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THE MULTIPLE HEMOGLOBINS OF COHO SALMON, *Oncorhynchus kisutch*.

by

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B.Sc. Hon., University of Manitoba, 1965

M.Sc., University of British Columbia, 1969

A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in the Department
of
Zoology

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February, 1973

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Date April 6, 1973

ABSTRACT

Studies were conducted to determine the ontogenetic changes in the number and relative concentration of the electrophoretically distinguishable hemoglobin polymorphs of coho salmon, *Oncorhynchus kisutch* and the influence of certain environmental factors upon the expression of the hemoglobin variants. In addition some of the oxygen equilibrium characteristics of the hemoglobin of freshwater fry and adult coho were investigated using both hemolyzates and whole blood.

Throughout the life cycle of coho salmon seventeen to nineteen distinct hemoglobin components were identified in micro-starch-gel electropherograms prepared in borate buffer at pH 8.5. These components formed three main electrophoretic hemoglobin patterns associated with different stages of the life cycle. Unhatched embryos and alevins possessed twelve anodic and one cathodic components. All except three anodic components had disappeared from the blood of free-swimming fry fourteen weeks after hatching. This three-component pattern was retained until the beginning of the presmolt period, approximately eleven months after hatching. At this stage, five new cathodic components, one new anodic component and one anodic component which had previously been visible in alevin electropherograms appeared. In presmolts and smolts these additional seven components accounted for less than 20% of the total hemoglobin of the blood while the three components observed in fry blood accounted for the remainder. Following migration to sea

water the relative concentration of these seven components gradually increased to 45 to 50% of the hemoglobin over a two-month period. No further change in either the number or relative concentrations of the hemoglobin components was observed during the remaining phases of the life cycle.

Since it was apparent that changes in hemoglobin pattern were temporally associated with changes in the characteristics of the environment occupied by the juvenile coho the effects of water temperature, dissolved oxygen concentration and salinity upon the physical development and electrophoretic hemoglobin pattern of underyearling coho were examined. Exposure to freshwater temperatures of 1.4 to 15.0 C, dissolved oxygen concentrations of 2.2 to 9.7 ppm and salinities of 0 to 30 $^{o}/oo$ for periods of 49 to 60 days had no influence upon the electrophoretic hemoglobin pattern of either 3 1/2-month-old fry or 11-month-old presmolts. Presmolts reared for 60 days in freshwater at 15 C and in 10 $^{o}/oo$ salinity at 9.2 C grew at a highly accelerated rate and were equal or greater in size than 16 1/2-month-old postsmolts which had been residing in sea water for one month. These large presmolts retained the hemoglobin pattern characteristic of normal presmolts of the same age. Postsmolts maintained in aerated freshwater rather than sea water underwent changes in the electrophoretic hemoglobin pattern characteristic of seawater residents. The foregoing observations suggest that age rather than physical size or environmental factors is the main determinant in the expression of the polymorphic hemoglobins of coho salmon.

The oxygen equilibrium characteristics of adult coho hemoglobin and hemoglobin components A6-8 (fry hemoglobin) isolated from adult hemolyzates by ion-exchange chromatography were investigated. Adenosine triphosphate concentrations ranging from 0.0 to 0.76 moles/mole hemoglobin had no influence upon the oxygen equilibrium of adult hemolyzates whereas at a concentration of 7.56 moles/mole P_{50} increased by 1 to 2 mm Hg. Since erythrocyte ATP concentrations of freshwater adult coho ranged between 0.8 and 1.3 moles/mole hemoglobin this organic phosphate is probably not a modifier of oxygen affinity in coho salmon.

The hemoglobin of adult coho was relatively insensitive to variations in pH and temperature with $\phi = -0.172$ at 9.8 C over the pH range of 6.95 to 8.20 and $\frac{\Delta \log P_{50}}{\Delta T} = 0.019$ between 5 and 15 C. The Bohr effect of fry hemoglobin was nonlinear so that ϕ = -0.033, -1.729 and -0.182 in the pH ranges of 6.82 to 7.08, 7.08 to 7.50 and 7.50 to 8.50, respectively. The estimate of $\frac{\Delta \log P_{50}}{\Delta T}$ was 0.056 for fry hemoglobin. Thus at 9.8 C the oxygen affinity of fry hemoglobin exceeded that of adult hemoglobin at pH greater than 7.3 but was lower at values of pH less than 7.3. At pH 7.4, the P_{50} of fry and adult coho hemoglobin was 8.4 and 17.9, respectively. In neither case was a Root effect observed.

Heme-heme interaction was similar for both adult and fry hemoglobin and the value of n always exceeded 1.0. The estimate of n was generally less than 2.0 at pH greater than 7.0

and tended to decrease as the pH or the equilibration temperature increased.

Studies with fry and adult whole blood equilibrated with 0.2 and 3.4 mm Hg of carbon dioxide generally confirmed the qualitative differences observed between the oxygen equilibria of fry and adult hemolyzates. The estimates of P_{50} at 9.3 C and P_{CO_2} of 0.2 and 3.4 mm Hg were 5.5 and 12.5 mm Hg respectively for fry blood and 10.7 and 15.6 mm Hg, respectively, for the blood of freshwater adults.

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ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to the Fisheries Research Board of Canada, West Vancouver, B.C. for providing experimental facilities and financial support throughout this investigation. Also I would like to express individual thanks to Drs. J.C. Davis, W.S. Hoar, J.D. Randall, and W.E. Vanstone for their guidance and helpful criticisms during the preparation of this manuscript.

INTRODUCTION

The major function of hemoglobin in the blood of vertebrates and most invertebrates possessing this respiratory pigment is the extraction from the environment and subsequent delivery to the tissues of molecular oxygen. In many instances hemoglobin may perform secondary functions which include the transport of carbon dioxide from the tissues to the respiratory surface (Guyton, 1961), and acting as one component in the buffer system of the blood (Rossi-Bernardi and Roughton, 1967; Reeves, 1972). The characteristics of the reversible combination of oxygen with hemoglobin and the effects of carbon dioxide, pH, temperature and certain allosteric effector substances are prime considerations in the analysis of the *in vivo* transport of oxygen. Substantial variation in these characteristics has been observed in the hemoglobin from a wide range of animals (Prosser and Brown, 1961).

In fish the oxygen equilibrium characteristics of hemoglobin are functionally adapted to maintain efficient oxygen transport under the prevalent environmental conditions. Thus fish which inhabit hypoxic water generally possess hemoglobin with a high oxygen affinity (Krough and Leitch, 1919; Black, 1940; Riggs, 1970). Willmer (1934) asserted that in fish residing in water at high carbon dioxide tensions the oxygen affinity of the hemoglobin is relatively insensitive to variations in carbon dioxide but additional evidence makes this generalization somewhat questionable (Lenfant,Johansen

and Grigg, 1966). This variation in oxygen affinity with changes in PCO_2 or pH is termed the Bohr shift and it, in certain active fish, acts to mitigate somewhat, the increase in oxygen affinity of hemoglobin which occurs at reduced temperatures (Black, Kirkpatrick and Tucker, 1966 a, b; Black, Tucker and Kirkpatrick, 1966a) thereby maintaining high oxygen tensions and rate of release of oxygen to the tissues. In other instances this difficulty is resolved by a relatively temperature-insensitive hemoglobin (Lenfant, *et at.*, 1966). In tuna, *Thunnus thynnus*, the deeper body temperatures are maintained above ambient and the oxygen affinity is completely temperature-independent (Rossi-Fanelli and Antonini, 1960). One of the most outstanding features of the hemoglobin of certain fish is the presence of a Root effect (Root, 1931) which is the decrease in oxygen capacity associated with decreases in pH or elevated PCO_2. Recent evidence suggests that this effect may be an important feature in the functioning of the gas gland of teleost swimbladders (Steen, 1969).

On the molecular level fish hemoglobins are unusual in the prevalence of multiple forms of this protein occurring in most species. Although early observations suggested that only two to four electrophoretically distinct polymorphs existed in most fish blood (Buhler and Shanks, 1959; Hashimoto and Matsuura, 1959a; Schumann, 1959) more recent investigations employing improved electrophoretical techniques have demonstrated that two to nineteen hemoglobin polymorphs may be present (Vanstone, Roberts and Tsuyuki, 1964; Yamanaka,

Yamaguchi and Matsuura, 1965; Kock *et al.*, 1967; Grigg, 1969; Tsuyuki and Ronald, 1971). Subunit analysis of the multiple hemoglobins of Atlantic salmon and certain species of Pacific salmon have indicated that the high degree of polymorphism in salmonids is probably a reflection of the tetraploid genetic condition believed to occur in these fish (Wilkins, 1970; Tsuyuki and Roberts, 1971).

Ontogenetic variations in the number and relative concentrations of the multiple hemoglobins have been described for the lamprey *Petromyzon planerii* (Adinolfi, Chieffi and Siniscalco, 1959), rainbow trout *Salmo gairdneri irideus* (Iuchi and Yamagami, 1969), herring *Clupea harengus* (Wilkins and Iles, 1966), coho salmon *Oncorhynchus kisutch* and sockeye salmon *O. nerka* (Vanstone *et al.*, 1964) and Atlantic salmon *Salmo salar* (Kock, Bergstron and Evans, 1964a, b; Wilkins, 1968; Westman, 1970). The detailed ontogenetic changes in the electrophoretically distinct hemoglobin polymorphs have been described for the latter species only and demonstrate that in some fish these changes may be extremely complex. Westman (1970) observed eight distinct electrophoretic hemoglobin patterns involving thirteen components in Atlantic salmon 3.5 to 95.0 cm in length. Although considerable overlap was reported, the particular hemoglobin pattern exhibited by these salmon was correlated with the size and presumably the age of the fish.

The question arises as to the function of the multiple hemoglobins in fish and to the control of their synthesis. Ultimately the genotype limits the number and structure of the various polymorphs. Many examples exist, however, in

both plants and animals in which environmental factors influence the expression of certain genes (Gardner, 1960). Baldwin and Hochachka (1970) have demonstrated that two electrophoretically distinct forms of acetylcholinesterase are synthetized in the brain of rainbow trout kept at 12 C. At 2 and 17 C only one polymorph was present with the slower migrating form occurring at the lower temperature. The two forms of the enzyme were functionally different with the Km of the "cold" form being much lower than that of the "warm" form (*ibid.*). A similar process apparently occurs in the regulation of the synthesis of lactate dehydrogenase isoenzymes in trout (Hochachka and Somero, 1968). Thus although these salmonids are capable of synthetizing different isoenzymes it is apparent that environmental temperatures regulate the expression of the appropriate genes.

Coho salmon are anadromous fish and therefore reside in at least three distinct environments during the course of their development: the stream-bed as embryos and alevins, the stream as fry and smolts and the sea as grilse and maturing adults. Vanstone *et al.* (1964) observed three distinct electrophoretical hemoglobin patterns associated with the fry, smolt and seawater postsmolt stages of the coho life-cycle. Although the precise timing of the transition from one pattern to another was not described these observations indicated that the different hemoglobin patterns could be associated with either environmental or ontogenetic changes or a combination of both factors. The present investigation was conducted, therefore, to define precisely the ontogenetic changes in the

electrophoretic hemoglobin pattern of coho salmon from the embryonic to the adult stages of the life-cycle and to test the hypothesis that at certain stages of development environmental temperature, dissolved oxygen concentration and salinity exert some form of control upon the number or relative concentration of the hemoglobin polymorphs. A second aspect of this thesis was to investigate some of the functional characteristics of the hemoglobins representative of fish at different stages of development in relation to their respective environmental regimes.

GENERAL METHODS

EXPERIMENTAL ANIMALS

All fish studied were coho salmon, *Oncorhynchus kisutch*, the majority of which had been raised in the aquarium facilities of the Fisheries Research Board of Canada in West Vancouver, B.C. Adult coho were trapped during their spawning migration at the Big Qualicum River on Vancouver Island, B.C. and transported in large aerated tanks to the aquarium. These fish were held in 1.8 or 3.0 m diameter tanks provided with aerated well water or Cypress Creek water, until sexually mature and then artificially spawned by removing the eggs from the female through a ventral slit in the abdomen and thoroughly mixing the eggs and sperm in a plastic bucket. The fertilized eggs were buried under gravel in troughs, 4.9 m by 0.4 m by 0.2 m and provided with flowing water at a temperature of 3.4-8.0 C. After hatching, the alevins were collected as they emerged from the gravel and placed in 1.8 or 3.0 m diameter aquaria. These fish were fed to satiation, five times daily on a diet composed of beef liver, 4500 g; beef heart, 4500 g; canned salmon, 4500 g; salt, 110 g; and Pablum (Meade-Johnson Canada Ltd), 40 g. After the fish had begun to grow, the feeding was gradually reduced to once daily. The fry were placed in an artificial creek (104 m by 0.6 m by 0.2 m), containing

rocky riffles, pools and aquatic vegetation and supplied with flowing water from Cypress Creek. The following spring, after approximately one year in fresh water, the smolts were transferred to 3 m aquaria and gradually exposed to increasing concentrations of sea water (salinity, ca. 27-30 $^{o}/oo$). These fish were maintained in sea water for approximately 19 months, at which time they were considered to be adults and were transferred to fresh water where they became sexually mature. Since this procedure had been initiated two years before the experiment began, fish of various ages were available at any particular time.

In the autumn of 1971, adult coho were caught at the entrance to Great Central Lake on Vancouver Island, British Columbia, during their spawning migration and transported, live, to the aquarium facilities in West Vancouver. These fish were used in the majority of studies on the oxygen equilibria of adult coho blood. The adult salmon were not fed during their residence in fresh water.

