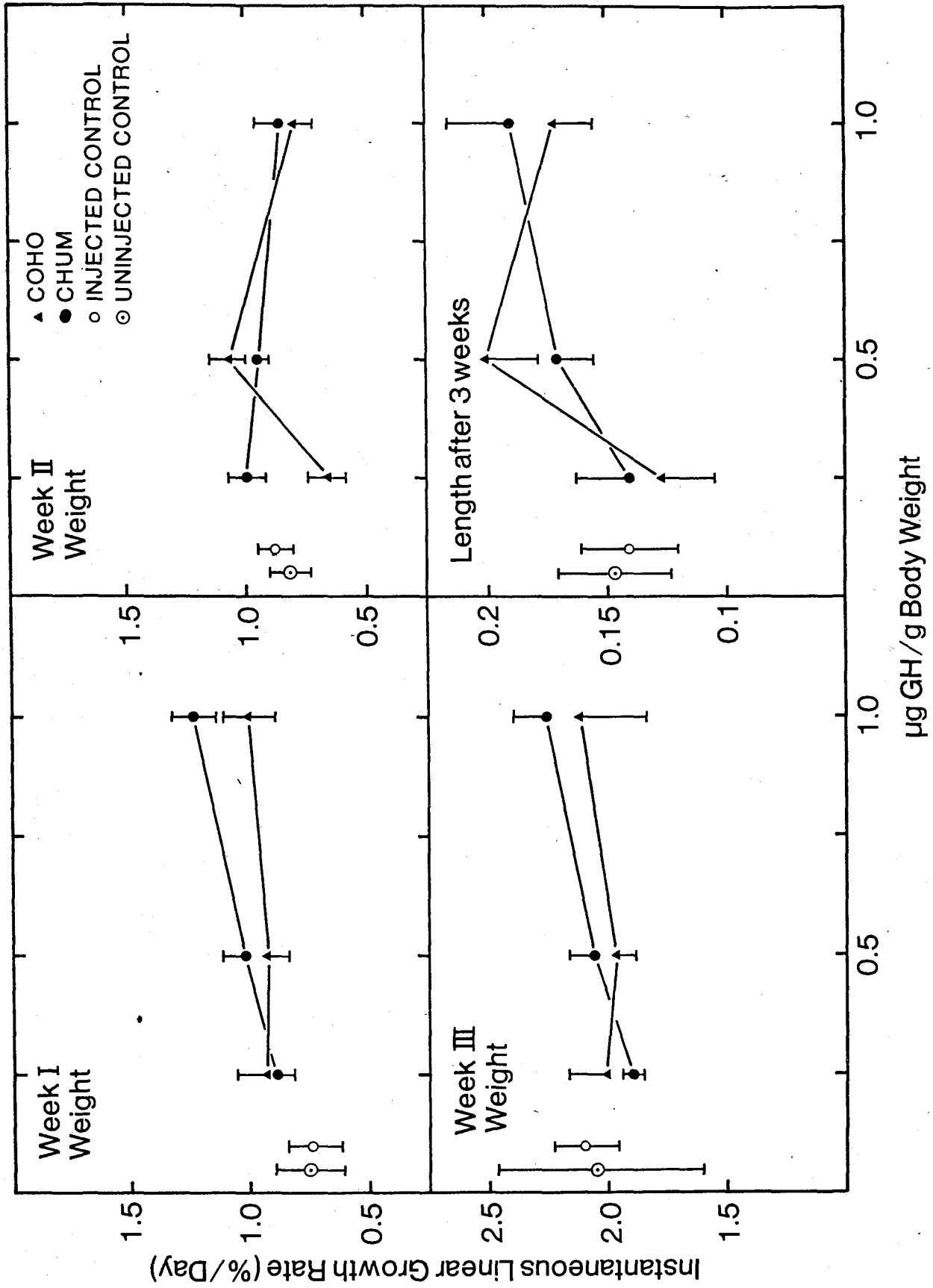
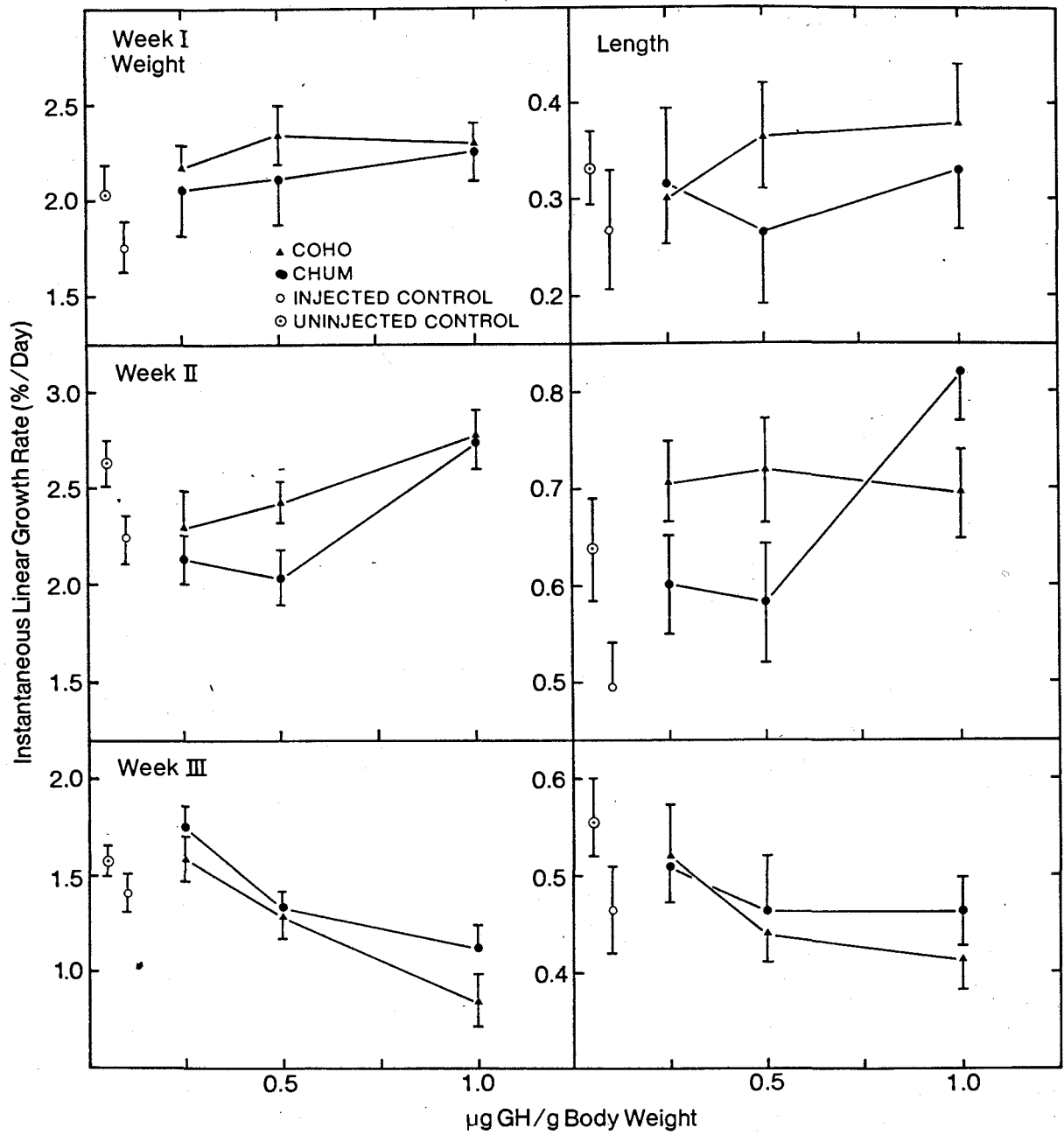


Figure 8. The bioassay of chum and coho growth hormone in rainbow trout (Experiment 3). Weekly instantaneous linear growth rates of intact rainbow trout following bi-weekly administration of chum and coho GH. Each data point represented consists of the mean  $\pm$  S.E.M. of the untransformed data. Significant differences (weight) are as follows; Week 2. Coho and chum GH (1.0 ug) higher than injected controls, coho GH (0.25 ug) and chum GH (0.25, 0.5 ug). Uninjected control higher than chum GH (0.25, 0.5 ug). Week 3. Coho GH (1.0 ug) lower than all other groups. Chum GH (1.0 ug) lower than coho GH (0.25 ug) and chum GH (0.25 ug). Coho GH (0.5 ug) lower than chum GH (0.25 ug). Chum GH (0.5 ug) lower than chum GH (0.25 ug). There was also a significant effect of week ( $P < 0.005$ ) and a significant interaction between GH therapy and week ( $P < 0.005$ ) upon growth.

Significant differences (length) are as follows; Week 2. Coho (0.25, 0.5 and 1.0 ug) and chum GH (1.0 ug) higher than injected controls. Chum GH (1.0 ug) higher than non-injected controls. Chum GH (1.0 ug) higher than chum GH (0.25, 0.5 ug). There was no significant effect of week upon length gain ( $P = 0.059$ ) and no interaction between week and GH therapy ( $P = 0.485$ ).



significantly different ( $P > 0.1$ ) than the controls.

The results of experiments two and three, the comparative bioassays of chum and coho GH are illustrated in Figures 7 and 8. During week 1 of experiment two (Fig. 7) both chum and coho GH induced significantly greater growth rates than the two control groups at a dosage of 1.0 ug/g body weight (BWT). There were no other differences observed at this time. In week 2 on the other hand, neither preparation enhanced growth when compared with the injected control group. In week 3, there were again no significant differences between the GH treated and the injected control groups. Growth rates with respect to length were not significantly different over the three week period and at no point in the experiment were the injected and non-injected control groups significantly different from one another. The analysis of variance revealed a highly significant effect of week upon growth with respect to weight ( $P < 0.0005$ ). There was no interaction however between GH treatment and week upon this growth ( $P = 0.12$ ). There was also no difference between the slopes of the various groups when the data were plotted as the cumulative percent weight gains of each group over the course of the experiment. For unknown reasons, fourteen mortalities occurred two days after the end of the experiment. There were no differences in body water content between any of the eight groups.

In experiment three (Fig. 8) only one mortality was observed during the experiment. During the first week, no

significant differences were observed between the treatment and control groups with respect to weight and length. In week 2 however both chum and coho GH (1.0 ug/g/BWT) again induced significantly greater growth rates (weight) than the injected controls as well as their respective low dose groups (0.25 ug/g/BWT). In addition, coho GH (1.0 ug/g/BWT) caused a significantly greater growth rate than chum GH (0.25 and 0.5 ug/g/BWT) while chum GH (1.0 ug/g/BWT) induced significantly greater growth rates than coho GH (0.25 and 0.5 ug/g/BWT). With respect to length in week 2, both chum and coho GH (1.0 ug/g/BWT) induced significantly greater growth rates than the injected control group. In the case of coho GH, both lower dose groups (0.25 and 0.5 ug/g/BWT) also caused significantly greater growth rates than the injected controls, although there was no indication of a dose-related response.

In week 3, there was a trend towards a negative effect of increasing GH dosage upon growth rates. With respect to weight, coho GH (1.0 ug/g/BWT) significantly reduced the growth rate compared to the injected controls and all other groups. In addition, chum GH (1.0 ug/g/BWT) caused a significant reduction in growth rate when compared with chum and coho GH at a dosage of 0.25 ug/g/BWT. Fish receiving chum GH at 0.5 ug/g/BWT also had a significantly lower growth rate than fish receiving chum GH at 0.25 ug/g/BWT. Although the same negative effects of GH treatment are suggested with respect to length, no significant differences were observed. The analysis of variance revealed a

highly significant effect of week upon growth rate (weight) in experiment 3, as well as a highly significant interaction between week and GH treatment upon growth rate ( $P < 0.0005$  in both cases). There is an indication of a possible interaction between week and growth rate with respect to length ( $P = 0.059$ ), but no interaction between week and GH treatment. No differences were observed between the slopes of the various groups, when the data were plotted as the cumulative percent weight gains of each group over the three weeks of the experiment.

When the growth rates (weight only) of the fish from experiment 2 were monitored for a further fifteen weeks, a pronounced cyclical pattern of growth was observed (Fig. 9). Four distinct cycles of growth were recorded during the fifteen weeks of observation, including one cycle during the three weeks of experiment 2 (weeks 1-3 of Fig. 9). The growth rate peaked every three or four weeks and in each case were followed by periods of significantly reduced growth. Analysis of variance revealed a highly significant effect of week upon this pattern of growth ( $P < 0.0025$ ). A total of ten mortalities were recorded during the fifteen weeks of observation.

#### The Bioassay of Salmon Growth Hormones in the Rat Tibia Test

The results of the rat tibia bioassays for chum and coho GH are listed in Tables II and III respectively. In the first bioassay, all preparations induced significant tibial growth.

Figure 9. Weekly growth rate of rainbow trout (n=70) on a natural photoperiod. There was a significant effect of week upon the observed cyclical pattern of growth ( $P < 0.0025$ ).



INSTANTANEOUS LINEAR GROWTH (%/DAY)

WEEKLY GROWTH RATE OF RAINBOW TROUT  
ON A NATURAL PHOTO PERIOD

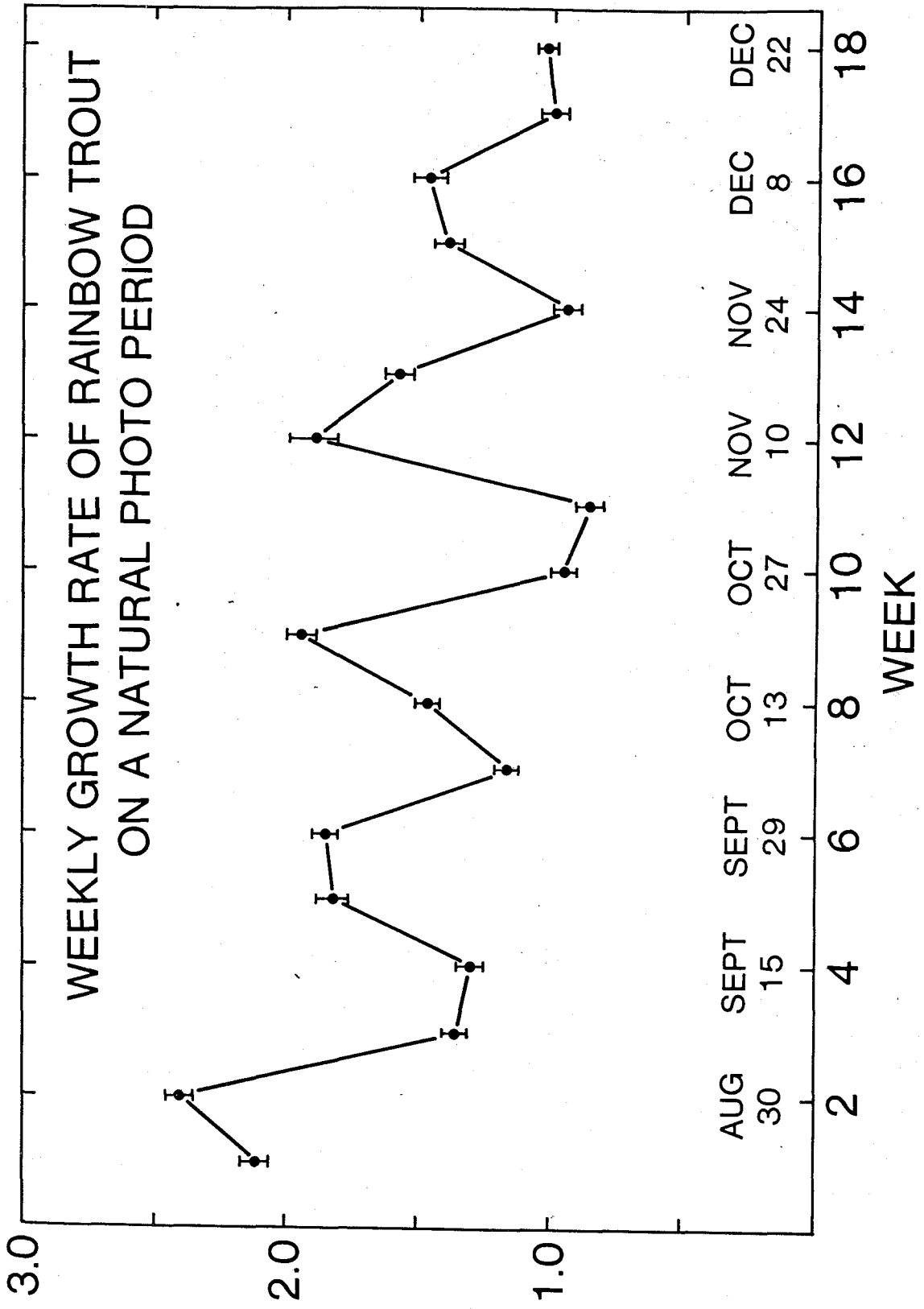


Table III

Rat Tibia Bioassay of Chum Salmon GH, Ovine GH and a Sockeye Salmon Pituitary Extract (P.E.)

TREATMENT (Daily Dose)	TIBIA WIDTH ( $\mu\text{m} \pm \text{SEM}$ )	N	P
Saline	135.0 $\pm$ 5.3	5	--
Sockeye P.E. (5mg)	172.9 $\pm$ 10.0	4	<0.01
Chum GH(70ug)	185.4 $\pm$ 17.9	5	<0.025
Ovine GH(70ug)	208.7 $\pm$ 7.5	5	<0.001

Table IV

Rat Tibia Bioassay of Coho and Ovine GH

TREATMENT (Daily Dose)	TIBIA WIDTH ( $\mu\text{m} \pm \text{SEM}$ )	N	P
Saline	149.4 $\pm$ 28.1	6	--
Ovine GH(80ug)	260.8 $\pm$ 51.2	6	<0.005
Coho GH(40ug)	148.8 $\pm$ 32.9	7	N.S.
Coho GH(80ug)	159.3 $\pm$ 30.2	7	N.S.

Table V

Comparative Amino Acid Composition of Chum, Coho Salmon and Carp  
Growth Hormone and Chum Salmon Prolactin (PRL)

	ChumGH	CohoGH	CarpGH <sup>1</sup>	ChumPRL <sup>2</sup>
Lys	14.1	10.1	10.4	13.0
His	6.6	4.3	4.8	6.6
Arg	7.7	8.1	9.3	12.4
Asx	31.1	25.3	24.8	21.2
Thr	7.7	8.0	10.1	8.0
Ser	18.1	16.7	16.5	25.8
Glx	22.0	25.2	23.6	13.7
Pro	7.2	13.0	8.9	13.4
Gly	10.9	9.8	8.8	9.5
Ala	7.9	7.3	10.6	6.2
1/2Cys	3.8	3.7	3.7	3.7
Val	7.9	9.3	11.8	4.6
Met	2.6	2.8	4.5	9.1
Ile	6.3	8.8	7.0	10.0
Leu	24.0	26.0	22.8	26.4
Tyr	6.0	5.4	4.5	2.2
Phe	6.2	7.1	8.0	6.6
Trp	N.D.*	0.6	1.0	1.0

1. Cook *et al.* (1983)

2. Kawauchi *et al.* (1983)

\*N.D. (Not Determined)

Figure 10. The immunocytochemical localization of growth hormone cells in the pituitary gland of four species of Pacific salmon, using an antiserum raised against chum salmon growth hormone.

A). Spawning pink salmon. Note the absence of immunoreactive cells in the prolactin follicles.

B). Spawning sockeye salmon. Note the growth hormone cells in the pars intermedia on the top side of the photograph.

C). Juvenile coho salmon. Elements of the pars nervosa (n) can be seen invaginating the caudal pars distalis.

D). Spawning chum salmon. Note the manner in which the growth hormone cells are lined up along the capillary.

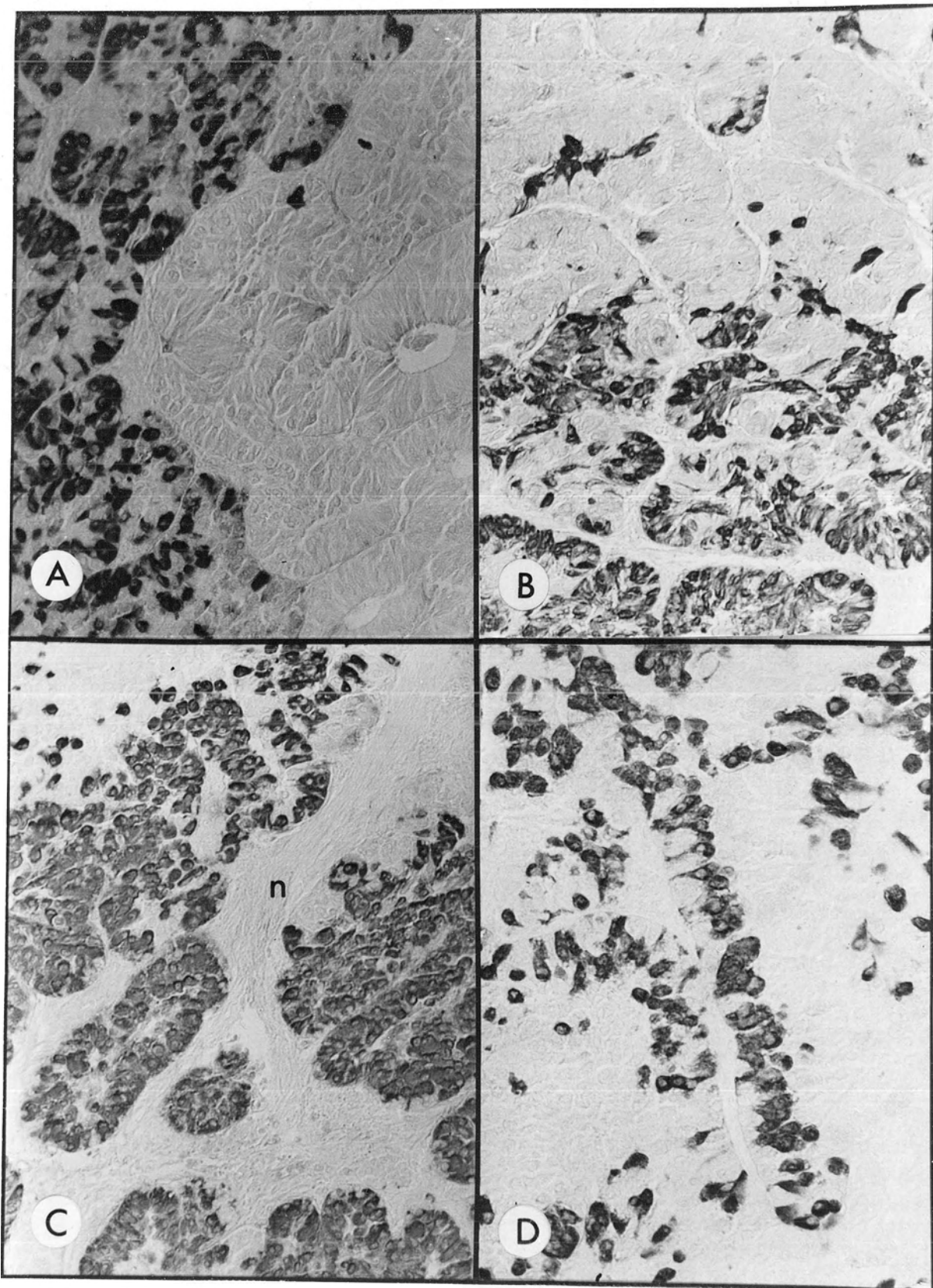


Figure 11. Fractionation of freshly iodinated chum salmon growth hormone on a 1 x 7 cm, Sephadex G-50 column. Ten drop fractions were collected in test tubes containing 1 ml of phosphate-buffered saline containing 1% bovine serum albumin. The first peak represents  $^{125}\text{I}$ -GH while the second peak consists of unreacted  $^{125}\text{I}$ iodine. Under these conditions, the labelled growth hormone co-elutes with the void peak.