The photoperiod regime in the aquarium building was equivalent to the natural photoperiod at 45 degrees north latitude whereas the fish maintained in the outdoor facilities experienced the natural photoperiod of 49.5 degrees north latitude. Thus all experimental coho and newly emerged alevins which were maintained in the aquarium building encountered a slightly different pattern of light and darkness than the majority of the stocks of salmon.

PREPARATION OF BLOOD FOR ELECTROPHORESIS

The blood of small fish, (alevins to smolt stages), was collected in heparinized microhematocrit tubes, (I.D. 0.55, or 1.2 mm., Clay-Adams Inc.), from the severed caudal peduncle. Blood was obtained from larger fish, (grilse and adults), by aortic puncture in the tail region with an 18 or 20 gauge needle fitted with a heparinized (40 U.S.P. units/ml of blood) syringe.

The microhematocrit tubes were sealed with Seal-ease (Clay-Adams Inc.), and centrifuged at 3000 r.p.m. for 10 minutes in a clinical centrifuge (Buchler Instruments Inc.). Hematocrits were measured and the plasma and white blood cells removed by suction with a finely drawn capillary tube. The microhematocrit tube was filled with 1.0% NaCl from a finely drawn capillary. The open end was sealed by flame and the erythrocytes centrifuged through the saline. After repeating this washing procedure twice, the packed erythrocytes were placed in a desiccator containing ice. The desiccator was evacuated, then filled with carbon monoxide gas (research grade, Matheson of Canada Ltd.). This gassing procedure was repeated twice and following the final flooding with carbon monoxide, the dessicator valve was closed and a few pounds pressure of gas trapped in the vessel. After 20 minutes equilibration, twice the packed erythrocyte volume of cold, carbon monoxide gassed distilled water was placed in each microhematocrit tube and the open end of the tube flamed shut. The erythrocytes were hemolyzed by repeated

centrifugation through the distilled water in a cold room at 0-2 C. A final centrifugation at 3000 r.p.m. for 20 minutes at 0-2 C served to separate the carboxyhemoglobin solution from the cell debris. Larger blood samples were prepared in centrifuge tubes in essentially the same manner.

ELECTROPHORESIS

Eighteen grams of hydrolyzed starch (lots 261-1 and 283-1, Connought Medical Research Laboratories), were suspended in 150 ml of 0.023 m borate buffer, pH 8.5, in a 500 ml vacuum flask and heated with constant swirling over a naked flame until the suspension was clear (Smithies, 1959). The gel was degassed under vacuum during the last minute of heating, poured into plexiglass moulds, 14.0 by 3.7 by 2.5 cm, covered with a sheet of mylar and allowed to set at 0-2 C for 2-3 hours. Slices of gel, 1.6 mm thick were removed from the cooled gel and transverse slots cut at the mid-line as described by Tsuyuki *et al.*, (1966). The hemoglobin solution was introduced into the slots from finely drawn capillary tubes and the gel covered with a strip of mylar. The electrophoresis chamber contained 0.3 m borate buffer, pH 8.5, in both compartments, which were separated by a distance of 10.5 cm. A constant potential difference of 200 volts was applied for 90 minutes. During electrophoresis the starch-gels were maintained at 0-2 C in a cold room and the current did not exceed 2.5 milliamps per gel.

STAINING AND IDENTIFICATION OF HEMOGLOBIN BANDS

Immediately after electrophoresis, the starch gels were stained in a 0.1% solution of Amido Schwartz B, (w/v), in acetic acid: methanol: water, (1:5:5, v/v/v), for approximately 10 minutes and then cleared by repetitive washing in a similar solution without the stain. The identity of the hemoglobin bands was ascertained by staining certain gels with 3,3'-dimethoxybenzidine dihydrochloride, (Owen, Silbermann and Got, 1958; O'Brien, 1961). prior to staining with Amido Schwartz. Gels were stored for future analysis in plastic bags containing a small amount of clearing solution. No deterioration in the gel or the precipitated proteins was observed over a 2 1/2-year period.

DETERMINATION OF THE RELATIVE CONCENTRATION OF HEMOGLOBIN COMPONENTS

The cleared starch gels were positioned on glass plates and covered with a sheet of Mylar in such a manner that no air bubbles were trapped around the gel. The starch-gel sandwich was placed over a 30 by 30 cm piece of flashed opal glass which was illuminated from below by four 25 cm-long 40 watt light bulbs. The extraneous light was eliminated and the gels were photographed using only the light transmitted through the gel.

The photographic negatives of the starch gels were scanned at 660 mμ in a Gilford Model 2400 spectrophotometer fitted with a linear transport carriage and a 0.05 by 2.36

mm slit plate. The changes in absorbance were recorded on a variable span chart recorder coupled with a disc integrator (Disc Instruments Inc., Model 201-B).

The chart area represented by each hemoglobin component was expressed as a percentage of the total area of all the bands. This latter area was an estimate of the relative amount of hemoglobin applied to each starch gel. Since it was possible that the estimate of the relative concentration of the hemoglobin components was influenced by the relative amount of hemoglobin applied to the gel, a Spearman rank correlation coefficient test was conducted on six electropherograms from each of six groups of presmolt coho. The individual fish within each group were all of the same age and had been reared under the same environmental conditions. No correlation between the relative concentrations of the hemoglobin components and the relative amount of hemoglobin applied to the gel was observed at the 99% confidence level.

PART ONE

ONTOGENETIC CHANGES IN THE MULTIPLE HEMOGLOBINS OF COHO SALMON

INTRODUCTION

Despite the widespread occurrence of hemoglobin polymorphism in fish, relatively little work has been done on the ontogeny of multiple hemoglobins in these animals. Observations as to the electrophoretic pattern of hemoglobins at certain distinct phases of the life-cycle have been made for the lamprey *Petromyzon planerii* (Adinolfi, Chieffi and Siniscalco, 1959), rainbow trout *Salmo gairdneri irideus*, (Iuchi and Yamagami, 1969), and coho and sockeye salmon, *Oncorhynchus kisutch* and *O. nerka*, (Vanstone, Roberts and Tsuyuki, 1964). In all of the foregoing studies frequent observations of the multiple hemoglobin pattern was not made although in most cases it appeared that there was a general progression from the patterns observed in juvenile fish to the pattern observed in mature fish. Fish between these two general age groups had a hemoglobin pattern containing elements from both groups.

The detailed ontogeny of the multiple hemoglobins has been described for only two species of fish. Wilkins and Iles (1966) have demonstrated that the blood of the herring, *Clupea harengus*, contains four hemoglobin polymorphs which, based upon different relative concentrations, form up to nine electrophoretical patterns during the life-cycle of this

fish. Similarly, Atlantic salmon, *Salmo salar*, exhibit up to nine anodic and eight cathodic hemoglobin components during their life-cycle (Wilkins, 1968). Earlier studies on this salmon had only demonstrated two components (Schumann, 1959), but, using improved electrophoretical techniques, first thirteen (Koch, Bergstrom, and Evans, 1964 a,b) and then seventeen different hemoglobin polymorphs were observed (Koch, Wilkins, Bergstrom and Evans, 1967). No single electrophoretical technique however, could separate all seventeen components. Westman (1970), employing a micro starch-gel method, has demonstrated that the thirteen hemoglobin polymorphs of Atlantic salmon which can be separated with this technique form eight different electrophoretical patterns depending upon the size and presumably the age of the fish. This latter study did not include salmon less than 3.5 cm in length.

In view of the complex ontogenetic changes in the multiple hemoglobins of certain salmonids it appeared probable that the observations of Vanstone *et al.* (1964) were not sufficiently detailed to outline the complete ontogeny of the multiple hemoglobins of coho salmon. To this end the present studies were undertaken using an improved micro starch-gel electrophoresis method (Tsuyuki *et al.*, 1966) to separate the hemoglobin components of coho salmon from the embryonic to adult stages of the life-cycle. This information was also required prior to the investigation of the effects of certain environmental factors upon the multiple hemoglobin pattern and ontogenetic changes in the oxygen equilibrium of the blood of

coho salmon.

METHODS I

In order to observe early changes in hemoglobin pattern just after hatching, it was necessary to determine the precise date of hatching. This was accomplished by removing a sample of eggs of the 1971 brood year, from the gravel about a week prior to the expected date of hatching. These eggs were placed in plastic screen boxes, returned to the incubation trough and observed twice daily for evidence of hatching. Thus the time of hatching was ascertained to within 16 hours. The majority of eggs hatched within one day and these young alevins were then divided into ten groups of 10-12 fish and placed into individual cages composed of a 4-inch piece of 2-inch diameter PVC plastic pipe with both ends capped with plastic screen. A few pieces of gravel were also placed in the cages which were then returned to the incubation trough. Thus, coho alevins of known age could be easily obtained at later dates. The ages of older fish are known to within 2-3 weeks, since the precise time of hatching was only determined for the 1971 brood year.

Blood samples were generally obtained as described in general methods. The blood from unhatched embryos was obtained by cutting the outer egg case and expelling the embryo onto a piece of tissue paper. The tail region was carefully dried with tissue and the caudal peduncle severed. Blood was collected in a finely drawn microhematocrit tube containing

heparin, (50 USP units per ml), in 1.0% NaCl. The use of heparinized saline during blood collection was continued until the stage when the yolk sac had been adsorbed since the blood tended to clot more quickly in these young fish. The erythrocytes were then washed, hemolyzed and electrophoresed as described previously. The gels were stained and photographed and the photographic negatives scanned in a densitometer as described in the general methods.

RESULTS I

A summary of the ontogenetic changes in the multiple hemoglobins of coho salmon is presented in Figure 1. For reference purposes the hemoglobin components have been labelled as migrating either toward the anode (A) or the cathode (C) from the central origin. The number following the anodic or cathodic designation represents the relative position of the component in order of increasing distance from the origin toward either the anode or cathode. In this respect the electrophoretical mobilities of all the components observed throughout the life-cycle were ranked prior to the assignment of each label (Figure 1). The code designating the various hemoglobin components presented in Figure 1 will be used in the discussion of the photographic plates 1 to 10.

Twelve closely-spaced anodic and one diffuse cathodic carboxyhemoglobin bands were observed in coho embryos approximately two days prior to hatching, (Plate 1-A). Blood sampled within 14 hours and 15 days after hatching, (Plate 1-B,C) had

identical electrophoretic patterns to that observed prior to hatching, suggesting that the environmental changes experienced by the embryos during hatching and early inter-gravel residence were not associated with changes in the composition of the hemoglobin.

Thirty days after hatching, bands A6, 7, and 8 became relatively more dense (Plate 1-E), while the slower migrating anodic bands, A1, 2, 4, became quite faint. The remaining anodic components also decreased in relative concentration. At six-weeks post-hatch when the fish were emerging from the gravel, bands A6-8 represented the large majority of the hemoglobin (Plate 1-F). At this stage, a second very faint cathodic component corresponding to C3 (Figure 1) appeared. The apparent dark band at the origin in Plate 1-F is an artifact representing a small amount of cell debris inadvertently applied with the hemoglobin solution.

Fourteen weeks after hatching, the free-swimming fry stage was well established and components A6-8 comprised virtually all the hemoglobin (Plate 1-G, Figure 1). The electropherogram presented in Plate 1-H represents the hemoglobin pattern of a fry from the 1970 brood year at a similar time, thus demonstrating that fish from both brood years had identical hemoglobin patterns at this stage of their life cycle.

Coho fry remain in fresh water for approximately one year and then migrate to sea during the period from April to June as smolts. Salmon undergo several biochemical and behavioural changes during the smoltification process (Baggerman,

FIGURE 1:

Schematic representation of the relative electrophoretic mobilities of the multiple hemoglobin components of coho salmon during the alevin, fry and adult stages of the life cycle. No attempt has been made to reproduce the relative concentrations of the various components observed at different ontogenetic stages.

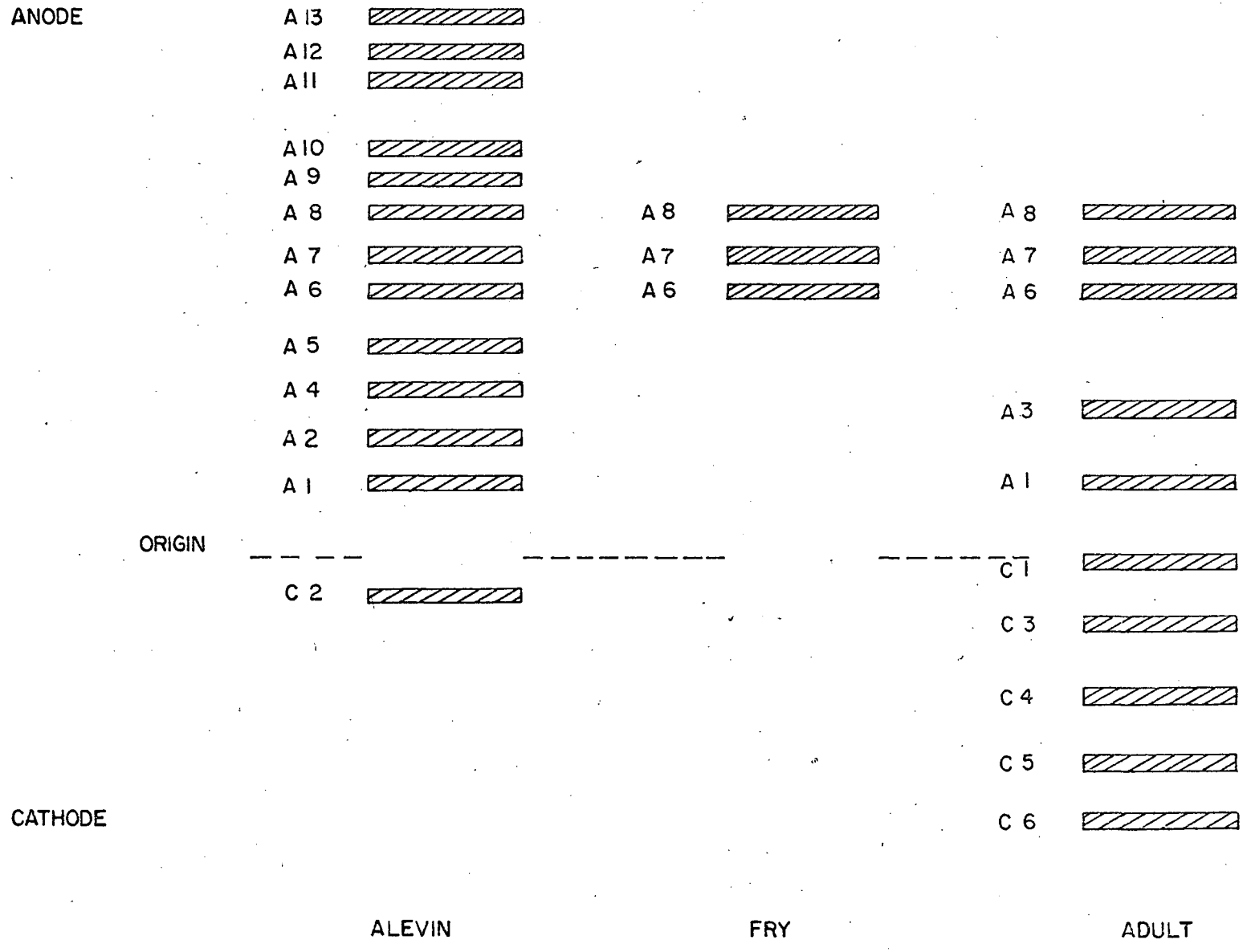

ANODE
A 13
A 12
A 11
A 10
A 9
A 8
A 7
A 6
A 5
A 4
A 2
A 1
A 8
A 7
A 6
A 8
A 7
A 6
A 3
A 1
ORIGIN
C 2
C 1
C 3
C 4
C 5
C 6
CATHODE
ALEVIN
FRY
ADULT