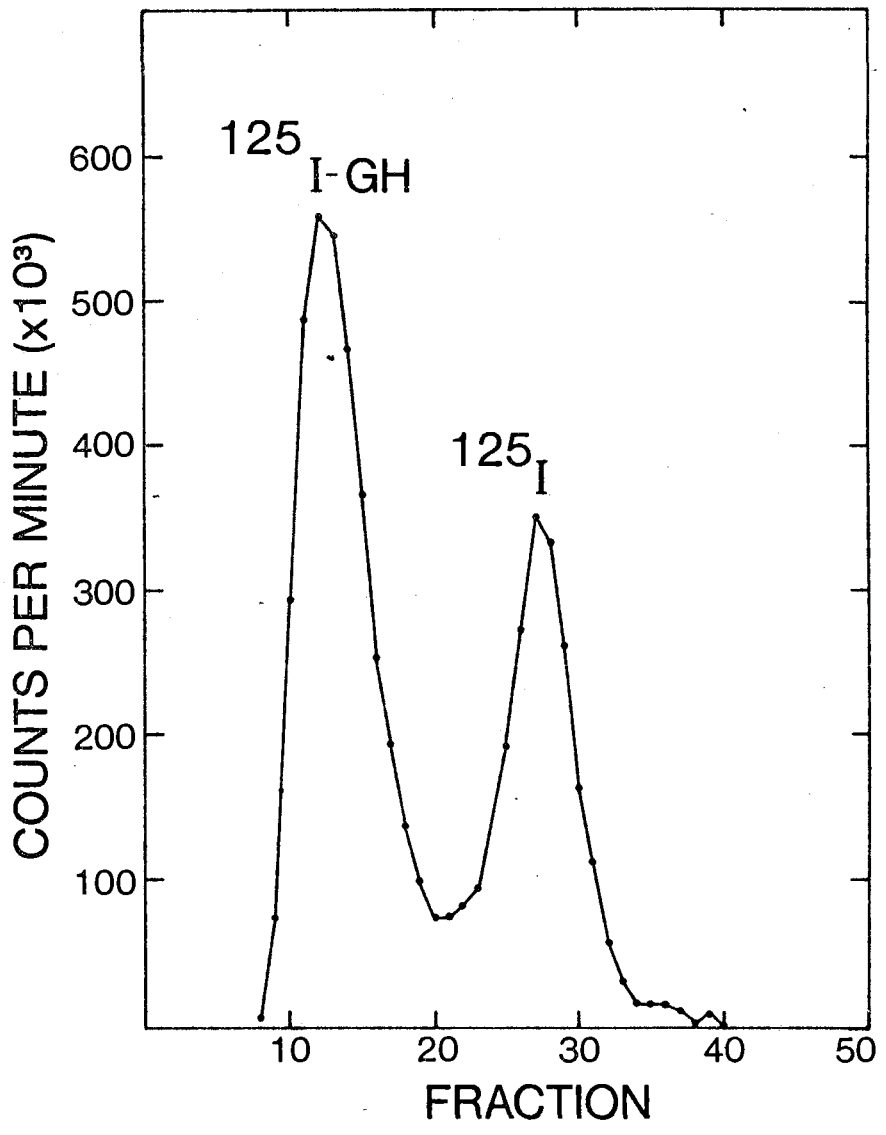
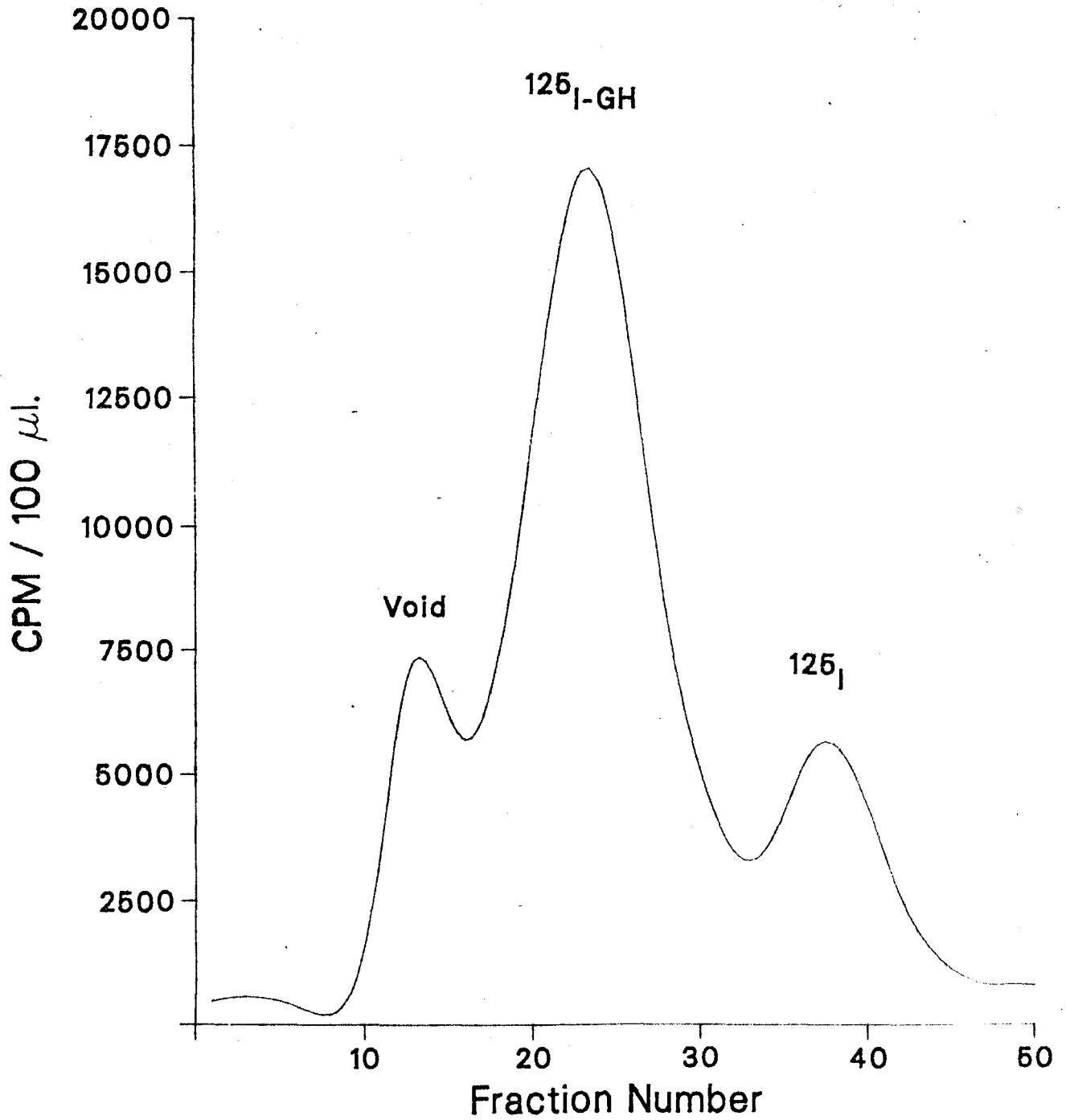




Figure 12. Re-purification of  $^{125}\text{I}$ -chum salmon growth hormone on a 1 x 29 cm column of Sephadex G-100. The labelled hormone was eluted from the column with 0.015 M ammonium acetate-buffered saline, pH 8.1. The fraction size was approximately 1.1 milliliters. The first peak consists of contaminants of higher molecular weight and the second peak comprises the monomeric  $^{125}\text{I}$ -GH. The last peak consists of dissociated  $^{125}\text{I}$ iodine. The labelled growth hormone eluted with a  $K_{av}$  of 0.47. This figure was drawn by computer, assuming a gaussian distribution of the points comprising the three peaks.

# Repurification of $^{125}\text{I}$ -Chum GH on Sephadex G100.



When compared with the saline-injected control group ( $135 \pm 5.3$  um,  $x \pm \text{SEM}$ ), ovine GH caused the largest growth increment ( $208.7 \pm 7.5$  um,  $P < 0.001$ ), followed by the sockeye pituitary extract ( $172.9 \pm 10$  um,  $P < 0.01$ ) and chum GH ( $185.4 \pm 17.9$  um,  $P < 0.025$ ). One rat in the group injected with the pituitary extract had severe skeletal deformities by the end of the experiment and was excluded from the data analysis.

In the second bioassay, only ovine GH induced significant tibial growth ( $260.8 \pm 51.2$  um,  $P < 0.005$ ) when compared with the saline-injected control group ( $149.4 \pm 28.1$  um). Coho GH illicited no response at either dosage.

#### Amino Acid Analysis of the Salmon Growth Hormones

By amino acid analysis (Table IV), both chum and coho GH were shown to have similar characteristics of a vertebrate GH; a low histidine and methionine content, two disulphide bridges, a high content of leucine and glutamic acid and one tryptophan residue (Wilhelmi, 1974). The two species of GH have a similar amino acid composition and differ most notably in the higher proline content of coho GH.

Immunocytochemical and Immunodiffusion Studies with an Antiserum to Chum Salmon Growth Hormone

The immunocytochemical results are illustrated in Figure 10. In each of the four species investigated, a distinct population of cells was stained by the procedures employed. The staining of these cells was not an artifact of the procedure, as no reaction was obtained when the primary antiserum was saturated with chum GH or substituted with normal rabbit serum. When adjacent sections of coho salmon pituitary were stained with hematoxylin and eosin, the eosinophils corresponded in both appearance and distribution to the immunoreactive cells. The distribution of the immunoreactive cells was mainly confined to the caudal pars distalis, with single cells scattered throughout the pars intermedia. In no instances were the prolactin follicles stained. In the juvenile coho salmon (Fig. 10;C) the immunoreactive cells were densely packed and were the predominant cell type in the caudal pars distalis. The cells were oval in appearance and contained densely packed granules. The nuclei remained unstained. The dense packing of the immunoreactive cells was interrupted only periodically by invaginations of the pars nervosa into the caudal pars distalis. In the sexually mature, adult sockeye, pink and chum salmon (Fig. 10; A, B and D), the immunoreactive cells were less densely packed due to the proliferation of gonadotrops. The isolated cells which were stained in the pars intermedia,

occurred in both juveniles and adults and were generally more densely stained than the cells in the caudal pars distalis.

The pituitary extracts of all species tested in Ouchterlony (1968) double gel diffusion plates cross-reacted with the antiserum to chum GH (not shown). A single precipitin line was observed in each case and all species showed immunological identity with one another, by an absence of spurs between adjacent lines. The pituitary extracts also showed immunological identity with purified chum GH.

#### Development of a Radioimmunoassay for Salmon Growth Hormone

Following iodination (Fig. 11), chum salmon GH was very stable. After each label re-purification on Sephadex G-100, the areas underlying the various peaks (Fig. 12) were calculated and totalled. The area of each peak was then expressed as a percentage of the total. The percentages represented by the various peaks did not change after the label had been stored for one month at  $-20^{\circ}\text{C}$ . Despite the apparent purity of chum GH by electrophoresis however, minor contaminants became evident following its iodination. The re-purification of the labelled chum GH on Sephadex G-100, demonstrated that contaminants of high molecular weight, which eluted in the void volume, were present in this preparation. However, as this preparation had been previously gel filtered on G-100 during the purification procedure, these high molecular weight substances should not

have been present in the final yield. It is concluded therefore, that these contaminants may have arisen as a result of either the freezing or lyophilization processes following the final gel filtration step in the purification.

The sensitivity of the radioimmunoassay (RIA), defined as the smallest quantity of GH which results in a significant ( $P < 0.05$ , Student's T-test) displacement of the labelled GH, was 250 picograms or 2.5 ng/ml (Fig. 13). The coefficients of variation (CV) for intraassay variability were 7.9 and 5.8% for 5 and 20 ng/ml solutions of GH respectively. The CV for interassay variability was 6.61%. The results of the parallelism studies are shown in Figures 14 and 15 and the regression equations are listed in the captions to these figures. Plasma from rainbow trout and coho salmon ( $P > 0.1$ ) and a coho pituitary extract ( $P > 0.05$ ) all had slopes that were not significantly different than the slope of the standard curve. The gel filtration profile of coho plasma on Sephadex G-50 is shown in Figure 16. The immunoreactive component of the plasma eluted as a symmetrical peak with the same  $K_{av}$  (0.23) as  $^{125}\text{I}$ -GH. The recovery of 250 picograms of GH from 10, 20 and 30  $\mu\text{l}$  of trout plasma were 90, 112 and 108% respectively, with a mean recovery of 103.3%. Whereas the plasma from intact killifish contained measureable quantities of immunoreactive-GH (8.4 ng/ml,  $\%B/B_0=87.7\%$ ), plasma (10-40  $\mu\text{l}$ ) from hypophysectomized killifish contained non-detectable levels of this hormone. The range of the  $\%B/B_0$  values for the various volumes measured was

Figure 13. The standard curve developed for the radioimmunoassay of salmon growth hormone. The sensitivity of the assay, defined as the smallest quantity of growth hormone which results in a significant ( $P < 0.05$ , Student's T-test) displacement of the labelled hormone is 250 picograms/100 ul or 2.5 ng/ml.

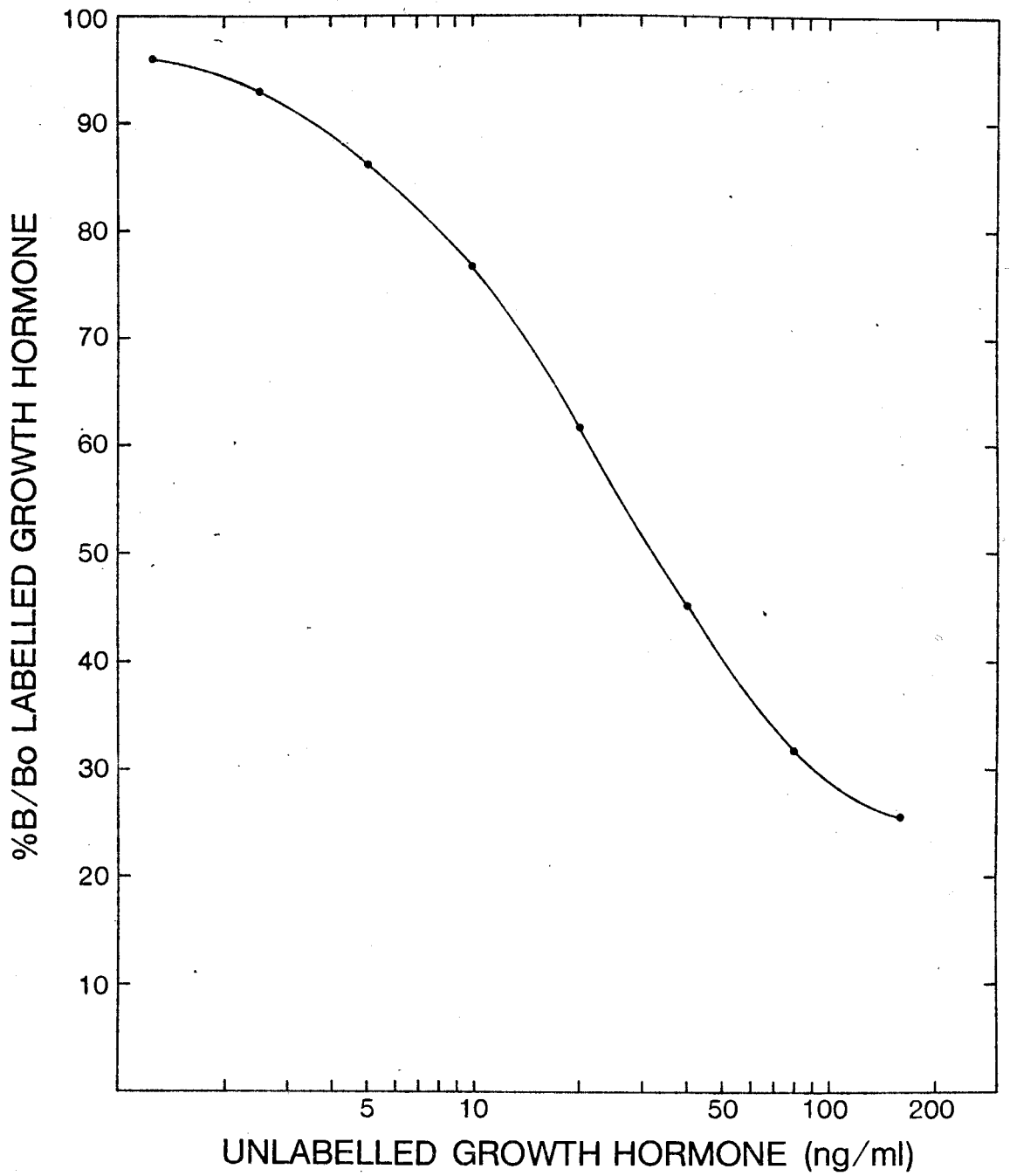




Figure 14. Parallelism of rainbow trout plasma versus a salmon growth hormone standard curve. Because the transformation of the % B/B<sub>0</sub> values to logits resulted in negative numbers in some instances, one logit unit was added to all y-values to facilitate slope computations and statistical comparisons. The slopes of the two lines are not significantly different ( $P > 0.1$ , Student's T-test).

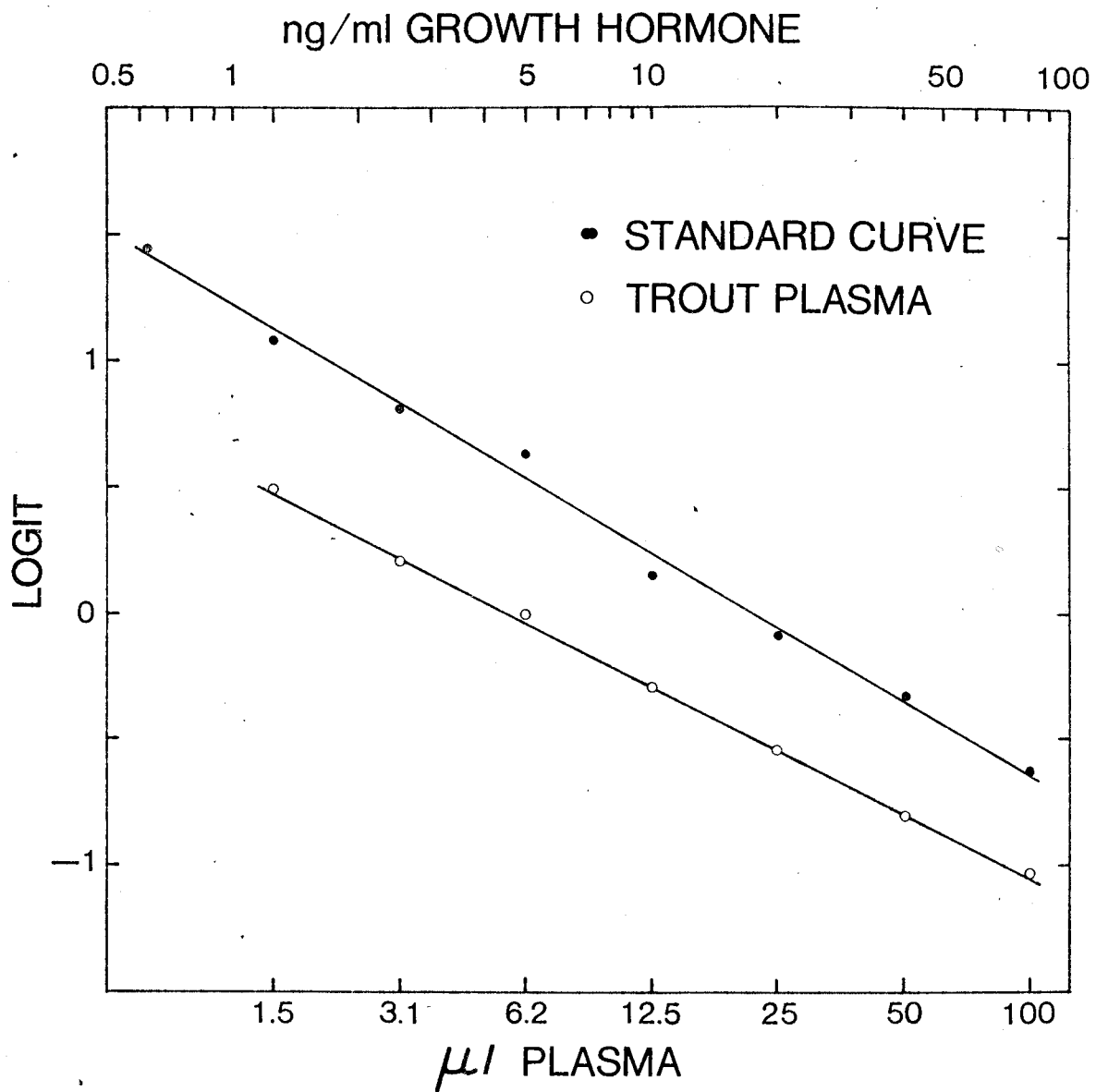


Figure 15. Parallelism of coho salmon plasma and a coho pituitary extract versus a salmon growth hormone standard curve. Because the transformations of the  $\%B/B_0$  values to logits resulted in negative numbers in some instances, one logit unit was added to all y-values to facilitate slope computations and statistical comparisons. The slopes of the plasma ( $P>0.1$ ) and the pituitary extract ( $P>>0.05$ ) are not significantly different than the standard curve (Student's T-test).