PLATE 1:

Electropherograms of the multiple hemoglobins of coho salmon from the embryo to the fry stages of development. The blood samples were obtained 2-3 days before hatching (A) and > 14 hours (B), 6 days (C), 15 days (D), 30 days (E), 42 days (F), and 98 days (G) after hatching from the 1971 brood stock. Electropherogram H illustrates the electrophoretic hemoglobin pattern of a 6-month-old coho fry from the 1970 brood stock. The electropherograms are enlarged to 1.4 X actual size.

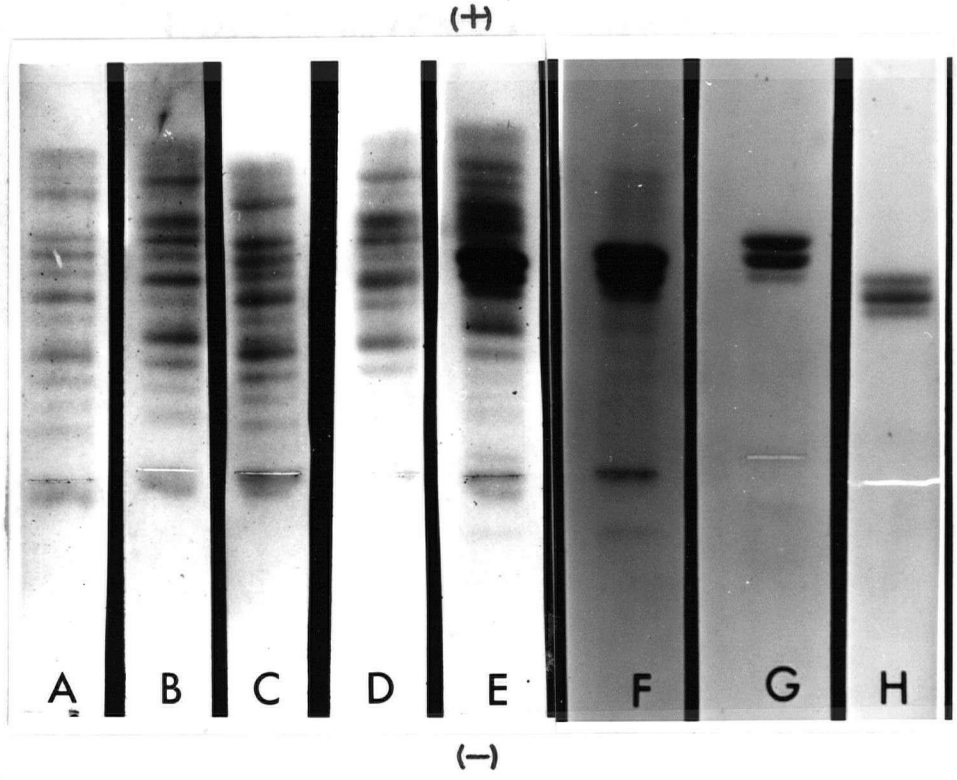

(+)
A
B
C
D
E
F
G
H
(−)

PLATE 2

Electropherograms of the multiple hemoglobins of coho salmon from the fry to adult stages of development. The blood samples were obtained from a 4 1/2-month-old fry (A), 11 1/2-month-old (B), 14-month-old (C), 15-month-old (D) presmolts, 15 1/2-month-old smolts (E), a 16 1/2-month-old postsmolt in seawater for one month (F), a 2-year-old seawater grilse (G) and a 3-year-old spawning female (H). The electropherograms are enlarged to 1.4 X actual size.

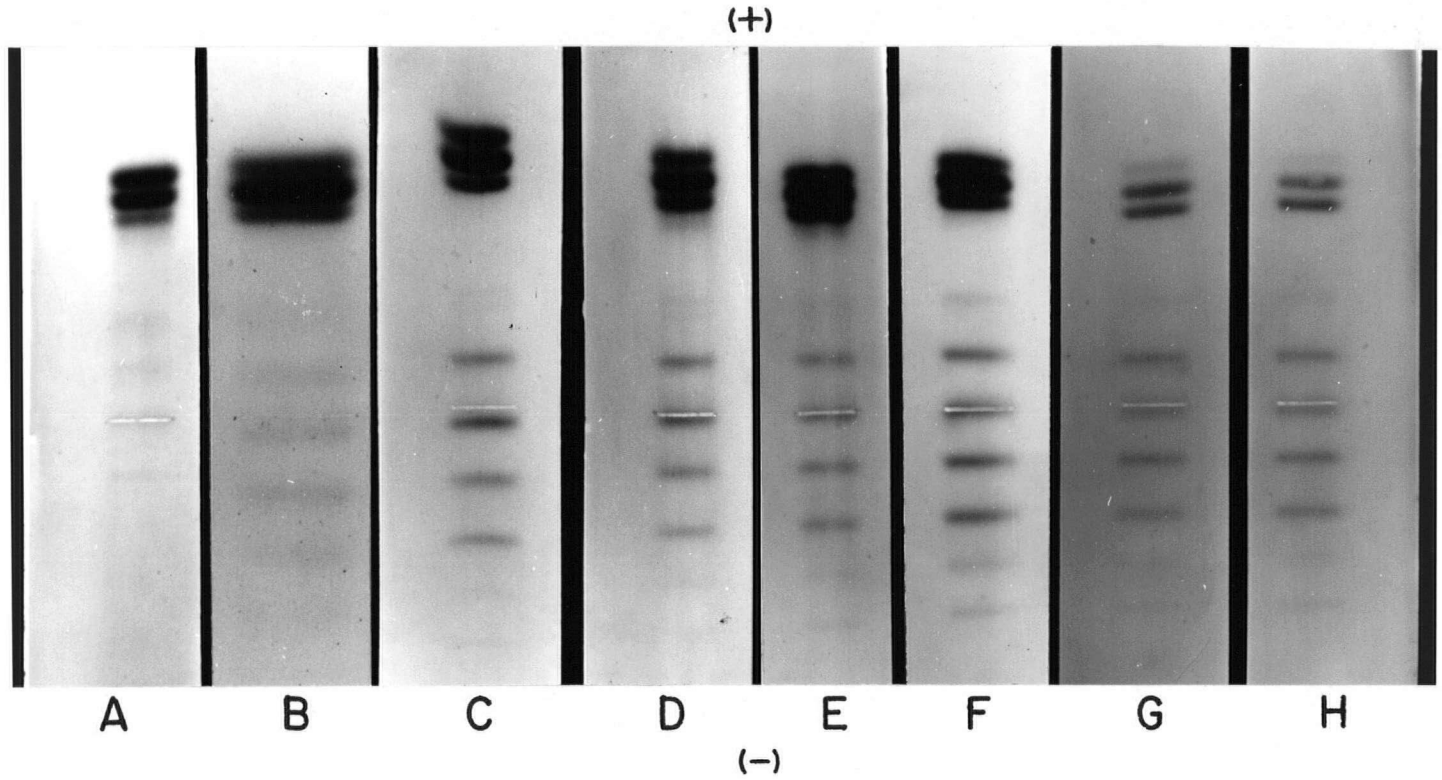

(+)
A
B
C
D
E
F
G
H
(−)

TABLE I:

The relative concentration of the components of the multiple hemoglobins of the blood of coho salmon from the fry to the freshwater adult stages of the life cycle. The age is given from the time of hatching.

SAMPLE DATE	DEVELOPMENTAL STAGE	AGE	YEAR HATCHED	RELATIVE CONCENTRATION OF HEMOGLOBIN COMPONENTS (%) A6-8	A3	A1	C1	C3	C4	C5	C6
1/6/71	F.W. Fry	3 1/2 mos.	1971	100.							
29/6/71	F.W. Fry	4 1/2 mos.	1971	94.3	2.3	1.4	1.7	0.3	0	0	0
2/2/71	F.W. Presmolt	11 1/2 mos.	1970	90.5	0.8	2.0	3.8	1.0	2.0	0	0
2/3/71	F.W. Presmolt	12 1/2 mos.	1970	*86.7	1.1	1.7	4.9	1.9	1.6	1.4	0.7
16/3/71	F.W. Presmolt	13 mos.	1970	*80.4	1.5	2.6	5.5	3.7	3.2	1.9	1.2
22/4/71	F.W. Presmolt	14 mos.	1970	78.0	0.6	4.6	6.9	4.1	4.3	1.0	0.6
12/5/71	F.W. Presmolt	15 mos.	1970	80.4	1.6	3.7	5.4	3.5	3.4	0.8	1.2
1/6/71	F.W. Smolt	15 1/2 mos.	1970	78.4	0.7	5.4	4.9	4.9	4.8	0.6	0.5
29/6/71	S.W. Postsmolt	1 1/3 yrs.	1970	68.7	1.5	6.2	8.2	6.3	6.2	1.5	1.4
3/8/71	S.W. Postsmolt	1 1/2 yrs.	1970	50.9	3.3	10.5	13.3	9.0	9.1	0.9	3.1
Oct/Nov.70	S.W. Grilse	1 3/4 yrs.	1969	*55.4	5.8	8.8	9.3	8.7	6.7	2.8	2.5
10/1/72	S.W. Grilse	2 yrs.	1970	61.7	4.7	8.3	7.7	8.5	6.2	2.3	0.8
10/1/72	F.W. Adult	3 yrs.	1969	53.1	2.7	10.1	6.9	10.8	11.3	1.4	3.6

F.W. - Fish residing in fresh water.

S.W. - Fish residing in salt water.

* Average values of individual electropherograms of hemoglobins from 6 fish.

1965; Giles, 1969, Hoar, 1965; Vanstone and Markert, 1968), which may begin several months before the onset of seaward migration. In the present study, the smolts were simply adapted to seawater in early June and did not undergo a seaward migration as such. It was impossible, therefore, to use seaward migration as a criterion for completion of the smolting process. Since the fish used in this part of the study had hatched in mid-February, 1970, their approximate age from hatching can be determined from the dates on which the blood was sampled. In addition to the electropherograms, the relative concentration of the hemoglobin components from densitometric scans of the photographic negatives are presented (Table 1).

Plate 2-A presents the typical electrophoretic pattern of hemoglobin from 4 1/2-month-old fry. Components A6-8 comprised over 94% of the total hemoglobin (Table 1) while A3, A1, C1, and C3 (Figure 1), account for the remainder. Component A3 was the most prominant of the minor components (2.3%). Components C4-6 (Figure 1) were not present at this age. By early February components A6-8 had decreased slightly in relative concentration and component C4 had appeared (Plate 2-B). Components C5 and C6 had appeared by early March when the relative concentration of components A6-8 had decreased to approximately 87% (Table 1).

No substantial changes in either the electrophoretic pattern or the relative distribution of the components occurred during the period from mid-March to early June (Plates 2-C,

D,E). At this time the fish were adapted to seawater. After approximately 30-days in seawater (Plate 2-F) components A6-8 had decreased to approximately 68% of the total hemoglobin concomitant with increases in the remaining seven components. Although there was considerable variation, the minor hemoglobin components were normally ranked in the following order of decreasing relative concentration; C1, C3, A1, C4, A3, C5, and C6. In older saltwater fish C1 tended to decrease in relative concentration and ranked fourth in this series. This reduction in the faster-moving anodic bands continued until by August components A6-8 comprised only 55% of the total hemoglobin, (Table 1). The distribution of hemoglobin components in fish after four to five months in seawater remained relatively unchanged throughout the remainder of the life cycle (Plate 2-G, H, Table 1).

Although Figure 1 indicates that nineteen hemoglobin components can be separated with micro starch-gel electrophoresis it should be pointed out that the designation of components A4 and C2 of the alevin blood as distinct from A3 and C1, respectively, of adult blood may be erroneous. Component C1 of the 15-month-old presmolt presented in Plate 2-D was quite similar in position to C2 of alevin blood (Plate 1-A, B,C). In most cases, however, C1 was much nearer the origin of the electropherogram (Plate 2-D to H) and was therefore considered to be distinct from C2.

The uniqueness of A3 and A4, however, is much more questionable. The leading edge of A3 overlapped the trailing

edge of A4 and minor differences in gel preparation could have caused small changes in electrophoretic mobility of the hemoglobin components. It is quite possible, therefore that the nineteen components illustrated in Figure 1 should be reduced to seventeen or more probably eighteen.

DISCUSSION I

The analysis of the electrophoretical data indicates that the multiple hemoglobins of coho salmon are composed of seventeen to nineteen different components which undergo a relatively complex series of ontogenetic changes. Although no variation in the number or distribution of hemoglobin components was observed in fish of the same age minor variation in the relative proportions of the components did occur.

Various uncertainties are encountered in the interpretation of electropherograms of organic compounds, especially those composed of two or more similar subunits. Multiple electrophoretic zones can arise from protein-buffer interaction (Cann and Goad, 1965; Cann, 1966) or from the binding of inorganic or organic compounds such as adenosine triphosphate or 2,3-diphosphoglycerate to a portion of the protein (Chanutin and Curnish, 1964). This latter factor may be of some importance in ontogenetic studies of hemoglobin since in many animals the concentrations of certain organic phosphates in the erythrocyte do change during the life cycle (Mission and Freeman, 1972; Wood, 1972). In this respect removal of over 90% of the total phosphate from hemolyzates of adult coho blood did not decrease

the number or electrophoretic mobilities of the hemoglobin components (Plate 10, Part III).

Subunit dissociation or aggregation may occur under the stresses imposed by electrophoresis which would lead to spurious results when interpreting the electropherograms. Although this factor was not investigated in the present study, it has been demonstrated that the molecular weights and subunit composition of Atlantic salmon hemoglobins are unchanged during a second electrophoretic run, after an initial electrophoretic separation (Wilkins, 1970). There is no reason to assume that Pacific salmon hemoglobins are less stable than Atlantic salmon hemoglobins in this respect. Also, the uniformity of relative concentrations and distribution of hemoglobins observed in fish of similar age argues against the occurrence of a significant amount of subunit dissociation.

The oxidation of the iron molecule from the ferrous to ferric state would obviously change the electrophoretic mobility of the hemoglobin molecule since one to four positive ionic charges would be added to the molecule. Hemoglobin can be oxidized to the ferric state (methemoglobin) more readily when it is in the deoxygenated state (Benesch, Benesch, and Macduff, 1964; Mahler and Cordes, 1966). Since oxyhemoglobin and carboxyhemoglobin have identical electrophoretic mobilities (Tsuyuki and Ronald, 1971) carbon monoxide was employed to stabilize the hemoglobin prior to electrophoresis. The dissociation constant of carbon monoxide from hemoglobin is

approximately 200 times less than that of oxygen and the former gas therefore forms a much more stable complex with the respiratory pigment. Methemoglobin, when present in significant amounts (1-5%) forms secondary bands visible adjacent to each hemoglobin component (Plate 10, Part III). No such secondary bands were observed in the present hemoglobin samples which were less than 24 hours old.

The seventeen to nineteen hemoglobin components of coho salmon exhibit a complex series of ontogenetic changes although only four distinct electrophoretic patterns were observed compared to the eight different patterns described for Atlantic salmon (Westman, 1970). A striking similarity was evident between the hemoglobin electrophoretic pattern of newly hatched coho alevins and the eight to nine anodic components of trout alevin hemoglobin reported by Iuchi and Yamagami (1969). While 25-day-old trout alevins possessed three anodic and three cathodic components (*ibid.*), the reduction in the number of anodic components of coho alevin blood did not begin until 30 days after hatching and an additional 10 weeks was required to develop the fry hemoglobin pattern consisting of three anodic components (A6-8). Although the difference in the timing of the change in hemoglobin pattern may reflect species differences or different rearing conditions it is interesting to note that in both species, the change in pattern was associated with the timing of the emergence of the alevins from the gravel.

Vernidub (1966) during an extensive study of

cytological changes in the blood of Atlantic salmon during the embryonic to free-swimming stages, demonstrated the occurrence of two general periods of erythropoeisis. The first ended just prior to hatching and was characterized by large numbers of normoblasts and basophilic erythrocytes, which were derived from blood islands on the periblast. The second period of erythropoeitic activity began 200 degree days after hatching at a length of 21 to 22 mm and continued until after 700 degree days at a length of 33 to 37 mm, 95% of the red blood cells consisted of mature erythrocytes, derived from the new source. Vernidub suggested that middle kidney was the erythropoeitic organ during the second phase of erythrocyte production. The close correspondence between the timing of erythropoeitic activity in the alevins of Atlantic salmon and the timing of changes in the multiple hemoglobin pattern of rainbow trout and coho salmon suggest that these two processes are in some way linked and may be characteristic of salmonids at this stage of development.

The final major changes in hemoglobin pattern of coho salmon was associated with the period of smolting, and resulted in an electrophoretic pattern of five anodic and five cathodic components. During the period of January to mid-February five cathodic and two anodic hemoglobin components appeared in presmolt hemoglobin while the relative concentration of components A6-8 decreased to 80%. At this time components A3, A1, C1, C3, C4, C5, and C6, accounted for 1.5, 2.6, 5.5, 3.7, 3.2, 1.9 and 1.2 %, respectively, of the total

hemoglobin. This pattern remained essentially unchanged during the next 2 1/2 months of freshwater residence. Following transfer to sea water, however, the relative proportion of A6-8 began to decrease and was only 51% of the total hemoglobin of coho postsmolts after approximately 2 months in sea water. Coupled to this decrease in the concentration of A6-8 was an increase in the seven minor components of smolt hemoglobin. Thereafter little variation in the distribution of the ten hemoglobin components was observed even when the mature coho were returned to freshwater prior to spawning.

Thus throughout the entire life cycle of the multiple hemoglobins of coho under three periods of relatively rapid change in composition while the number and relative concentration of the components remain almost constant between these three periods. The relative proportion of anodic components decreased during the life cycle but the change was intermittent rather than continuous as observed in Atlantic salmon (Westman, 1970).

The molecular basis for hemoglobin polymorphism in salmonids appears to reside in the presence of eight structural genes coding for eight different polypeptide chains (Tsuyuki and Ronald, 1971; Wilkins, 1971). In Atlantic salmon, these eight polypeptides appear to be divided into two groups of four "α-like" and four "non α-like" chains. Two chains of each group are involved in the formation of the nine anodic hemoglobins and the remaining pair of each

group form the seven to eight cathodic components and these sets of complementary pairs are mutually exclusive (Wilkins, 1971). Each polymorphic form of hemoglobin is a tetrameric combination of two "non-α-like" and two "α-like" chains. Basically, the same system has been found to occur in the five species of Pacific salmon (Tsuyuki and Ronald, 1971), although the latter workers used a different method of notation for each subunit of the hemoglobin molecule. In the latter study it was found on the basis of tryptic digests, that hemoglobins from different species with identical electrophoretic mobilities were composed of different subunits. Since both of these investigations only include blood from the juvenile to adult stages, it is possible that additional subunits may be discovered which occur only in the embryonic stages as is observed in certain mammals (Ingram, 1963).

At the present time, the results observed in this study are compatible with the concept of eight structural genes coding for the hemoglobin subunits if the previously discussed limitations on the possible tetrametric combinations are applied. In such a system, a total of eighteen unique tetrameric combinations are possible. Since there were serious doubts as to the differentiation of components A3 and A4, it is probable that only eighteen instead of nineteen multiple forms of hemoglobin occur during the life cycle of coho salmon.

SUMMARY I

1) The ontogenetic changes in the multiple hemoglobin of coho salmon, *Oncorhynchus kistuch*, from the embryonic to freshwater adult stages of the life cycle were investigated using micro starch-gel electrophoresis to separate the hemoglobin polymorphs.

2) A total of twelve to thirteen anodic and five to six cathodic hemoglobin polymorphs were observed during the life cycle of coho salmon.

3) The hemoglobin of embryos, just prior to hatching, and of alevins for a period of approximately two to three weeks after hatching, is composed of twelve anodic and one cathodic component. All except three of the anodic components (A6-8) disappeared during subsequent development to the free-swimming fry stage.

4) At the age of approximately eleven to twelve months, five new cathodic and one new anodic component, C1, C3, C4, C5, C6, and A3, respectively, appeared in the hemoglobin of presmolt coho. The uniqueness of A3 and C1 may be questionable. Component A1 of alevin hemoglobin also reappeared, resulting in a ten-component pattern. In presmolt coho components A6-8 comprised over 80% of the total hemoglobin.

5) Following transfer to seawater, a further reduction in the relative concentration of A6-8 was observed and after approximately two months in seawater, A6-8 accounted for

approximately 50 to 55% of the hemoglobin. The remaining two anodic and five cathodic components accounted for the remainder of the hemoglobin. Generally, no significant change in the electrophoretic pattern or relative concentrations of components was observed during the portion of the life cycle following the first two months of marine existence.

PART TWO

INFLUENCE OF WATER TEMPERATURE, DISSOLVED OXYGEN CONCENTRATION AND SALINITY UPON GROWTH, DEVELOPMENT AND ELECTROPHORETIC HEMOGLOBIN PATTERN IN UNDERYEARLING COHO SALMON

INTRODUCTION II

During the ontogenetic studies of the multiple hemoglobins of coho salmon it was observed that major changes in the electrophoretic pattern were associated with periods of change in behaviour and habitat of the fish. The alevin hemoglobin pattern was transformed into the fry pattern upon emergence from the stream bottom and the presmolt pattern appeared during a period when the fish are known to be undergoing a variety of physiological and biochemical changes in preparation for marine residence (Baggerman, 1965; Vanstone and Markert, 1968; Giles, 1969). The full expression of the postsmolt or adult hemoglobin pattern was not complete until the fish had been in sea water for at least two months, again implicating environmental factors in the elicitation of a change in hemoglobin pattern.

Although the foregoing considerations suggest that environmental factors may be responsible for ontogenetic variations in the coho hemoglobin polymorphs it may still be argued that the size or the age of the fish is the important criterion in the control of the timing of the hemoglobin changes. Growth in fish is dependent upon environmental

conditions and it is conceivable that both factors may exert a combined action in control of hemoglobin polymorphism. Since the juvenile stage of the coho salmon life cycle is characterized by considerable changes in both the number and relative concentrations of the hemoglobin polymorphs as well as variations in the pattern of growth the following experiments were conducted to determine the effects of temperature, dissolved oxygen concentration and salinity upon the growth and hemoglobin pattern of 3 1/2-month old fry and 11-month old presmolt coho.

METHODS II

EXPERIMENTAL ANIMALS

Eleven-month old presmolts from the 1970 brood stock were anesthetized in 2-phenoxyethanol and measured for fork length on January 28, 1971. Fish in the 8.5 to 8.9 cm length class (mean length, 8.6 ± 0.1 cm; mean weight, 6.6 ± 0.4 g) were removed and allowed to recover in aerated fresh water for one day prior to exposure to the different environmental conditions. Eighteen fish were placed in each fibreglass aquarium, maintained under the conditions described in the following methods. Fish were transferred directly to the experimental conditions with no period of adaptation.

On June 6, 1971, 3 1/2-month old coho fry of the 1971 brood stock were separated randomly into groups of

approximately thirty individuals, without the use of anesthetic. With certain exceptions, individual groups of thirty fish were transferred directly to aquaria maintained at the constant environmental conditions described in the following Section. Fry to be exposed to salinities of 20 and 30 °/oo were maintained in dilute seawater, salinity 10°/oo for two days and then transferred to 20°/oo seawater. Fish to be exposed to full-strength seawater were maintained in 20°/oo seawater. In addition, fish to be exposed to water temperatures of 15 °C were exposed to increasing water temperatures from approximately 9.5 to 15 °C over a 2 day period.

BLOOD SAMPLING

The presmolt coho were sampled at approximately 2-week intervals over a period of 60 days. Three fish were removed from each treatment and anesthetized with 2-phenoxyethanol. The individual lengths and weights were measured and duplicate blood samples collected in microhematocrit tubes from the severed caudal peduncle. Hematocrits were measured and the hemoglobin samples prepared and electrophoresed as described in the General Methods Section. Coho fry were treated in an identical manner, except that a single sample of 4-6 fish was taken from each experimental environment after 49-50 days of treatment. The electropherograms were photographed and scanned in a densitometer as described previously. In some cases, photographic prints of the gels

are presented rather than the densitometer scans.

WATER SUPPLY

Well water (hardness (hardness 18-40 mg/1 $CaCO_3$) was used as a source of fresh water in all experiments. During the experimental period the fresh water temperature was 9 to 10 °C and dissolved oxygen concentration was 2.2 ppm before aeration. Water aspirators were fitted to the valve outlets to aerate the water and compressed air was supplied to the aquaria when necessary to maintain high oxygen tensions. Seawater (salinity ca. 30°/oo, range 27-32 °/oo, temperature 8.4 °C) was supplied to some aquaria as required. The sea water was generally more than 90% saturated with oxygen.

TEMPERATURE CONTROL

Insulated, 60-1 fibreglass aquaria were fitted with thermo-switches (micro-set Model 62542, Precision Scientific Co.) which controlled the operation of electronic relays (Lapine Scientific Co.). To maintain temperatures above ambient, stainless steel immersion heaters, (250-750 watts) were placed in the aquaria and connected to the relays. To maintain temperatures below ambient, 4m aluminum cooling coils were placed in the aquaria and connected through a pump to a reservoir of ethylene glycol-water mixture maintained at -10°C with a cooling unit (Bendin Westinghouse Ltd.). The pump was controlled through the relay and when the water

temperature rose above the desired temperature, the pump was activated to run coolant through the coils.