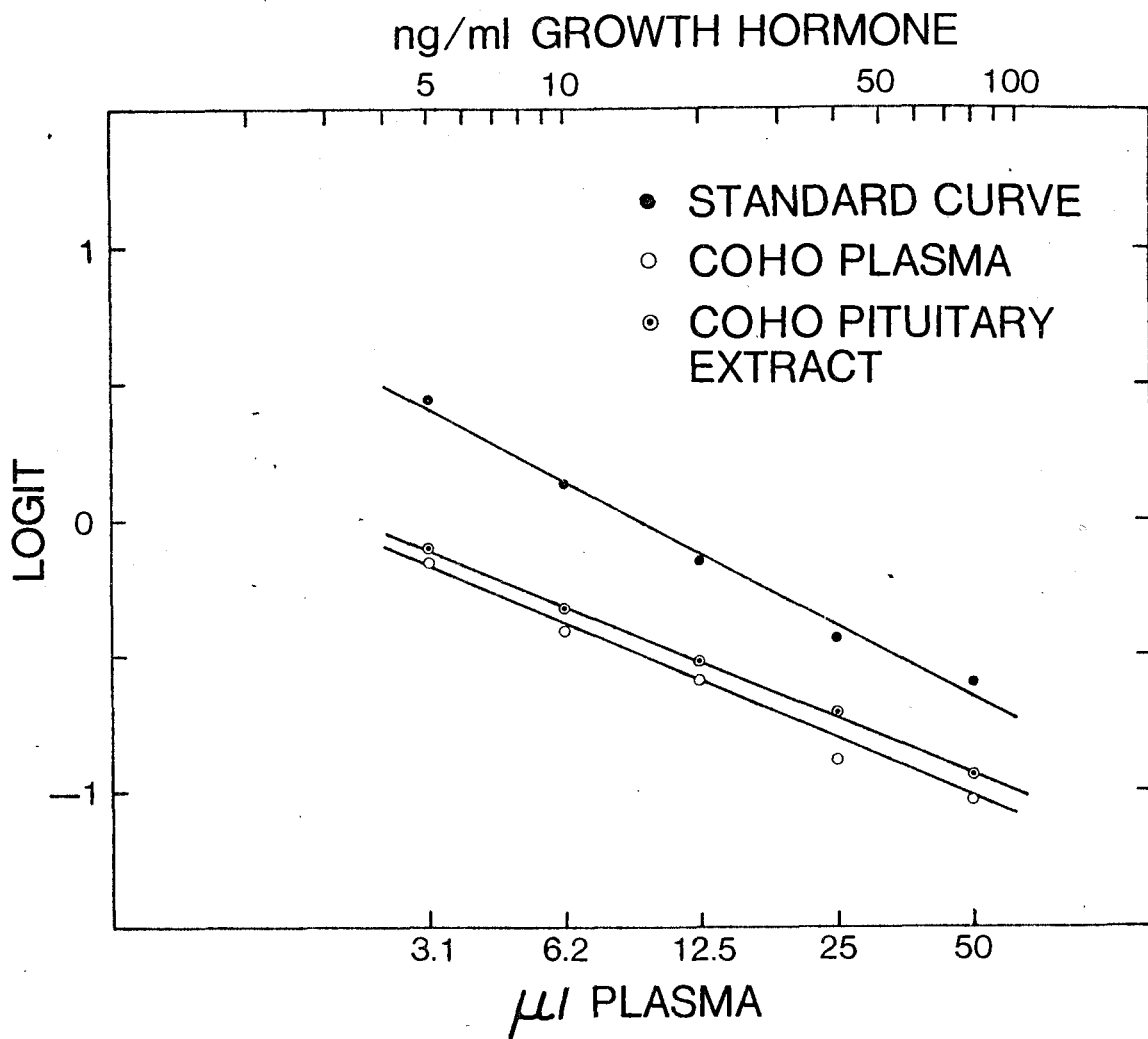
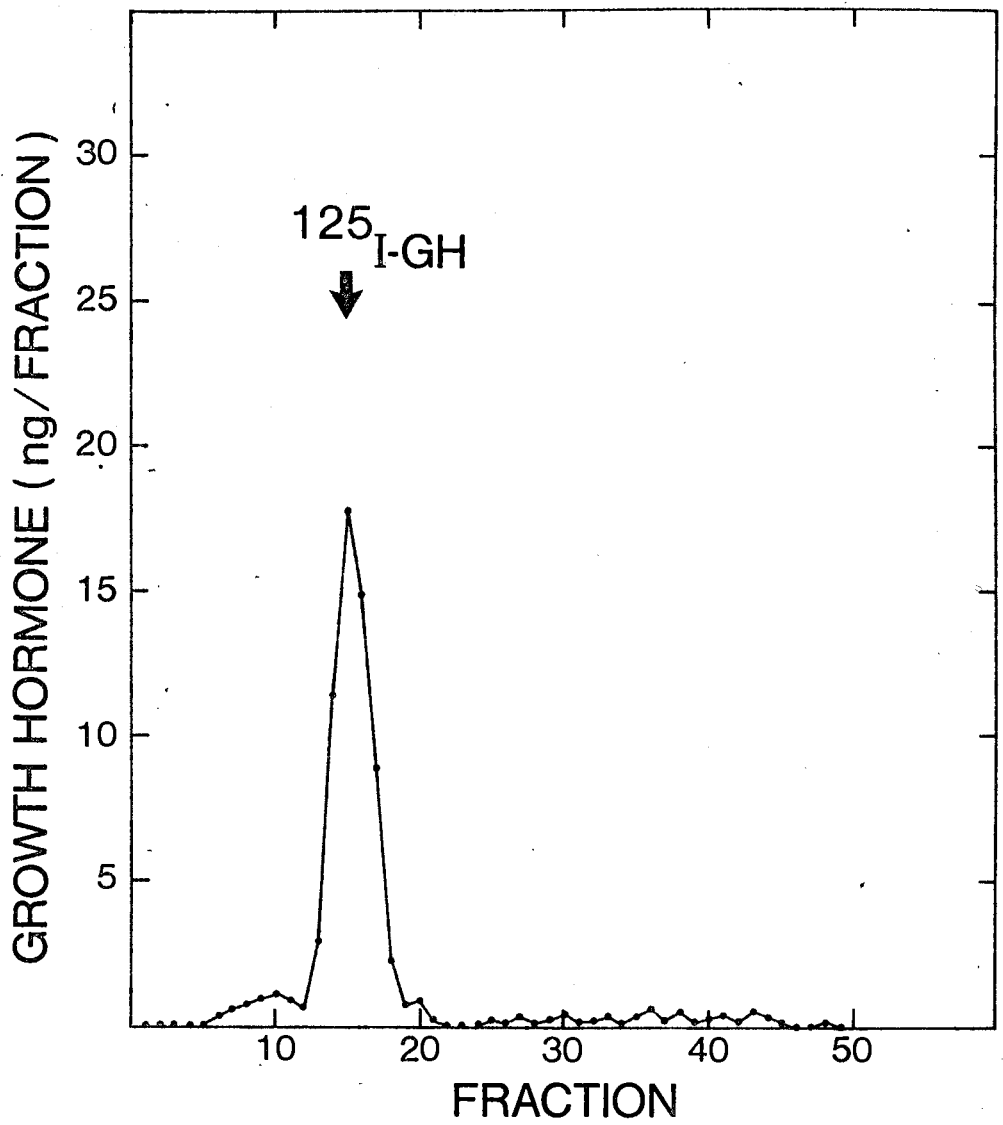


Figure 16. Fractionation of coho salmon plasma on a 1.6 x 58 cm column of Sephadex G-50. The column was first calibrated with  $^{125}\text{I}$ -growth hormone before the application of one milliliter of coho plasma. The immunoreactive component of the plasma eluted as one peak with the same  $K_{av}$  (0.23) as  $^{125}\text{I}$ -growth hormone. The void peak eluted at fraction nine.



99.4-106.6%. Plasma from intact killifish has not been tested for parallelism however.

#### The Effects of Handling Stress on Plasma Growth Hormone Levels in Rainbow Trout

One hour following the induction of handling stress (Table V), the plasma GH levels in stressed rainbow trout were  $54.0 \pm 15.85$  ng/ml ( $x \pm$  SEM) compared with  $47.07 \pm 13.90$  ng/ml for the non-stressed control group. After seven hours, plasma GH levels in the stressed fish were  $32.08 \pm 5.80$  ng/ml compared with  $26.57 \pm 7.34$  ng/ml in the control group. At neither time were the groups significantly different than one another ( $P > 0.5$  in both cases). The hematocrits of the stressed fish were significantly lower than the control group after one hour ( $P < 0.01$ ) and significantly higher after seven hours ( $P < 0.02$ ).

#### The Effects of Long-Term Starvation (Three Weeks) on Plasma Growth Hormone Levels in Rainbow Trout

Following three weeks of starvation (Table VI), the unfed rainbow trout weighed less ( $11.12 \pm 3.3$  g,  $x \pm$  S.D.) than the fed fish ( $27.92 \pm 2.9$  g). The plasma GH levels in the starved fish ( $765 \pm 60.27$  ng/ml,  $x \pm$  SEM) were nine times higher ( $P < 0.001$ ) than the plasma GH levels in the control group ( $85.45 \pm 14.71$  ng/ml).

Table VI

The Effects of Handling Stress on Plasma Growth Hormone Levels  
and Hematocrits in Rainbow Trout

	Plasma GH (ng/ml, $\bar{x} \pm \text{SEM}$ )	P	Hematocrit ( $\bar{x} \pm \text{SEM}$ )	P
<u>One hour</u>				
Control (7)*	47.07 $\pm$ 13.90		42.71 $\pm$ 0.83	
Stressed(6)	54.0 $\pm$ 15.85	>0.5	38.83 $\pm$ 1.01	<0.01
<u>Seven Hours</u>				
Control (7)	26.57 $\pm$ 7.34		35.71 $\pm$ 1.20	
Stressed (6)	32.08 $\pm$ 5.80	>0.5	39.66 $\pm$ 0.71	<0.02

\* Numbers in parentheses indicate sample size.



Table VII

The Effects of Long-Term Starvation (Three Weeks) on Plasma Growth Hormone Levels in Rainbow Trout

Treatment	Weight ( $\bar{x} \pm \text{SEM}$ )	Plasma GH (ng/ml, $\bar{x} \pm \text{SEM}$ )	P
Fed (10)*	27.92 $\pm$ 2.90g	85.45 $\pm$ 14.71	
Starved (12)	11.12 $\pm$ 0.96g	765 $\pm$ 60.27	<0.001

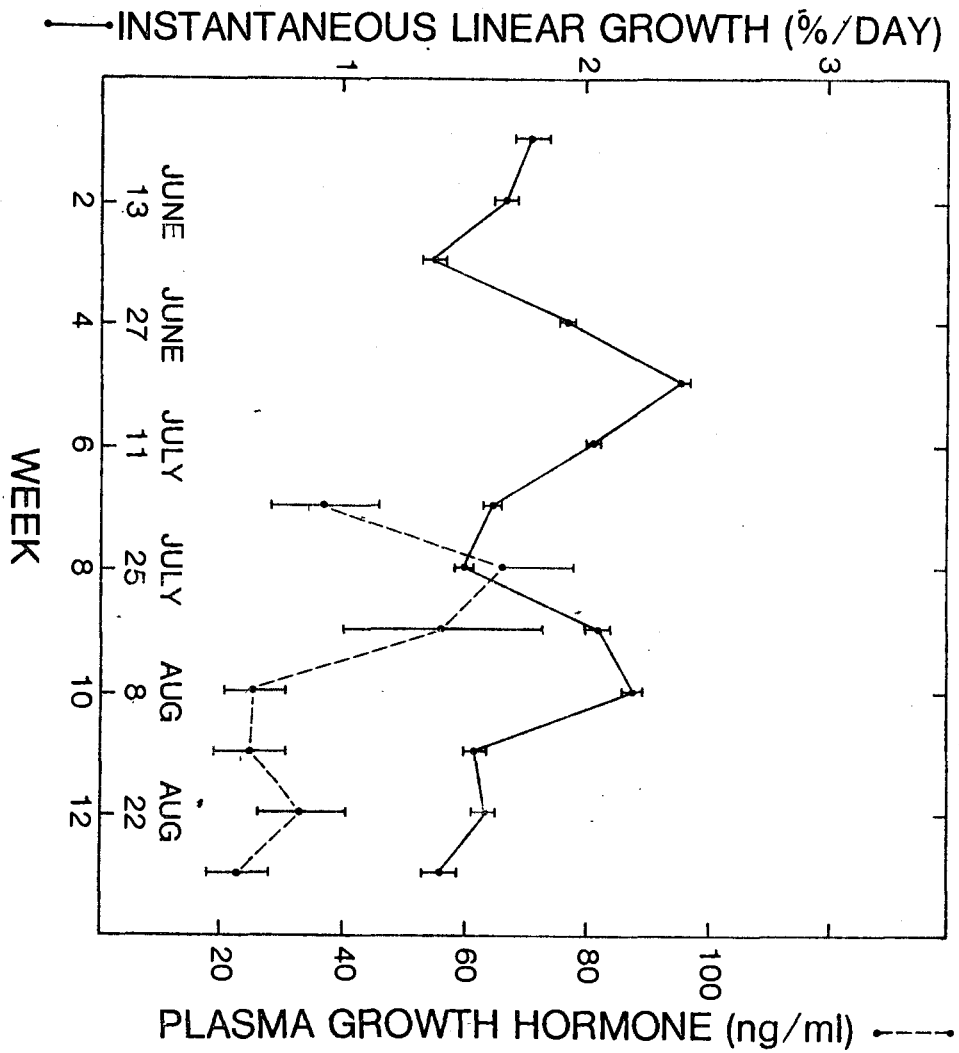
\* Numbers in parentheses indicate sample size.