Aerated well water was supplied to these aquaria at the rate of 200 l/minute. Each aquaria was fitted with a submersible recirculation pump (Little Giant Pump Co.) to provide a circular water flow and maintain uniform temperatures throughout the tank.

OXYGEN CONCENTRATION CONTROL

Three, 225-l fibreglass aquaria were supplied with flowing well water which was maintained in circular flow by means of submersible recirculation pumps. The valves controlling water inflow were fitted with water aspirators. The air intake of the aspirators was connected to a gas flow-meter (Gilmont Instruments Inc.) and the air flow through the meter was controlled by a needle valve. By a trial and error method, the flow of air to each aspirator required to produce desired dissolved oxygen concentrations was determined and the flows were maintained at these levels. Oxygen concentrations were normally determined every two days, using the modified Winkler method (Strickland and Parsons, 1968) or by measuring the partial pressure of oxygen with a Radiometer thermostated oxygen micro-electrode.

When coho fry were exposed to low oxygen concentrations (2.2-3.2 ppm) considerable gulping behaviour was observed at the water surface. To prevent this behaviour from interfering with the experiment, these fish were placed in 25 cubic

centimeter boxes constructed of netting on a stainless steel frame, positioned 5 cm below the surface. Since this gulping behaviour was not observed in presmolt coho, these fish were not confined in the net enclosures.

SALINITY CONTROL

Two constant head devices, one for seawater and one for well water, were constructed to permit a uniform water pressure to be maintained. A controlled flow of water was drawn from each header and supplied in various ratios to 250 litre fibreglass aquaria to produce the desired salinities. Each aquaria was provided with a recirculation pump to maintain a circular flow and uniform mixing of the two water supplies. The temperature of the seawater and the well water differed by approximately 1.2-1.6 C, but no attempt was made to adjust this differential since the maximum difference in temperature between the three experimental tanks was 1.0 C.

The oxygen concentrations, water temperature and salinities maintained through the experimental period of January 29, 1970 to March 29, 1971, are presented in Table II. The control fish represent presmolt coho which were maintained in aerated well water (dissolved oxygen concentration, 9.7 ppm = 87% air saturation) and thus serve as controls for both the salinity and oxygen concentration experiments.

TABLE II:

A summary of the water temperature and dissolved oxygen concentrations maintained in the various environmental regimes employed in this study. Values given are the mean $\pm$ 1 standard deviation of measurements recorded throughout the experimental period.

ENVIRONMENTAL VARIABLE	WATER TEMPERATURE (C)	OXYGEN CONCENTRATION (ppm)
Temperature	1.3 ± 0.1	10.0 ± 0.9
	4.1 ± 0.2	9.6 ± 0.9
	8.0 ± 0.1	9.1 ± 0.6
	10.1 ± 0.1	9.4 ± 0.6
	15.0 ± 0.0	9.3 ± 0.7
Dissolved Oxygen Concentration	9.7 ± 0.2	3.1 ± 0.3
	9.8 ± 0.2	5.8 ± 0.3
	9.7 ± 0.2	7.9 ± 0.6
Salinity 1/3 SW	9.2 ± 0.5	9.9 ± 0.6
2/3 SW	8.7 ± 0.9	9.9 ± 0.6
3/3 SW	8.5 ± 0.1	9.2 ± 0.8

EFFECT OF LONG-TERM EXPOSURE TO LOW-OXYGEN LEVELS

On February 5, 1971, a group of coho presmolts from the 8.5-8.9 cm length class were placed in a 225-l fibreglass aquaria provided with flowing aerated well water. The aeration was gradually reduced over a 20-day period to a final dissolved oxygen concentration of 2.2 ppm and the fish maintained under these conditions throughout the smolting period until August 3, 1971. A second group of fish was maintained under similar conditions except that the water was aerated to oxygen levels at least 85% of air saturation. On August 3, 1971, blood was obtained from one fish of each group and the hematocrits and electrophoretic hemoglobin pattern compared to a fish which had undergone normal smoltification and was living in seawater. Unfortunately, soon thereafter the water supply to the freshwater tanks was interrupted and the fish died within a few minutes. The results therefore, represent the blood of a single fish from each experiment.

CALCULATIONS EMPLOYED IN THIS STUDY

The instantaneous rate of growth was calculated using the relationship:

$$\text{Growth rate} = \frac{\log_e W_T - \log_e W_t}{T - t} \times 100$$

where $\log_e W_T$ and $\log_e W_t$ are the natural logarithms of the wet weights of fish at time t and some later time T and both

T and t are in days (LeBrasseur and Parker, 1964; Brett, Shelbourne and Sloop, 1969). These instantaneous rates of growth were calculated on the mean weights of each sample of fish since it was not possible to determine rates of growth of individual fish over the experimental period.

The weight-length relationship, $W = \alpha L^{3.25}$, where W is the wet weight in mg and L is the fork length in cm was used to determine the α intercept. This value is quite similar to the so-called condition factor $\frac{W}{L^3}$ but it has been found that the slope, b, of a double logarithmic plot of weight on length is approximately 3.20 to 3.25 in Pacific salmon (LeBrasseur and Parker, 1964; Vanstone and Markert, 1968, Giles, 1969). The value of α is given in mg and was calculated from the lengths and weights of individual fish rather than from the mean length of each sample.

RESULTS II

GROWTH AND HEMATOCRIT OF UNTREATED PRESMOLT COHO

The changes in mean length, weight and α intercept of the weight-length relationship $W = \alpha L^{3.25}$ as well as hematocrit of coho salmon reared in the artificial creek environment from February 16, 1971 to June 1, 1971 and in seawater from June 1, 1971 to June 29, 1971 are presented in Table III. The mean instantaneous rates of growth in

TABLE III

Growth parameters and hematocrit of juvenile coho salmon reared in an artificial stream during the presmolt to smolt stages of the life cycle and of presmolts in seawater. The $\propto$ intercept was calculated for individual fish from the weight-length relationship $W = \propto L^{3.25}$, where W is the wet weight in mg and L is the fork length in cm. The instantaneous growth rates were calculated at $\frac{\log_e W_T - \log_e W_t}{T - t} \times 100$ from the mean wet weight of 6 to 9 fish. With the exception of growth rate, all data are presented as the mean ± 1 standard deviation.

DATE	N	LENGTH (cm)	WEIGHT (g)	α INTERCEPT (mg)	INSTANTANEOUS GROWTH RATE (% / DAY)	HEMATOCRIT (% RBC)
16/2/71	6	8.70 ± 0.7	7.5 ± 2.05	6.42 ± .49	--	31.3 ± 3.3
2/3/71	6	9.20 ± 0.7	8.5 ± 1.70	6.23 ± .34	0.894	34.8 ± 5.2
16/3/71	6	9.00 ± 0.4	8.3 ± 1.20	6.50 ± .30	-0.170	28.7 ± 1.8
29/3/71	6	9.50 ± 0.8	9.6 ± 2.60	6.25 ± .27	1.119	39.1 ± 3.0
22/4/71	9	10.40 ± 0.7	11.5 ± 2.5	5.67 ± .49	0.683	37.9 ± 6.0
12/5/71	6	11.10 ± 0.8	13.5 ± 2.8	5.44 ± .31	0.385	29.2 ± 4.0
1/6/71	6	11.60 ± 1.0	16.1 ± 4.0	5.35 ± .14	1.381	33.7 ± 3.2
29/6/71*	6	11.90 ± 1.3	17.0 ± 5.3	5.38 ± .35	0.194	33.2 ±10.8

*In seawater since June 1, 1971.

weight have been calculated for each sampling period.

The mean length and weight of coho presmolt increased from 8.7 cm and 7.5 g to 9.5 cm and 9.6 g, respectively, during the period of January 16, to March 29. This represents a mean instantaneous growth rate of 0.614%/day during this period. The α intercept remained relatively constant, ranging from 6.23 to 6.50 mg. Hematocrits averaged about 30% with the exception of the March 29 sample which was 39.1%.

From March 29, to June 1, the fish continued to grow at a slightly accelerated rate. The mean growth rate during this period was 0.816%/day but was highly variable, ranging from 0.385 to 1.381%/day. The value of α, however, steadily declined from 6.25 to 5.35 mg during this two-month period. This indicates that the fish were becoming more streamlined, a phenomenum observed in salmon prior to seaward migration (Giles, 1969; Vanstone and Markert, 1968). Hematocrits also declined slightly over this period.

On June 1, 1971, after obtaining a final sample of freshwater coho smolts the fish were transferred to seawater. Although the growth rate had declined to 0.194%/day by June 29, no change was observed in either the hematocrit or the value of the α intercept.

Thus the most consistent change in the pattern of growth observed in coho salmon during the progression of the smolting period was the decrease in the value of α in the weight relationship $W = \alpha L^{3.25}$. This streamlining effect was

utilized as a physiological indicator in determining whether or not the experimentally manipulated variation in certain environmental factors resulted in an acceleration of the smolting process in underyearling coho. A decrease in the value of α as well as maintenance or acceleration of growth rate would be indicative of acceleration of the smolting process. If the value of α remained relatively unchanged but variations in growth rate were related to the environmental factors occurred, then it could be concluded that the environmental variable was influencing the rate of growth but not the rate of smoltification.

EFFECT OF WATER TEMPERATURE, DISSOLVED OXYGEN CONCENTRATION AND SALINITY UPON GROWTH, HEMATOCRIT AND HEMOGLOBIN PATTERN OF COHO PRESMOLTS AND FRY

The fork lengths wet weights, α intercepts, instantaneous growth rates and hematocrits are presented for coho presmolts and fry reared in fresh water at five temperatures (Table IV) and three dissolved oxygen concentrations (Table V) and in three dilutions of sea water (Table VI). In general, water oxygen saturation ranged between 80 to 90% in the controlled temperature experiments and 95 to 100% in the various dilutions of sea water. The temperature of the water containing the three levels of dissolved oxygen was 9.7 to 9.8 C. The details of the conditions maintained in each experimental environment are presented in Table II.

TABLE IV

Growth parameters and hematocrit of 3 1/2-month old coho fry and 11-month old presmolts exposed to fresh water of various temperatures. The α intercept was calculated for individual fish from the weight-length relationship $W = \alpha L^{3.25}$, where W is the wet weight in mg and L is the fork length in cm. The instantaneous growth rate calculated as $\frac{\log_e W_T - \log_e W_t}{T - t} \times 100$ from the mean wet weight of 2 to 3 fish. With the exception of growth rate, all data are presented as the mean ± 1 standard deviation, for a sample size N of 3.

AGE GROUP	WATER TEMPERATURE (C)	TREATMENT TIME (DAYS)	N	LENGTH (cm)	WEIGHT (g)	α INTERCEPT (mg)	INSTANTANEOUS GROWTH RATE (% / DAY)	HEMATOCRIT (% RBC)
Presmolt	1.3	19	3	8.8±0.2	6.9±0.7	5.89±0.34	0.234	48.1±2.3
Presmolt	4.1	19	3	9.1±0.2	8.2±0.1	6.17±0.28	1.142	33.6±1.3
Presmolt	8.0	19	3	9.1±0.4	8.8±1.5	6.68±0.22	1.514	32.7±1.5
Presmolt	10.2	19	3	9.2±0.3	8.8±0.7	6.85±0.33	1.514	35.6±2.3*
Presmolt	15.0	19	3	9.9±0.3	10.9±2.1	6.36±0.60	2.640	32.9±7.7
Presmolt	1.3	33	3	8.3±0.1	6.1±0.2	6.17±0.16	-0.239	32.5±2.8
Presmolt	4.1	33	3	9.4±0.3	9.1±1.3	6.27±0.27	0.973	39.6±1.4
Presmolt	8.0	33	3	9.8±0.1	11.2±0.5	6.73±0.37	1.603	32.6±1.5
Presmolt	10.2	33	3	10.1±0.3	12.0±0.9	6.62±0.11	1.812	35.7±5.7
Presmolt	15.0	33	3	10.6±0.3	13.8±1.1	6.50±0.14	1.932	33.1±6.1
Presmolt	1.3	47	3	8.8±0.3	7.6±1.3	6.35±0.59	0.300	35.8±8.5
Presmolt	4.1	47	3	9.4±0.2	9.7±1.2	6.63±0.47	0.819	30.1±5.7
Presmolt	8.0	47	3	10.2±0.2	12.5±0.7	6.53±0.41	1.146	28.9±4.6
Presmolt	10.2	47	3	10.7±0.4	13.5±1.5	6.09±0.30	1.310	32.5±3.8
Presmolt	15.0	47	3	11.5±0.3	17.2±1.8	6.12±0.17	2.038	33.2±3.6
Presmolt	1.3	60	3	8.7±0.3	7.5±0.5	6.52±0.40	0.213	33.7±5.2
Presmolt	4.1	60	3	9.8±0.1	9.8±0.3	5.98±0.23	0.659	38.4±1.1
Presmolt	8.0	60	3	10.9±0.7	14.5±2.8	6.08±0.12	1.312	39.2±4.2
Presmolt	10.2	60	3	11.3±0.4	16.2±1.8	6.10±0.08	1.497	37.4±2.3
Presmolt	15.0	60	3	11.9±1.1	19.5±6.0	6.10±0.19	1.806	34.5±7.5
Fry	1.3	49	6	4.0±0.1	0.6±0.1	7.31±0.78	--	37.5±6.3
Fry	4.1	49	6	4.3±0.2	0.8±0.1	7.39±0.28	--	32.2±5.4
Fry	8.0	49	6	5.1±0.4	1.5±0.3	7.72±0.71	--	34.3±6.3
Fry	10.2	49	6	5.7±0.5	2.5±0.7	8.80±0.99	--	35.0±5.5
Fry	15.0	49	6	6.3±0.8	3.7±1.2	9.29±1.02	--	26.4±2.6

*Two measurements only: values given are mean ± 1/2 difference between two observations.

TABLE V

Growth parameters and hematocrit of 3 1/2-month old coho fry and 11-month old presmolts exposed to fresh water containing various concentrations of dissolved oxygen at 9.7 to 9.8 C. The $\propto$ intercept was calculated for individual fish from the weight length relationship $W = \propto L^{3.25}$, where W is the wet weight in mg and L is the fork length in cm. The instantaneous growth rate calculated as $\frac{\log_e W_T - \log_e W_t}{T - t} \times 100$ from the mean wet weight of 2 to 3 fish. With the exception of growth rate, all data are presented as the mean ± 1 standard deviation, for a sample size N of 3.

AGE GROUP	DISSOLVED OXYGEN (ppm)	TREATMENT TIME (DAYS)	N	LENGTH (cm)	WEIGHT (g)	α INTERCEPT (mg)	INSTANTANEOUS GROWTH RATE (% / DAY)	HEMATOCRIT (% RBC)
Presmolt	3.1	19	3	9.0±0.3	7.8±0.3	6.22±0.37	0.879	34.0±3.0
Presmolt	5.8	19	3	9.2±0.5	8.7±0.9	6.35±0.40	1.454	36.4±1.4
Presmolt	7.9	19	3	9.6±0.1	10.3±0.1	6.55±0.05	2.343	32.4±1.4
Presmolt	9.7	19	3	9.2±0.1	7.9±0.1	5.80±0.72	1.514	36.2±6.9
Presmolt	3.1	33	3	9.2±0.3	8.5±1.1	6.22±0.37	0.767	31.7±3.1
Presmolt	5.8	33	3	9.9±0.3	11.1±1.2	6.35±0.36	1.575	34.3±4.0
Presmolt	7.9	33	3	9.7±0.5	10.0±2.1	6.16±0.46	1.259	39.2±3.1
Presmolt	9.7	33	3	9.8±0.1	10.2±0.1	6.17±0.06	1.812	28.9±4.2
Presmolt	3.1	47	3	9.8±0.4	9.8±1.5	5.92±0.13	0.841	39.3±1.4
Presmolt	5.8	47	3	10.4±0.1	12.8±0.5	6.36±0.21	1.197	34.4±1.0
Presmolt	7.9	47	3	10.4±0.1	12.5±0.9	6.19±0.31	1.146	33.5±9.1
Presmolt	9.7	47	3	10.3±0.2	12.3±1.1	6.20±0.26	1.310	34.4±3.6
Presmolt	3.1	60	3	10.0±0.3	10.2±0.9	5.73±0.10	0.726	41.4±4.7
Presmolt	5.8	60	3	10.6±0.5	12.3±1.7	5.66±0.09	0.871	41.8±5.4
Presmolt	7.9	60	3	10.7±0.1	13.4±0.2	6.06±0.17	1.186	38.5±3.8
Presmolt	9.7	60	3	11.0±0.3	14.2±0.9	5.81±0.19	1.497	35.8±4.5
Fry	2.2	49	4	4.2±0.4	0.6±0.2	6.08±0.57	--	39.0±2.1
Fry	5.5	49	6	4.7±0.3	1.0±0.2	6.21±0.41	--	35.8±3.5
Fry	8.1	49	6	5.8±0.6	2.4±0.5	7.88±1.24	--	30.4±3.6
Fry	9.3	49	6	5.7±0.5	2.5±0.7	8.80±0.99	--	35.0±5.5

TABLE VI

Growth parameters and hematocrit of 3 1/2-month old coho fry and 11-month old presmolts exposed to water of various salinities. The water temperature varied from 8.5 to 9.2 C. The $\propto$ intercept was calculated for individual fish from the weight-length relationship $W = \propto L^{3.25}$, where W is the wet weight in mg and L is the fork length in cm. The instantaneous growth rate calculated as $\frac{\log_e W_T - \log_e W_t}{T - t} \times 100$ from the mean wet weight of 2 to 3 fish. With the exception of growth rate, all data are presented as the mean $\pm$ 1 standard deviation for a sample size N of 3.

AGE GROUP	SALINITY (°/oo)	TREATMENT TIME (DAYS)	N	LENGTH (cm)	WEIGHT (g)	α INTERCEPT (mg)	INSTANTANEOUS GROWTH RATE (% / DAY)	HEMATOCRIT (% RBC)
Presmolt	0	19	3	9.2±0.1	7.9±0.9	5.80±0.72	0.946	36.2±6.9
Presmolt	10	19	3	9.4±0.1	9.2±0.4	6.57±0.20	1.748	30.0±1.1
Presmolt	20	19	3	9.7±0.3	11.6±1.2	6.71±0.14	2.698	28.7±2.0*
Presmolt	30	19	3	9.2±0.4	9.0±1.2	6.60±0.32	1.632	33.6±3.3
Presmolt	0	33	3	9.0±0.1	10.2±0.1	6.17±0.06	1.319	28.9±4.2
Presmolt	10	33	3	9.9±0.3	10.6±1.1	6.23±0.05	1.436	30.7±5.1
Presmolt	20	33	3	9.8±0.3	10.8±1.4	6.55±0.16	1.492	28.3±3.5
Presmolt	30	33	3	9.9±0.4	10.7±1.8	6.49±0.40	1.464	27.1±1.5
Presmolts	0	47	3	10.3±0.2	12.3±1.1	6.20±0.26	1.112	34.4±3.6
Presmolts	10	47	3	11.0±0.6	15.7±1.7	6.49±0.40	1.844	30.8±4.7
Presmolts	20	47	2*	11.0±0.5	14.6±2.3	5.95±0.09	1.689	34.4±4.4
Presmolts	30	47	3	10.7±0.4	15.7±1.4	7.11±0.40	1.844	33.1±3.4
Presmolts	0	60	3	11.0±0.3	14.2±0.9	5.81±0.19	1.277	35.8±4.5
Presmolts	10	60	3	11.7±0.4	18.0±2.2	6.04±0.16	1.672	39.6±1.7
Presmolts	20	60	3	11.2±0.5	14.4±1.8	5.57±0.17	1.300	36.1±4.7
Presmolts	30	60	3	10.8±0.4	14.9±1.6	6.43±0.13	1.190	34.1±5.9
Fry	10	50	6	5.8±0.4	2.3±0.5	7.51±0.37		33.9±7.3
Fry	20	50	6	5.9±0.8	2.6±0.9	7.97±0.56		29.6±3.3
Fry	30	50	6	4.7±0.3	1.18±0.3	7.72±0.57		32.1±3.6

*Two measurements only: values given are mean ± 1/2 difference between two observations.

In the presmolt coho the α intercept of the weight-length relationship did not exhibit a consistant relationship to any of the environmental variables examined during the period of 19 to 47 days of treatment and generally exceeded 6.0 in those fish reared at various water temperatures for the entire 60-day experimental period. The intercept of presmolts exposed to 3.1 and 5.8 ppm dissolved oxygen and to $20^{o}/oo$ salinity, however, did decrease substantially after 60 days to 5.73, 5.66, and 5.57mg, respectively.

The final sample (60-days treatment time) of the presmolts exposed to the various environmental regimes was obtained on March 29, 1971. A comparison of these fish with presmolts reared in the artificial creek (Table III) demonstrates that, with the exception of the presmolts reared in fresh water at 1.3 C and in full-strength sea water, the α intercept was consistently less in the experimental fish at this time. The differences in the food ration and daily photoperiod experienced by these two groups of fish probably accounts for this disparity.

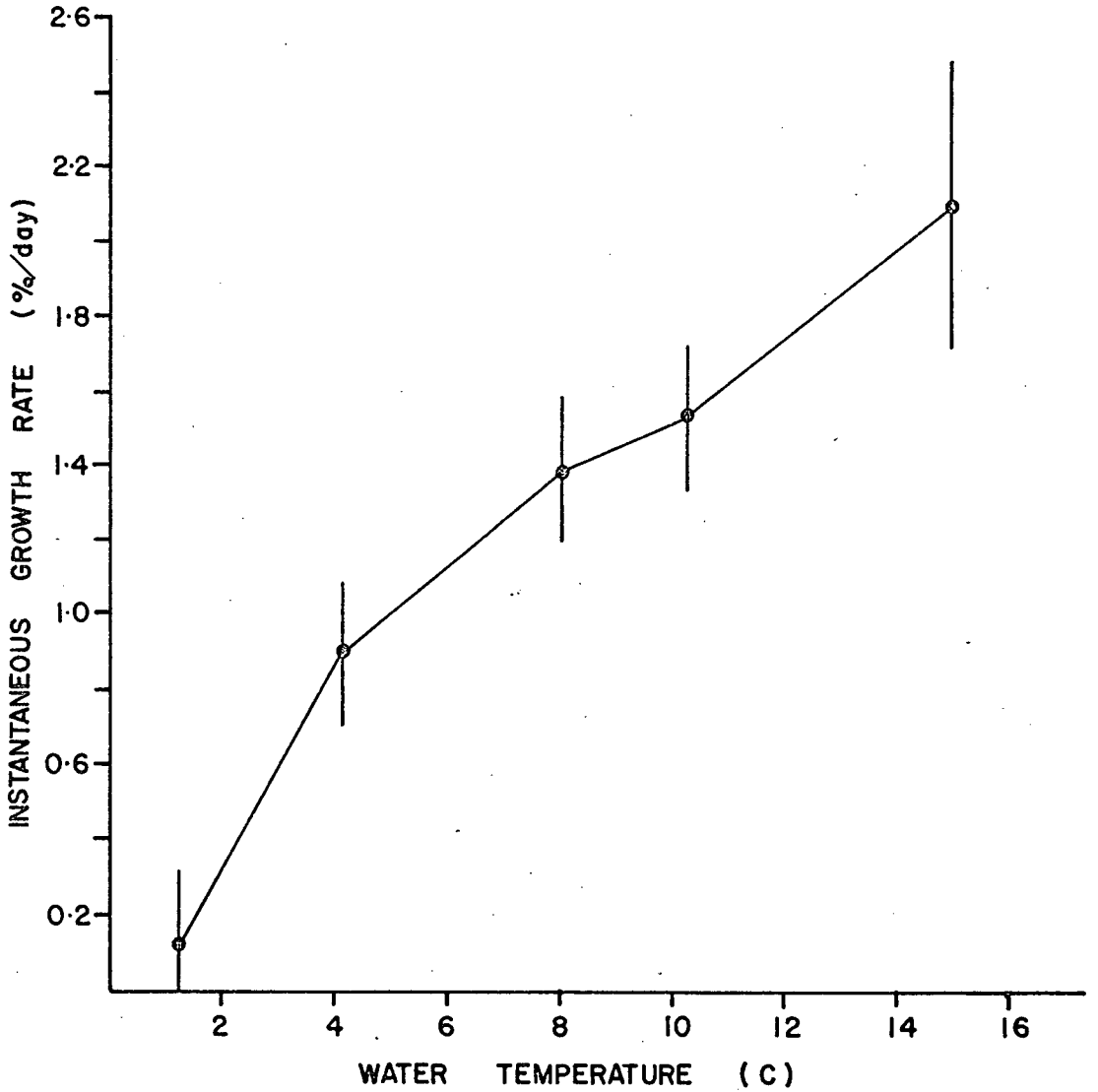
The α intercept of coho fry was generally greater than 7.3 mg indicating that the fry were considerably less streamlined than the presmolts. The value of α increased with elevated water temperature (Table VI) and with increasing dissolved oxygen concentration (Table V), but was unaffected by variations in salinity. The values of 6.08 and 6.21 mg recorded in fry reared at 2.2 and 5.5 ppm dissolved oxygen suggest that these fish were growing relatively faster in length than in weight, although growth in general was retarded.

The instantaneous growth rate of presmolt coho, expressed as percentage increase in weight per day, was markedly dependent upon the water temperature. From 4.1 to 15.0 C the mean instantaneous growth rate calculated over the 60-day experimental period increased by 0.112%/day for each degree increase in water temperature (Figure 2). The maximum mean growth rate recorded was 2.104%/day in presmolts reared in freshwater at 15C. Presmolts reared in freshwater at 10.2 C (Table IV) and at 9.7 C with 7.9 to 9.7 ppm dissolved oxygen (Table V) and in water of 10 to 30 ^{o}/oo salinity all exhibited mean growth rates of 1.5 to 1.7%/day over the experimental period. Dissolved oxygen concentrations of 3.1 and 5.8 ppm reduced the rates of growth to 0.8 and 1.3 %/day respectively.

Although growth rates of the coho fry could not be calculated it is evident that fish reared for 49 to 50 days at temperatures of 10.2 C, dissolved oxygen concentrations of 8.1 and 9.3 ppm and salinities of 10 and 20 ^{o}/oo all exhibited wet weights of 2.3 to 2.6 g (Tables IV, V, VI). Growth was substantially reduced at dissolved oxygen concentrations of 2.2 and 5.5 ppm and at a salinity of 30 ^{o}/oo. The severity of the stress imposed upon these fry was evident from the 85 to 50% mortalities recorded in fry held at 2.2 ppm oxygen and 30 ^{o}/oo salinity, respectively.

FIGURE 2

Mean instantaneous growth rates of presmolt coho held in aerated well water at various temperatures for 60 days. The points represent the mean of the measurements obtained after 19, 33, 47, and 60 days of exposure and the vertical bars represent ± 1 standard deviation of this mean.


INSTANTANEOUS GROWTH RATE (%/day)
2·6
2·2
1·8
1·4
1·0
0·6
0·2
2
4
6
8
10
12
14
16
WATER TEMPERATURE (C)

No consistent relationship between any of the environmental regimes and blood hematocrit was observed in either coho presmolts or fry (Tables IV, V, VI), although occasional significant differences did occur (presmolt at 1.3 C after 19 days treatment, Table IV). Although fry reared at 2.2 ppm dissolved oxygen exhibited a mean hematocrit of 39.0% those exposed to 5.5 and 9.3 ppm had almost identical hematocrits.