## Weekly Changes in Growth Rate and Plasma Growth Hormone Levels in Rainbow Trout

The weekly growth rates and plasma GH levels for rainbow trout are shown in Figure 17. The range of the growth rate over the thirteen weeks of observation was 1.37-2.4%/day and encompassed two complete cycles of growth. The plasma GH levels, which were monitored during the second cycle of growth, ranged between  $66.12 \pm 11.93$  ( $x \pm \text{SEM}$ ) and  $23.05 \pm 5.07$  ng/ml, in weeks 8 and 13 respectively. The GH levels fluctuated in a manner similar to the growth rate. However the cycle for plasma GH preceded the growth cycle by one week. Plasma GH levels were highest during week 8, one week prior to the onset of the rapid growth phase. Coincident with the peak in growth rate during week 10, plasma GH levels had declined and subsequently remained unchanged for the duration of the experiment. Analysis of variance followed by multiple range testing demonstrated that the plasma GH levels in week 8 were significantly higher than levels in weeks 10 ( $P < 0.05$ ), 11 ( $P < 0.05$ ) and 13 ( $P < 0.02$ ). The correlation equations and correlation coefficients of growth hormone levels versus growth rates are listed in the caption to Figure 15. The correlation equation calculated for the data in-phase was not significant and poorly correlated ( $r = 0.087$ ). However, when the plasma GH data was advanced one week, the regression equation was significant ( $P < 0.05$ ) and the

Figure 17.

Weekly changes in growth rate and plasma growth hormone levels in rainbow trout on a natural photoperiod (June-August). Analysis of variance followed by multiple range testing demonstrated that plasma GH levels were significantly higher in week 8, than levels in weeks 10 ( $P < 0.05$ ), 11 ( $P < 0.05$ ) and 13 ( $P < 0.02$ ). The correlation equation for growth rate versus plasma growth hormone levels in phase ( $y = (0.0005)x + 1.45$ ) was not significant ( $P > 0.5$ ) and poorly correlated ( $r = 0.069$ ). When the plasma GH data were advanced one week out of phase, the correlation equation ( $y = (0.006)x + 1.25$ ) was significant ( $P < 0.05$ ) and well correlated ( $r = 0.865$ ). When the plasma GH data were advanced two weeks out of phase, the regression equation ( $y = (0.003)x + 1.34$ ) was not significant ( $P > 0.5$ ) and poorly correlated ( $r = 0.56$ ).



data were well correlated ( $r=0.87$ ). When the plasma GH data was advanced two weeks, the correlation equation was not significant and the data were poorly correlated ( $r=0.56$ ).

## D. Discussion

### Purification of Salmon Growth Hormones

As a result of the purification procedures employed, growth hormone has been isolated from two species of Pacific salmon. Despite the differing yields that were obtained from chum and coho pituitaries, GH was obtained from each species in sufficient quantities to bioassay and characterize them comparatively. Structurally, the pituitary GH molecule is similar in the chum and coho salmon. In SDS electrophoresis gels (Fig. 4), both GH species migrate as single bands with calculated molecular weights of 23,500 and 22,500 daltons respectively. Chum PI and PII migrate as single bands in acid-urea electrophoresis gels at pH 4.5. Coho GH migrates as a single band under the same conditions. In alkaline (pH 8.3) gels both salmon GHs separate into two or three distinct bands with Rf's of 0.35, 0.41 and 0.45. What has been classified as PI, consists of a single band with an Rf of 0.35. The bands which arise after electrophoresis of PII have Rf's of 0.41 and 0.45. This behaviour of the salmon GHs in alkaline electrophoresis gels is also reflected in their elution sequence from DEAE Bio-gel (Fig. 2). The least negatively charged species, PI, elutes first from the ionic exchange column and has the lowest

Rf in alkaline gels. The two more negatively charged species which comprise PII elute later from the ionic exchange column and also migrate faster in alkaline gels. This differing behaviour of the salmon GHs in alkaline as opposed to acid gels has also been observed for all vertebrate GHs, including all of the fish GHs that have been isolated thus far (Cook et al., 1983; Farmer and Papkoff, 1979; Farmer et al., 1981). Several theories have been proffered as explanations. In the case of bovine GH, N-terminus heterogeneity has been identified as the cause (Wallis and Davis, 1976). Bovine GH has both alanine and phenylalanine on the N-terminal. It has been postulated that the cleavage of the GH molecule from a larger precursor is due to an endopeptidase which is specific for two sites. Depending upon where the cleavage occurs, the resulting N-terminal can be either alanine or phenylalanine (Wallis and Davis, 1976). The differing pKa's of the  $\alpha$ -amino groups then allows the alanyl and phenylalanyl chains of bovine GH to be easily separated by isoelectric focusing (Ellis et al., 1972) or by polyacrylamide gel electrophoresis (Wallis and Davis, 1976). In cases where a GH is composed of more than two bands, deamidization has been identified as the cause. The heterogeneity of most of the vertebrate GHs is due to deamidization (Farmer and Papkoff, 1979). In the case of GH isolated from the blue shark (Prionace glauca), a single GH band was observed after alkaline electrophoresis of a fresh pituitary extract. The purified hormone on the other hand, exhibited multiple bands in the same

electrophoretic system (Lewis et al., 1972). Deamidization is not the cause however, for the multiple bands exhibited by tilapia GH. Leucine, isoleucine and valine have each been identified on the N-terminus of the GH molecule in this species (Farmer et al., 1976). This is thought to be caused by single base mutations of the gene coding for phenylalanine, the most common N-terminal residue among the vertebrate GHs, resulting in the aforementioned amino acids being coded for instead (Farmer et al., 1976). The specific cause of the differentially charged forms of salmon GH has not yet been identified, although it may be due in large part to the freshness of the pituitary glands employed. The fresher coho pituitaries consistently yielded smaller quantities of PII than the chum pituitaries, which were often in an advanced state of necrosis. In fact, in one instance, the fastest migrating band of coho PII ( $R_f=0.45$ ) was entirely absent in the final yield (Fig. 4).

In spite of the charge differences, chum salmon PI and PII appear to be very similar. They share immunological identity according to the immunodiffusion study (Fig. 5) and they are both capable of promoting trout growth (Table 1, Fig. 6) at a dosage of 1 ug/g/body weight. In terms of absolute weight gain, chum PI appears to be more potent than chum PII (Table 1). However, by calculating growth on the basis of cumulative percent weight gain (Fig. 6), which eliminates the initial size differences of fish both within individual groups and between treatment groups, chum PI and PII appear to be equipotent. In an



earlier reported partial isolation of chum salmon GH, Idler et al. (1978) also identified two fractions eluting from the ionic exchange column, DEAE 2 and DEAE 3, each of which had growth-promoting activity in rainbow trout. These fractions eluted from the ionic exchange column between 0.016-0.024 and 0.024-0.04 M ammonium bicarbonate, pH 9.0. DEAE 2 was more potent than DEAE 3 in increasing the growth rate of rainbow trout. In this case growth rate was also calculated on the basis of percent weight gain. It was concluded that DEAE 2 was growth hormone and that the activity inherent in DEAE 3 was due to cross-contamination (Komourdjian and Idler, 1979). DEAE 2 consisted of three bands under both SDS and acidic (pH 4.5) electrophoretic conditions, whereas the electrophoretic characteristics of DEAE 3 were not reported. It is possible however that DEAE 2 corresponds roughly to what has been described here as PI and that DEAE 3 is synonymous with PII. The only possible comparison however is between DEAE 2 and chum salmon PI; DEAE 2 consists of at least three different proteins, whereas PI is comprised of only one. One other notable difference is in the molecular weight estimation of the chum "somatotropic principle", as it is referred to by Idler et al. (1978). The major protein in DEAE 2 has an estimated molecular weight of 18,400 daltons, with contaminants of 43,000 and 73,000 daltons, as estimated by SDS electrophoresis (Weber and Osborn, 1969). In this study, chum and coho GH migrated in SDS gels with calculated molecular weights of 23,500 and 22,500 daltons

respectively. The difference in the molecular weight estimates for chum GH by Idler and myself may be due to the different SDS procedures employed. Using the technique of Weber and Osborn (1969), I observed that coho and chum had the same apparent molecular weight. By the Laemmli (1970) procedure however, a difference of 1000 daltons became apparent. This may be due to the fact that the Weber-Osborn technique employs a 10% acrylamide gel, whereas the Laemmli technique employs a 12.5% gel.

In further corroboration of the results obtained in this study, two GH species have also been identified in the eel (Anguilla anguilla) pituitary gland by Ingleton and Stribley (1977). In this instance, GH1 and GH2 as they were classified, were separated by alkaline electrophoresis (pH 8.3) and compared immunologically. Antisera raised against each GH species reacted specifically with the GH-cells in the eel pituitary and both GH's shared immunological identity as well. It appears likely therefore, that the different molecular species which comprise a vertebrate GH are biologically and immunologically similar, despite the observed charge differences.

Nevertheless, in future purifications of salmon GH, it may be advisable to use only the Peak I fraction for physiological studies. For if the charge differences of Peak II are due to damage of some kind, this damage could result in subtle or even radical changes in the true physiology of the hormone.

## The Bioassay of Salmon Growth Hormones in Rainbow Trout

There is not a standardized bioassay for fish GHs. A variety of fish species have been employed, intact and hypophysectomized, to study the effects of both mammalian and fish GH preparations on fish growth (for a review, see Donaldson *et al.*, 1979). The parameters that have been monitored following GH therapy include body water content, total body lipid, condition factor, salinity tolerance and gains in length and weight to name a few. Due to my contrasting results obtained in the tibia tests, chum and coho GH were also compared in the intact trout bioassay. Initially, the bioassays were to be conducted with coho salmon obtained from Capilano Hatchery in North Vancouver. However, these fish became so stressed following the anaesthetization and weighing procedures that they would not feed for days afterwards. For this reason, rainbow trout were subsequently employed, as they do not respond as severely to the same handling procedures. A dose-response format was chosen for the comparative bioassays to determine the lowest dosage of salmon GH which would induce a significant rise in the rate of growth. One microgram/g/body weight (BWT) was chosen as the highest dosage, on the basis of the results obtained in the first trout bioassay of chum PI and PII (experiment 1). The results demonstrated that one microgram is the minimum dosage required to cause a significant rise in the growth rate under the specified assay conditions. In experiments two and three

however, the responses of the high dose (1 ug) groups to GH therapy were not as pronounced as those in experiment one. Two differences in particular between the experimental protocols may account for this fact. In experiment one, the hormone dosages were adjusted on each weighing day; that is twice weekly, while in experiments two and three the dosages were adjusted only once a week, as this was how often the fish were weighed. Therefore the fish in experiment one received more GH on a weekly basis than the fish in the later experiments. Secondly, the fish in experiments two and three were maintained on a fixed ration level of 15% BWT/week, whereas the fish in experiment one were fed ad libitum. As GH treatment is known to improve the appetite of young salmonids (Donaldson et al., 1979), it is possible that the fish in experiment one had a greater food availability and consequently grew at a faster rate.

It appears that despite the inactivity of coho GH in the rat tibia test, it has demonstrable bioactivity in rainbow trout. Experiment two (Fig. 7) showed that both salmon GH preparations induced a significant rise in the growth rate during the first week, but not thereafter. For this reason, the bioassay was repeated. However, in experiment three (Fig. 8) growth hormone significantly stimulated the growth rate again only during one of the three weeks and only at the highest dosage. These experiments suggest that GH may not always be anabolic, at least in rainbow trout. In both of these bioassays and in the long-term growth study (Fig. 9), time (week in this

case) was a significant determinant of the growth rate ( $P < 0.005$ ). There was also a significant interaction between GH treatment and the week of administration upon growth rate in experiment three and a similar trend, while not significant ( $P = 0.12$ ) was also evident in experiment two. These results suggest that the receptiveness of rainbow trout to the anabolic effects of GH is week dependent. Furthermore, the negative effects of GH treatment which were observed in the third week of experiment three (Fig. 8) suggest conversely, that GH may also exert catabolic or other effects depending on which week this hormone is administered. What these other effects may be is not known. However, the possibility that these negative effects were due to contaminants in the GH preparations should not be ruled out.