Although no specific response to any of the environmental regimes was observed in the hematocrits of coho presmolts the average hematocrit of the grouped fish did exhibit a change during the 60-day experimental period. The means of the grouped hematocrits after 19, 33, and 47 days of treatment were 33.5, 32.5, and 33.4%, respectively, whereas after 60 days exposure, this value increased to 37.4%. This latter increase coincided with similar increases in hematocrit observed in the coho presmolts reared in the artificial creek during the same time period (Table III).

The relative concentration of hemoglobin components A6-8 of presmolts reared in the outdoor artificial creek and of presmolts exposed to various regimes of temperature, dissolved oxygen concentration and salinity for periods of 19, 33, and 47 days are presented in Table VII. It is evident that with the possible exception of the fish reared at 1.3 and 4.1 C no variation related to the experimental treatments occurred in the relative concentration of these three components.

TABLE VII

Relative concentration of hemoglobin components A6-8 of the blood of presmolt coho exposed to water of various dissolved oxygen concentrations, temperatures and salinities for periods of 19, 33 and 47 days and of coho presmolts maintained in an artificial creek during the same period. All data are presented as the mean ± 1 standard deviation for a sample of 3 to 6 fish.

TREATMENT	SAMPLE SIZE	RELATIVE CONCENTRATION OF HEMOGLOBIN COMPONENTS A6-8 (%)		
Treatment Time (Days)*		19	33	47
Dissolved Oxygen				
3.1 ppm	3	86.7 ± 6.2	89.3 ± 2.0	88.5 ± 1.8
5.8 ppm	3	83.0 ± 1.8	86.8 ± 6.3	85.2 ± 1.6
7.9 ppm	3	85.2 ± 5.9	89.0 ± 1.5	87.1 ± 0.5
Temperature				
1.3 C	3	90.5 ± 3.1	91.4 ± 0.5	86.4 ± 3.1
4.1 C	3	87.2 ± 1.8	89.5 ± 4.9	86.4 ± 2.4
8.0 C	3	84.7 ± 2.2	88.0 ± 0.7	83.6 ± 0.3
10.2 C	3	89.8***	86.3 ± 2.6	84.4 ± 2.5
15.0 C	3	84.0 ± 3.2	86.3 ± 0.2	83.7 ± 3.7
Salinity				
10 °/oo	3	84.7 ± 5.1	86.0 ± 1.5	84.1 ± 1.2
20 °/oo	3	86.8 ± 4.5	89.0 ± 0.6	83.5 ± 2.7
30 °/oo	3	83.4 ± 5.4	87.9 ± 0.9	86.6 ± 1.7
Controls	3	87.9 ± 2.4	87.9 ± 0.9	85.1 ± 1.4
Artificial Creek Fish**	6	84.9 ± 3.3	86.7 ± 3.5	80.4 ± 3.3

*The sampling date corresponding to the treatment times were: 19 days - 16/2/71; 33 days - 2/3/71; 47 days - 16/3/71.

**Fish from the artificial creek were sampled on the same dates as the treated fish.

***Two fish only: Range 88.5 to 91.2%.

The presmolts exposed to water temperatures of 1.3 to 4.1 C exhibited slightly elevated concentrations of A6-8 when compared to fish reared at higher temperatures, although the difference was only 2%.

Components A6-8 generally comprised a higher percentage of the total hemoglobin in the experimental fish than in presmolts in the artificial creek at the same time, although the differences were less than 5 to 6%, (Table VII). This difference is difficult to explain since the experimental fish experienced a slightly accelerated photoperiod which should accelerate the smolting process.

The relative concentrations of hemoglobin components A3, A1, C1, C3, C4, C5, and C6 of presmolt coho likewise did not exhibit any changes related to any of the eleven experimental treatments (Tables VIII, IX, X). These components were ranked in order of decreasing concentration C1:C3:C4: A1:A3:C5:C6. Although small variations occurred in individual fish the general ranking order of the minor components of the experimental fish was essentially identical with that of untreated coho presmolts as presented in Table I.

The electropherograms of the multiple hemoglobins of coho presmolts after 60 days exposure and of coho fry following 49 to 50 days exposure to the eleven environmental regimes are presented in Plates 3, 4, 5, 6, 7, and 8. These electropherograms are indistinguishable from the respective electropherograms of untreated presmolts (Plates 2A,B) and fry (Plate 1F,G) observed in Part I of this thesis, again

TABLE VIII

The relative concentrations of the hemoglobin components A3, A1, C1, C3, C4, C5, and C6 of the blood of coho presmolts reared in aerated well water at five temperatures for periods of 33 and 47 days. The values presented represent the mean ± 1 standard deviation for a sample of 3 fish.

WATER TEMPERATURE (C)	EXPOSURE TIME (DAYS)	RELATIVE CONCENTRATION (%)						
		A3	A1	C1	C3	C4	C5	C6
1.3	33	0.9 ±0.7	0.9 ±0.2	2.8 ±0.8	1.6 ±0.5	1.3 ±0.2	0.6 ±0.3	0.6 ±0.4
4.1	33	0.2 ±0.3	1.1 ±0.9	4.6 ±1.2	1.7 ±0.4	1.9 ±0.6	1.2 ±0.7	0.3 ±0.6
8.0	33	0.8 ±0.2	1.3 ±0.1	5.4 ±0.3	2.0 ±0.6	1.4 ±0.4	0.8 ±0.1	0.5 ±0.1
10.1	33	0.5 ±0.3	1.3 ±0.4	5.8 ±1.3	2.8 ±0.2	2.3 ±0.6	0.6 ±0.4	0.3 ±0.3
15.0	33	1.2 ±1.0	1.4 ±0.8	4.9 ±0.6	2.3 ±0.1	2.4 ±0.1	1.0 ±0.2	0.8 ±0.2
1.3	47	1.3 ±0.2	1.9 ±1.0	4.1 ±0.8	2.5 ±0.7	2.3 ±0.7	0.6 ±0.3	0.9 ±0.6
4.1	47	1.4 ±0.3	1.7 ±0.6	3.9 ±0.2	2.6 ±0.7	2.2 ±1.4	0.8 ±0.1	1.0 ±0.5
8.0	47	1.3 ±0.2	2.3 ±0.5	4.6 ±0.2	3.3 ±0.3	2.9 ±0.4	1.2 ±0.2	0.8 ±0.4
10.1	47	1.1 ±0.3	2.0 ±0.5	5.5 ±1.3	2.7 ±0.5	2.9 ±0.9	0.7 ±0.3	0.8 ±0.7
15.0	47	0.9 ±0.4	2.1 ±0.6	5.5 ±2.0	3.1 ±0.2	2.6 ±0.9	1.2 ±0.6	0.9 ±0.3

TABLE IX

The relative concentrations of the hemoglobin components A3, A1, C1, C3, C4, C5, and C6 of the blood of coho presmolts reared in well water at three concentrations of dissolved oxygen for periods of 33 and 47 days. The values presented represent the mean ± 1 standard deviation for a sample of 3 fish.

OXYGEN CONCENTRATION (ppm)	EXPOSURE TIME (DAYS)	RELATIVE CONCENTRATION (%)						
		A3	A1	C1	C3	C4	C5	C6
3.1	33	0.4 ±0.4	1.0 ±0.9	3.5 ±2.6	1.6 ±0.8	2.0 ±0.8	1.5 ±0.4	0.3 ±0.2
5.8	33	0.7 ±0.4	2.0 ±1.1	3.5 ±1.8	2.9 ±1.3	2.4 ±1.0	1.1 ±0.9	0.7 ±0.2
7.9	33	1.2 ±0.2	1.8 ±0.3	2.7 ±0.6	1.6 ±0.4	1.9 ±0.2	1.2 ±0.5	0.8 ±0.7
3.1	47	1.1 ±0.4	1.3 ±0.1	2.9 ±0.3	2.1 ±0.5	2.1 ±0.6	0.7 ±0.4	0.9 ±0.6
5.8	47	1.3 ±0.5	1.8 ±0.02	3.5 ±0.3	2.9 ±0.5	2.5 ±0.3	1.3 ±0.9	0.8 ±0.4
7.9	47	1.1 ±0.5	1.5 ±0.9	4.1 ±0.9	2.5 ±0.4	2.3 ±0.3	0.8 ±0.2	0.6 ±0.3

TABLE X

The relative concentrations of the hemoglobin components A3, A1, C1, C3, C4, C5, and C6 of the blood of coho presmolts reared at three water salinities for periods of 33 and 47 days. The values presented are the mean ± 1 standard deviation for a sample of 3 fish.

SALINITY (°/oo)	EXPOSURE TIME (DAYS)	RELATIVE CONCENTRATION (%)						
		A3	A1	C1	C3	C4	C5	C6
10	33	0.7 ±0.4	1.8 ±0.5	5.8 ±0.5	3.0 ±1.2	1.1 ±0.2	1.1 ±0.7	0.5 ±0.5
20	33	0.7 ±0.8	1.5 ±0.1	3.2 ±1.4	3.1 ±0.4	1.7 ±1.3	0.6 ±0.7	0.3 ±0.6
30	33	0.5 ±0.4	1.7 ±0.5	2.8 ±0.6	2.8 ±0.9	1.9 ±0.6	1.5 ±0.2	1.0 ±0.4
10	47	0.9 ±0.3	2.3 ±0.3	4.2 ±0.7	3.5 ±0.3	3.0 ±0.7	1.0 ±0.1	1.1 ±0.3
20	47	1.4 ±0.2	2.9 ±0.8	4.4 ±1.4	3.2 ±0.2	2.9 ±0.6	1.1 ±0.2	0.6 ±0.4
30	47	0.6 ±0.1	1.8 ±0.1	3.4 ±0.2	2.7 ±0.6	2.6 ±0.8	1.2 ±0.3	1.1 ±0.2

PLATE 3

Electropherograms of the multiple hemoglobins of presmolt coho salmon which had been exposed to aerated well water maintained at 1.3 C (A), 4.1 C(B), 8.0 C(C), 10.2 C(D), and 15.0 C(E), for 60 days. The electropherograms from two fish are presented for each temperature.

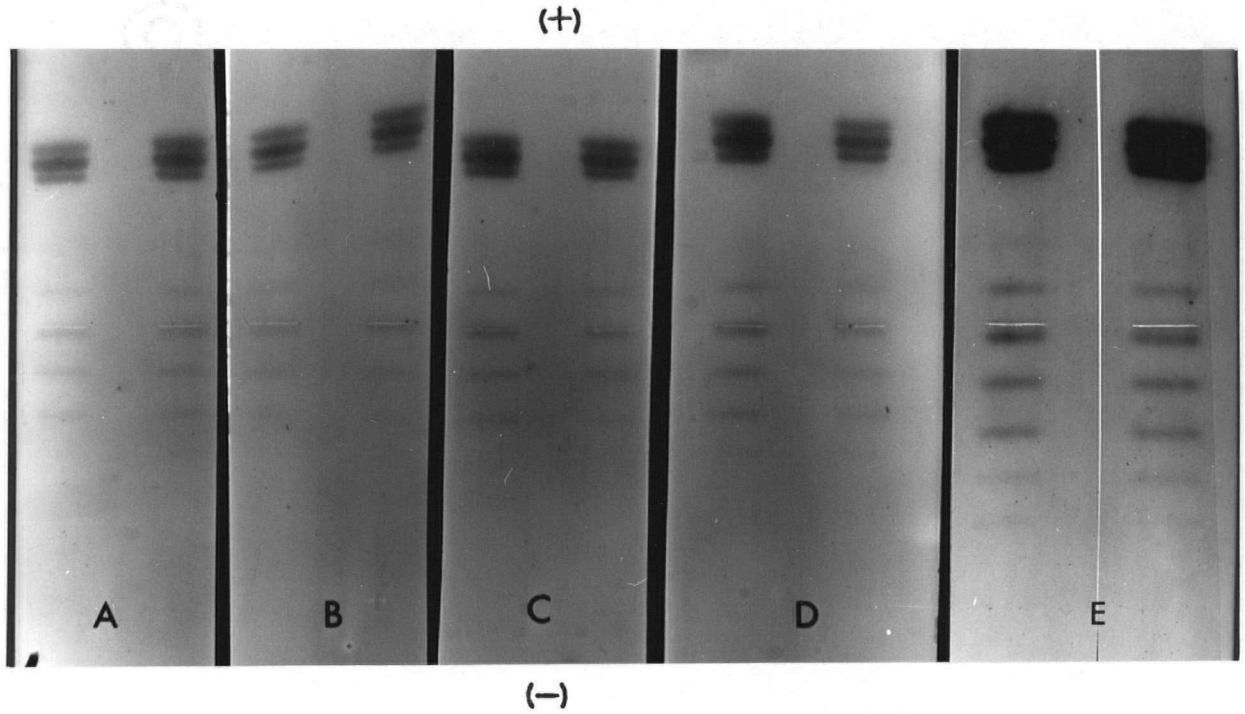

(+)
A
B
C
D
E
(–)

PLATE 4

Electropherograms of the multiple hemoglobins of coho fry which had been exposed to aerated well water maintained at 1.3 C (A), 4.1 C(B), 8.0 C(C), 10.2 C(D), and 15.0 C(E), for 49 days. The electropherograms from two fish are presented for each temperature.