In a previous bioassay of carp GH in intact goldfish (Carrasius auratus), Cook et al. (1983) observed what was described as a progressive reduction in the growth response to continued GH treatment. The cause was attributed to a plateauing of the response of goldfish to GH treatment and to handling stress associated with the injection procedures. However my observed responses of trout to salmon GH do not appear to be due to plateauing or a reduction in sensitivity to the hormone. In experiment two (Fig. 7), increased growth was observed in the first week. However in experiment three (Fig. 8), increased growth was not evident until week two. A major difference between my bioassays and those conducted by Cook et al. (1983)

was the photoperiod regime used. Photoperiod is a governing influence of GH cell activity in rainbow trout (Leatherland and Nuti, 1982), GH secretion in kokanee (O. nerka) salmon (Leatherland et al., 1974) and fish growth (Brett, 1979). The use of a fixed photoperiod by Cook et al. (1983) may possibly have eliminated or reduced the influence of this environmental cue upon growth and ultimately GH action. An alternate explanation for the periodicity of GH action may be found in the cyclical pattern of growth observed in the trout population following the second bioassay (Fig. 9). As most previous growth studies with fish have involved 'bi-monthly or monthly growth measurements and as the data have usually been expressed in a cumulative manner, this cyclical phenomenon has not been commonly observed before. A similar pattern of growth has only been observed in the brown trout (Salmo trutta), which exhibit a complete cycle of growth every four to six weeks (Brown, 1945). The results of this growth study have also demonstrated that rainbow trout do not grow at the same rate from week to week. They go through alternating phases of accelerated and reduced growth every three to six weeks, suggesting that the endocrine factors controlling growth may cycle as well. The data comprising the first three weeks of the growth cycle in Figure 9 represent the growth rates for the whole population during GH treatment in experiment three. The population was entering a high growth phase during the first week and subsequently achieved a maximum growth rate in week two. Although GH

treatment did not significantly accelerate the growth rate during week one (Fig. 8), a trend was nevertheless evident. In week two however, coincident with the peak in growth rate, the hormone exerted a significant anabolic effect. It was then between weeks two and three, coincident with the decline in growth rate, that the negative effect of GH was observed. It appears therefore that GH may only serve an anabolic function during periods of rapid growth. During periods of reduced growth however, it may then have an alternate function. What this function may be remains to be determined, but will be discussed in further detail in conjunction with the plasma GH studies later on in the discussion.

#### The Bioassay of the Salmon Growth Hormones in the Rat Tibia Test

In contrast to the similarities between chum and coho GH, a major difference became apparent in the rat tibia tests. Whereas chum GH and a sockeye salmon pituitary extract (although at a much higher apparent GH dosage than chum) significantly stimulated tibial growth, coho GH did not elicit a response at either dosage (Tables II and III). In a previous study, Hayashida and Lagios (1968) were also unable to stimulate tibial growth with a coho salmon pituitary extract, which supports the present results with purified coho GH. In any event, it is surprising to find that GHs from species of the same genus differ so radically in their bioactivities. This discrepancy

between chum and coho GH was not apparent in the trout bioassays (Fig. 6 and 7) and suggests that there may be sequence differences in the active sites of the two salmon GHs, which are only made manifest in a mammalian bioassay system. On the basis of previous studies with fish GHs however, the tibial bioactivity displayed by chum GH and the sockeye pituitary extract was entirely unexpected. Pituitary extracts and GHs purified from primitive fishes (elasmobranchs, chondrosteans, holosteans, crossopterygians and dipnoans) are all bioactive in the rat tibia test (Farmer et al., 1980; Hayashida, 1970, 1971, 1973; Hayashida and Lagios, 1969; Hayashida and Lewis, 1978) as are GHs from representatives of all the vertebrate classes (Farmer and Papkoff, 1979). Until now however, teleostean GHs and pituitary extracts have been found to possess little or no bioactivity in this same assay (Hayashida and Lagios, 1969; Farmer et al., 1976). Furthermore, immunodiffusion and radioimmunoassay (RIA) studies employing a rat GH antiserum have also confirmed the immunological uniqueness of teleostean GHs (Farmer et al., 1976; Hayashida and Lagios, 1969; Hayashida and Lewis, 1978). The results of these studies have prompted Hayashida to conclude that the teleosts are an evolutionary divergent group, at least with respect to the GH molecule. The main argument that can be raised against this hypothesis is simply that all of the biological comparisons were made using the rat tibia test and that most of the immunological comparisons were likewise conducted with a rat GH RIA. Although



this approach does permit comparisons to be made, it is narrow in scope and the results are not substantiated by similar studies employing GH RIA's from lower vertebrates.

When GHs from each of the vertebrate classes, including pituitary extracts from primitive fishes, were compared in a homologous RIA for shark GH, they all showed weak cross-reactivity and non-parallel slopes of inhibition compared to the shark GH standard curve, including rat GH. It could have been concluded from these studies that the sharks, like the teleosts, are also unique with respect to the GH molecule. These results were disclaimed however, 'as being due to radioiodination damage of the shark GH label (Hayashida and Lewis, 1978). Instead, the authors then employed rat GH as label and standards along with the same shark GH antiserum and thus obtained results which conformed with the previously outlined hypothesis (Hayashida and Lewis, 1978). In a similar study employing a homologous teleost GH RIA, chondrosteian and higher vertebrate GHs again showed weak cross-reactivity, as did a pituitary extract from a teleost fish, the perch (Phanerodon furcatus) (Farmer et al., 1976). More recently, Cook et al. (1983) has demonstrated the poor immunoreactivity of coho plasma GH in a homologous RIA for carp (Cyprinus carpio) GH. In view of these latter studies and the rat tibia test results with the salmon GHs, it appears rather that the teleost GHs are both biologically and immunologically diverse, and that this diversity is not necessarily phylogenically related.

## Amino Acid Analysis of the Salmon Growth Hormones

To date, three fish growth hormones have been successfully isolated to homogeneity and characterized with respect to electrophoretic mobility, molecular weight and amino acid composition; two from the superorder Teleostei (Cook et al., 1983; Farmer et al., 1976) and one from the superorder Chondrostei (Farmer et al., 1980). All of these fish GHs consist of three bands upon electrophoresis under alkaline conditions and one band under acidic conditions. The teleost GHs, tilapia and carp, have molecular weights of 22,200 and 22,500 daltons respectively, whereas sturgeon GH has a molecular weight of 23,500 daltons. By comparison, chum and coho GH have similar electrophoretic characteristics and similar estimated molecular weights. In terms of amino acid composition, chum and coho GH are also very similar and may be considered as indistinguishable, taking into account the error inherent in the amino acid analysis which was not corrected for hydrolytic destruction. They differ most notably in the higher proline content of coho GH (Table IV). Whereas most vertebrate GHs have between six and eleven proline residues, coho GH has thirteen and is only surpassed by chicken GH which has fourteen proline residues (Farmer and Papkoff, 1979). This differing proline content of chum and coho GH may be responsible, in part, for the differing bioactivities observed in the rat tibia tests (Tables

II and III), as this amino acid is known to be a helix interrupter. Although the high proline content of chicken GH has not affected its activity in the rat tibia test (Farmer and Papkoff, 1979), it can also be argued that the specific location of the proline residues in the primary sequence of the GH molecule may be equally as important. Such may be the case for coho GH, although unequivocal proof of this will only be forthcoming with the complete sequencing of the molecule. An alternate explanation for this bioassay discrepancy may be differing primary structures of the two salmon GHs, probably in the region of the active site as the two hormones appear to have common antigenic determinants. A consideration which must also be taken into account is that the higher proline content in coho GH might be due to contaminants in the preparation, although this is the only apparent discrepancy between the compositions of the two salmon growth hormones.

Comparatively, the salmon GHs are most similar in amino acid composition to carp GH (Table IV), which also has equimolar concentrations of leucine, aspartic and glutamic acid (Cook et al., 1983). The proline content of carp GH is low however, like that of chum GH. The main compositional criteria for the vertebrate GHs however, are those established by Wilhelmi (1974). These include a low histidine and methionine content, a high content of leucine and glutamic acid, two disulphide bridges and a single tryptophan residue. Both of the salmon GHs isolated in this study satisfy these criteria.

In comparison to chum salmon prolactin (Kawauchi et al., 1983), the salmon GHs display some major compositional differences (Table IV). The salmon GHs have approximately twice the number of tyrosine, valine and glutamic acid residues as salmon prolactin, in addition to lower quantities of serine, arginine and especially methionine. The two hormones are very similar in most other respects including the two disulphide bridges and a single tryptophan residue. The proline content of salmon prolactin is also high (Kawauchi et al., 1983), similar to coho GH.

Immunocytochemical and Immunodiffusion Studies with an Antiserum to Chum Salmon Growth Hormone

Teleost GH cells were first localized immunocytochemically in the sockeye salmon, using an antiserum raised against ovine GH (McKeown and van Overbeeke, 1971). The identity of these cells was suggested earlier on the basis of histophysiological studies (for a review, see Ball and Baker, 1969) and they are now known to comprise the acidophils in the caudal pars distalis of the pituitary gland. Since then, several laboratories have utilized immunocytochemistry to identify pituitary GH cells in a variety of fish species using antisera raised against both mammalian (Chadwick et al., 1976; Hansen and Hansen, 1975) and teleost GHs (Cook et al., 1983; Ingleton and Stribley, 1975, 1977; Komourdjian and Idler, 1979; Nagahama et al., 1981). This

study extends the earlier investigation of McKeown and van Overbeeke (1971) with the identification of GH cells in three other species of salmon, in addition to the sockeye. It also represents the first homologous immunocytochemical localization of GH cells in salmon. These cells are acidophilic as well, as confirmed in the coho pituitary by the staining of adjacent sections with hematoxylin and eosin. In the case of the sockeye salmon (Fig. 10, B), the cells identified with the chum GH antiserum correspond in both appearance and distribution to those identified by McKeown and van Overbeeke (1971) with an ovine GH antiserum. One notable exception however, was the observation of isolated immunoreactive cells in the pars intermedia of the sockeye and all other species investigated. Similar observations have been noted in the pars intermedia of the sturgeon (Acipenser guldensadti) pituitary, using a bovine GH antiserum (Hansen and Hansen, 1975). While the function of the GH cells here remains to be determined, it is noteworthy that they stained more intensely than cells in the caudal pars distalis, suggesting a different level of activity.

Morphologically, the pituitary GH cells are elliptical in appearance, with cytoplasmic extensions to nearby capillaries (Fig. 10, D) thus facilitating GH release into the circulation. The GH cells are more densely packed in juveniles (Fig. 10, C) than in sexually mature salmon (Fig. 10; A, B and D) due to the development and proliferation of gonadotropic cells in the latter instance. The GH cell population stained by the antiserum

should be homogenous, as the immunodiffusion of pituitary homogenates resulted in a single precipitin line for each species. The immunodiffusion study (not shown) has also demonstrated that the GH molecule is immunologically identical among all species of salmon under investigation, suggesting that the chum GH antiserum is likely suitable for radioimmunological measurements of plasma GH in all of these species.

#### Development of a Radioimmunoassay for Salmon Growth Hormone

The RIA developed in this study represents the first validated assay for the measurement of plasma GH levels in salmon and trout. The calculated coefficients of variation indicate that the assay is consistent, within a single assay and between separately conducted assays, in the measurement of plasma growth hormone. The recoveries of GH added to plasma, demonstrate that there is minimal interference by plasma constituents as well. The parallelism studies (Figs. 14 and 15) have affirmed that the plasma form of the GH molecule in salmon and trout is immunologically similar to the pituitary form of the hormone and that the assay is valid for plasma GH measurements in the genera Salmo and Oncorhynchus. Plasma GH levels in juvenile rainbow trout ranged between 10-240 ng/ml in the experiments conducted with the RIA. This range includes however only those trout which were maintained in a fed condition and which were not subjected to any experimental

manipulations. The Sephadex G-50 profile of fractionated coho plasma (Fig. 16) shows that the immunoreactive component in the plasma comprises either a single or similar molecular weight species of GH that are of approximately the same molecular weight as the pituitary form of the hormone. The sensitivity of the assay (2.5 ng/ml) also allows GH measurements to be conducted on as little as 10 ul of plasma. Comparatively, the salmon GH RIA has performance characteristics similar to those reported by Cook et al. (1983) for a carp GH radioimmunoassay. These two RIAs comprise the only available assays for teleost growth hormone. Prior to this, plasma GH levels in teleosts (salmon and goldfish) were monitored by an RIA employing ovine GH and an ovine GH antiserum (Leatherland et al., 1974; McKeown and van Overbeeke, 1972; Peter et al., 1976). Although this assay was reported to measure plasma GH-like immunoreactivity and the antiserum employed was specific for the GH cells in the salmon pituitary (McKeown and van Overbeeke, 1971), the limitations of a heterologous assay must also be recognized, especially in terms of lack of sensitivity and in its inability to measure absolute plasma GH levels. However, problems of this kind are not only confined to heterologous assays that cross vertebrate class boundaries. Although Cook et al. (1983) reported parallelism of trout plasma in a carp GH RIA, parallelism was not obtained with plasma from coho salmon, suggesting once more that the teleost GHs are extremely diverse. This particular observation by Cook et al. (1983) is even more

interesting as it contradicts the parallelism results obtained in this study; that coho and trout plasma GH are immunologically identical. The reason for this discrepancy may be that the carp and chum GH antisera were raised against different antigenic determinants of the two respective GH molecules. This would then imply that carp and trout GH share antigenic determinants which are not shared by trout and salmon, or that this particular antigenic sequence is hidden in salmon growth hormone.

#### The Effects of Handling Stress on Plasma Growth Hormone Levels in Rainbow Trout

Stress in fish is accompanied by changes in many blood parameters which have been viewed as physiological adaptations to a potentially harmful situation. In rainbow trout, stress of various types is associated with significant elevations in plasma glucose (Wagner and McKeown, 1982; Watson and McKeown, 1976a), plasma corticosteroids (Donaldson and Dye, 1975; Mazeud et al., 1977; Watson and McKeown, 1976b) and plasma catecholamines (Mazeud et al., 1977; Nakano and Tomlinson, 1967). Zinc stress in trout is also accompanied by a depletion in liver glycogen and concomitantly depressed plasma insulin levels (Wagner and McKeown, 1982) and a reduction in the rate of body growth (Watson and McKeown, 1976a). These responses are believed to occur for the mobilization of body reserves and are similar to the effects of stress in mammals (Porte and



Robertson, 1973). Plasma GH levels in rats are significantly depressed by a variety of stressors which include forced handling, forced immobilization, electric shock, injections, forced swimming and reduced temperatures (Arimura et al., 1976; Collu et al., 1973; Lenox et al., 1980; Martin, 1974; Miodusjewski and Critchlow, 1982; Terry et al., 1976). This depression of plasma GH can be reduced (Terry et al., 1976) or completely suppressed (Martin, 1974) by treatment of the animals with an antiserum to somatostatin. These results suggest that the inhibition of GH secretion during stress is mediated via an increased somatostatin release from the hypothalamus. In humans on the other hand, anaesthesia and surgical stress cause an elevation in plasma GH levels (Glick et al., 1965).

Rainbow trout do not respond to handling stress in either of these two ways. No changes were observed in plasma GH levels, one and seven hours following the induction of handling stress (Table V), suggesting that in rainbow trout, GH is not involved in the induction of the stress response. Alternatively, it might be suggested that as these were not wild trout and were somewhat used to handling, that they would not be expected to respond as severely to stress of this nature. In the eel (Anquilla anguilla), pituitary GH cells are also not stimulated by stressors such as anaesthesia and bleeding (Oliverreau, 1967a) but are activated by surgical stress (Oliverreau, 1967b; Oliverreau and Oliverreau, 1968). In mammals, the reasons for the changes in plasma GH levels have unfortunately not been pursued.

Most studies have rather been concerned with the mechanism by which GH secretion is reduced and consequently there is no information with which comparative assessments can be made. These results can be viewed positively however, in the sense that the handling associated with blood sampling will not effect plasma GH levels and thus influence experimental results with this species.

#### The Effects of Long-Term Starvation on Plasma Growth Hormone Levels in Rainbow Trout

In humans, fasting is accompanied by increased levels of plasma GH (Glick et al., 1965; Goschke, 1977; Marks et al., 1965; Roth et al., 1963; Unger et al., 1965). This response has been considered as counteractive to impending carbohydrate deficiency and subsequent hypoglycemia (Glick et al., 1965; Marks et al., 1965) by reducing both peripheral glucose utilization and hepatic glucose production (Adamson et al., 1977; Sherwin et al., 1983). In this manner, carbohydrate and protein reserves are spared and greater reliance is placed upon fat as a metabolic fuel (Glick et al., 1965). In contrast to humans, plasma GH levels and the episodic release of GH are suppressed in fasting rats (Dickerman et al., 1966; Tannenbaum et al., 1978).

The response of fish to prolonged fasting is similar to that observed in humans. There is a pronounced stimulation of

the GH cells in the carp, eel and goldfish as a result of prolonged food deprivation (Gas, 1975; Nagahama, 1973; Olivereau, 1970; cf. Nagahama et al., 1981). In corroboration with these early histological studies, rainbow trout responded to a three week fast with plasma GH levels approximately nine times higher than levels observed in trout on a normal feeding regime (Table VI). In a comparable study, McKeown et al. (1975) observed no changes in plasma GH levels of kokanee salmon starved for thirty days. This discrepancy may however be due to the different method employed for the measurement of plasma GH or to a differing response of this species to starvation. Insulin levels and thyroid activity are depressed in starved rainbow trout (Higgs and Eales, 1977; Leatherland et al., 1977; Thorpe and Ince, 1976) suggesting that these hormonal changes also occur in response to the altered metabolic state induced by food deprivation. A number of studies have been conducted with salmonids during starvation to elucidate the metabolic survival strategies employed. Following ten days of starvation, nitrogen excretion, which is an indicator of protein catabolism, is reduced in rainbow trout (Smith and Thorpe, 1977). With further starvation (3-4 weeks), there is a depletion of the free amino acid pool in the musculature (Timoshina and Shabalina, 1972) and visceral lipid reserves (Weatherly and Gill, 1981). In kokanee salmon, there is also a decline in plasma free fatty acids and liver glycogen and an increase in muscle glycogen (McKeown et al., 1975). With more prolonged starvation, muscle contractile

protein is then catabolized (Timoshina and Shabalina, 1972) and there is a rise in hepatic amino transferases to facilitate this protein catabolism (Gurss and Nicolai, 1977). The white musculature, which is largely anaerobic and used primarily for short bursts of swimming, is catabolized first, thus conserving the aerobic red musculature for sustained swimming (Love, 1980). Taken together, these studies suggest that during the initial stages of starvation, body protein is spared while amino acid and lipid reserves are mobilized to support fuel requirements. Amino acids are catabolized through gluconeogenic pathways to maintain adequate carbohydrate reserves while fats are catabolized by swimming and cardiac musculature (Love, 1980). With prolonged starvation and the subsequent depletion of these reserves, body protein is then mobilized. A similar course of events takes place during the spawning migration of the salmon (Duncan and Tarr, 1958; Idler and Bitners, 1958; Wood et al., 1960).

The dramatic rise in plasma GH levels of starved trout suggests that this hormone plays an important role in the mobilization of these reserves. However, as there have been no studies to date on the metabolic effects of fish GHs, the precise nature of this role can only be speculated on. Mammalian GH promotes nitrogen retention in fed rainbow trout, but has no effect on the increased nitrogen retention observed in this species during food deprivation (Smith and Thorpe, 1977). This suggests that protein conservation is maximally stimulated by

endogenous GH during starvation and cannot be augmented further by GH treatment. Mammalian GH also mobilizes lipid reserves and increases liver glycogen in kokanee salmon (McKeown et al., 1975) and reduces plasma amino acid levels in goldfish (Prack et al., 1980). Furthermore, plasma GH levels in salmon are elevated during enforced exercise (McKeown et al., 1975) and during the upstream spawning migration when the fish are not actively feeding (McKeown and van Overbeeke, 1972). These few studies indicate that the function of GH in fish is very similar to its role in mammals. Therefore, the elevated plasma GH levels in starved trout, may similarly act permissively on the sequential mobilization of metabolic reserves as outlined above, as is the case in humans (Glick et al., 1965).

#### Weekly Changes in Growth Rate and Plasma Growth Hormone Levels in Rainbow Trout

The weekly profiles of growth rate and plasma GH levels in trout are shown in Figure 17. The results have demonstrated that plasma GH levels are significantly correlated with the weekly growth rate in this species. However, this correlation is one week out of phase with the growth rate, implying that the effects of GH are not immediate, at least with respect to somatic growth. Growth hormone levels peaked during week 8, while the trout population was still in a low growth phase and then dropped progressively thereafter as the population entered

a high growth phase. The progressive decline in GH levels might be due to a decreased secretory activity of the GH cells or to an increased usage of the hormone, but may also be due to a combination of the two. As plasma GH levels and the growth rate cycle, it would follow that the pattern of GH secretion and tissue receptiveness are cyclical as well. Although the secretory pattern of these cells has not been investigated on a weekly basis, the nuclear diameters of these cells do cycle on a daily basis under photoperiodic influence (Leatherland and Nuti, 1982). Similarly, plasma GH levels in kokanee salmon fluctuate according to the photoperiod (Leatherland et al., 1974). In rats, plasma GH levels are also entrained to the light-dark cycle (Tannenbaum and Martin, 1976). In the present study, the photoperiod was adjusted weekly, resulting in a progressive rise in the light phase until June 22, followed by a progressive decline thereafter. Therefore, if the light-dark cycle does influence the weekly growth rate of rainbow trout, the cues may be the changing lengths of the light and dark phases, rather than when the lights turn on and off.

Weekly changes in growth rate have not been commonly observed principally because of the methods which have been employed to monitor growth. Fish growth has usually been expressed as the cumulative absolute weight or length gain or the cumulative percent gain over time, using the initial body size as the starting point of reference. Furthermore, the fish utilized have generally not been individually tagged for

identification. Data expressed in this manner does not allow any weekly changes in growth rate to be observed. The growth curves plotted for experiment one, the bioassay of chum PI and PII (Fig. 6) illustrate this point. Experiments two and three (Figs. 7 and 8) have shown that GH can be anabolic and possibly catabolic during different phases of the growth cycle. The anabolic effects were expected as GH is known to promote growth in all vertebrates. However the possible catabolic effects were unexpected and may in fact be non-physiological, in the sense that the hormone was administered at the end of a high growth phase, when plasma GH levels have subsequently been found to be low (Fig. 17). The cycling of the growth rate suggests that growth in trout involves at least two metabolic events, one of which is the assimilation of metabolites for somatic growth during the high growth phases. The events which occur during a low growth phase however, are less obvious. It is possible that the accumulation of growth precursors takes place during this time. In this manner, the low growth phase could be considered as preparatory to a period of accelerated growth. The observation that plasma GH levels are low during periods of reduced growth (Fig. 17) and that GH treatment reduced the growth rate at this time (Fig. 8) also suggests that the hormone may have an entirely different function here. This would likely involve intermediary metabolic processes, as is the case in humans (Glick et al., 1965). The uptake of growth precursors may be mediated by other hormonal factors, such as insulin. It is

then with the onset of a high growth phase that plasma GH levels rise, possibly for the mobilization of these reserves and subsequent growth. From this viewpoint, the catabolic effects of GH in experiment three may have been due to the mobilization of reserves at a time when the fish was not prepared or able to use them. However, as the physiological actions of salmon GH still remain largely unknown, these conclusions can only be considered as speculative.

Recent studies on GH physiology in rats provide some evidence suggesting that this proposed sequence of events could indeed be possible. Rats grow at a relatively constant rate and there is no correlation between growth rate and plasma GH levels (Eden, 1979). Surges in plasma GH occur approximately every three hours (<200 ng/ml), followed by nearly undetectable levels one-two hours later (Tannenbaum and Martin, 1976). These surges are correlated, following a lag phase of one hour, with increased amino acid transport and protein synthesis in diaphragm muscle (Albertsson-Wikland and Isaksson, 1978). The tissue then becomes refractory to GH by first slowing down amino acid transport and then protein synthesis (Albertsson-Wikland et al., 1980). The cycle of events then begins again with another GH surge and in this manner rats achieve somatic growth. Considering the lower metabolic rate of rainbow trout and the distinct manner in which they grow, phases of reduced growth may represent anabolic refractory periods, only protracted in length. In the same regard, the one week lag in the growth



response to increased GH levels may simply be the time necessary to achieve a full anabolic response in this species.

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