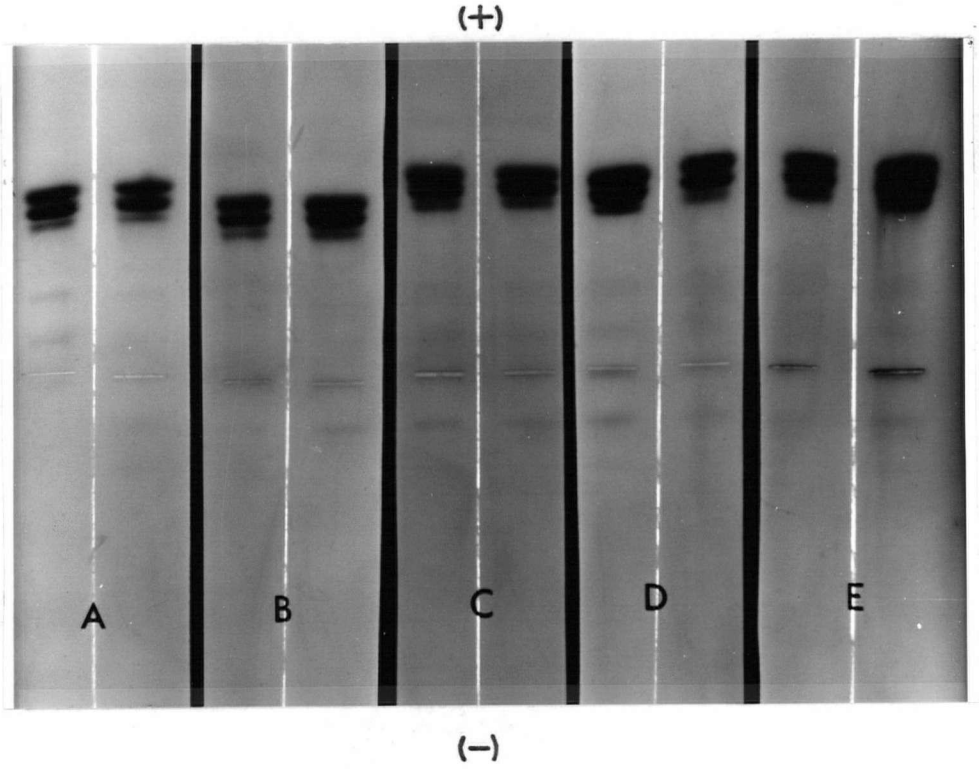

(+)
A
B
C
D
E
(–)

PLATE 5

Electropherograms of the multiple hemoglobins of presmolt coho which had been exposed to well water containing dissolved oxygen concentrations of 3.1 (A), 5.8 (B), and 7.9 (C) ppm for 60 days. The electropherograms of blood samples from two fish are presented for each dissolved oxygen concentration.

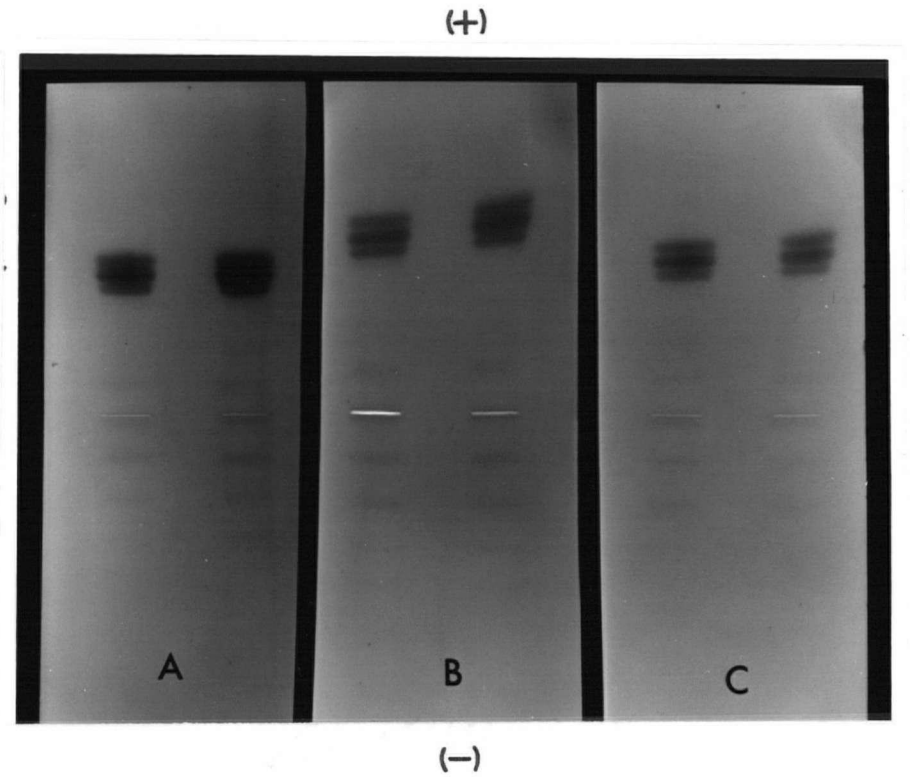

(+)
A
B
C
(−)

PLATE 6

Electropherograms of the multiple hemoglobins of coho fry which had been exposed to well water containing dissolved oxygen concentrations of 2.2 (A), 5.5 (B), and 8.1 (C), ppm for 49 days. The electropherograms of blood samples from two fish are presented for each dissolved oxygen concentration.

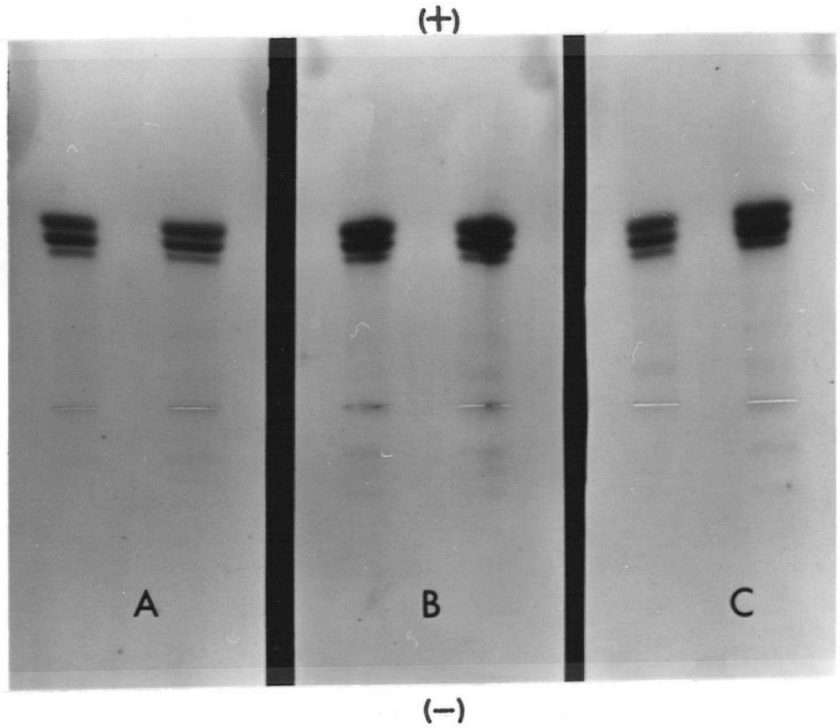

(+)
A
B
C
(−)

PLATE 7:

Electropherograms of the multiple hemoglobins of presmolt coho which had been exposed to salinities of 10 (A), 20 (B), and 30 (C) $^{o}/oo$ for 60 days. The electropherograms of blood samples from two fish are presented for each salinity.

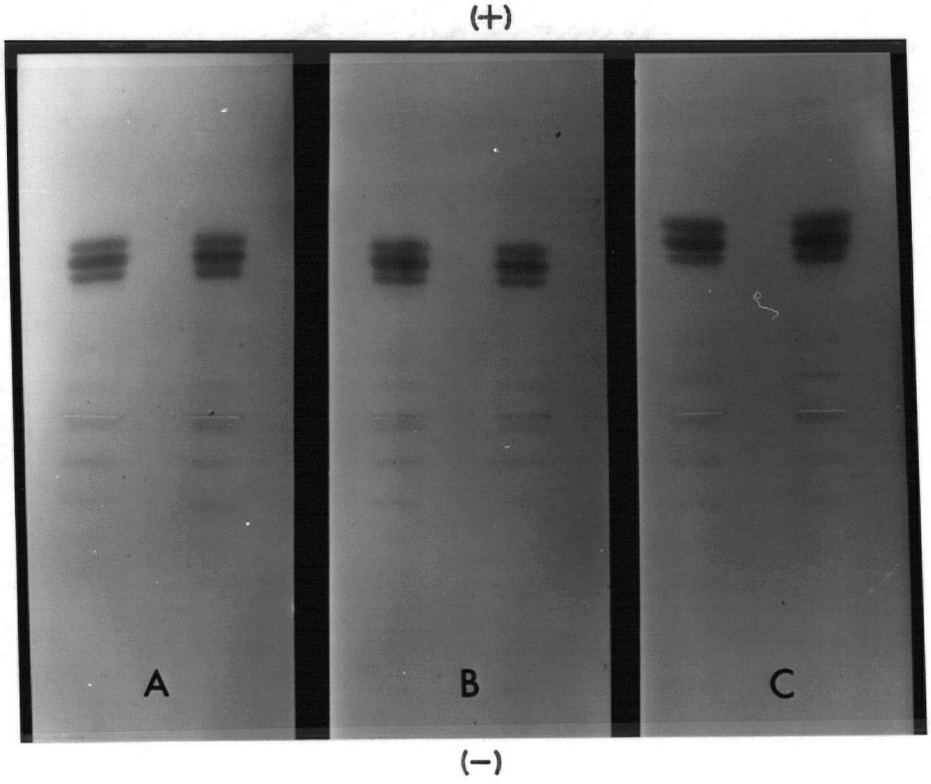
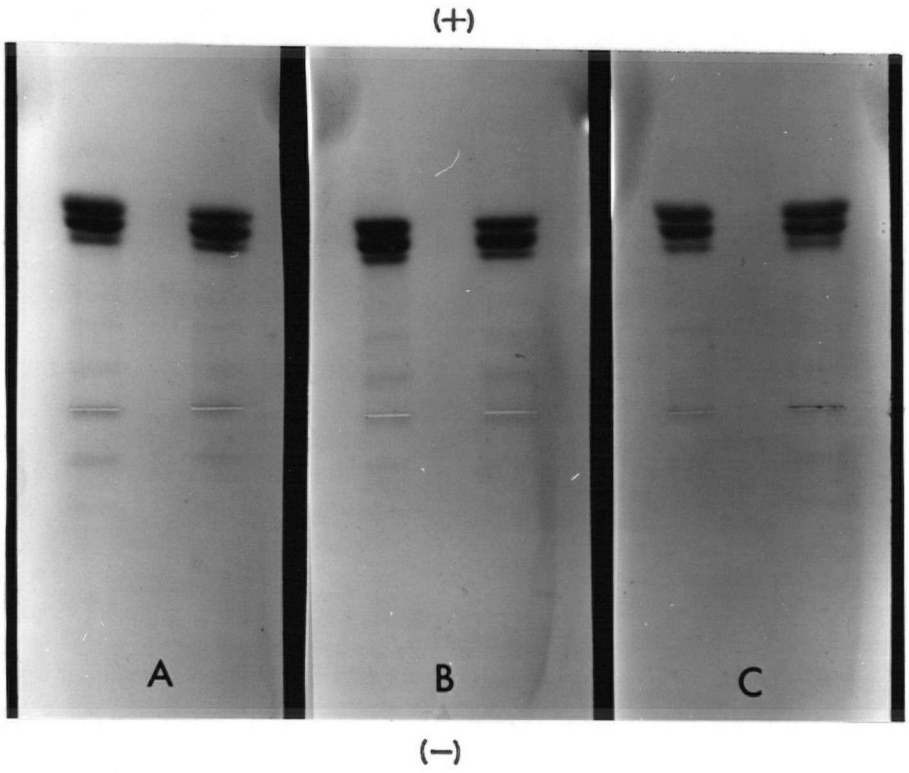

(+)
A
B
C
(−)

PLATE 8

Electropherograms of the multiple hemoglobins of coho fry which had been exposed to salinities of 10 (A), 20 (B), and 30 (C) $^{o}/oo$ for 50 days. The electropherograms of blood samples from two fish are presented for each salinity.


(+)
A
B
C
(−)

demonstrating that the environmental regimes to which these fish were exposed did not influence the electrophoretic pattern of the hemoglobin polymorphs.

EFFECT OF EXTENDED FRESHWATER RESIDENCE AND HYPOXIA UPON JUVENILE COHO.

Plate 9 and Table XI present the electrophoretic patterns and relative concentrations of the multiple hemoglobins of seventeen month-old coho salmon which had been reared for 5 1/2 months in fresh water at low (2.2 ppm) and high (9.7 ppm) dissolved oxygen concentrations and of normal seawater postsmolt of the same age. For reasons noted previously, the results represent the observations made on August 3, 1971 on one fish from each environment.

It is evident that the seawater and high-oxygen freshwater postsmolts are similar in respect to size, hematocrit and number and relative concentration of the ten hemoglobin components. Components A6-8 comprise approximately 7% more of the hemoglobin in the freshwater postsmolt but it is difficult to attach much significance to this relatively small difference without a large sample size.

The coho which had been reared under hypoxic conditions, however, was much smaller and has a much higher hematocrit than the former fish. This increase in hematocrit had occurred by the time the fish were fourteen months old when the mean hematocrit of three fish was 50.4%. The most significant effect of the hypoxic environment, however, was upon the

PLATE 9

Electropherograms of the multiple hemoglobins of 1 1/2-year-old coho presmolts which had been maintained in aerated (9.7 ppm O_2) well water (A), unaerated (2.2 ppm O_2) well water (B), or had been transferred to aerated (9.3 ppm O_2) 2 months previously (C). Two electropherograms are presented for the same blood sample from each fish.

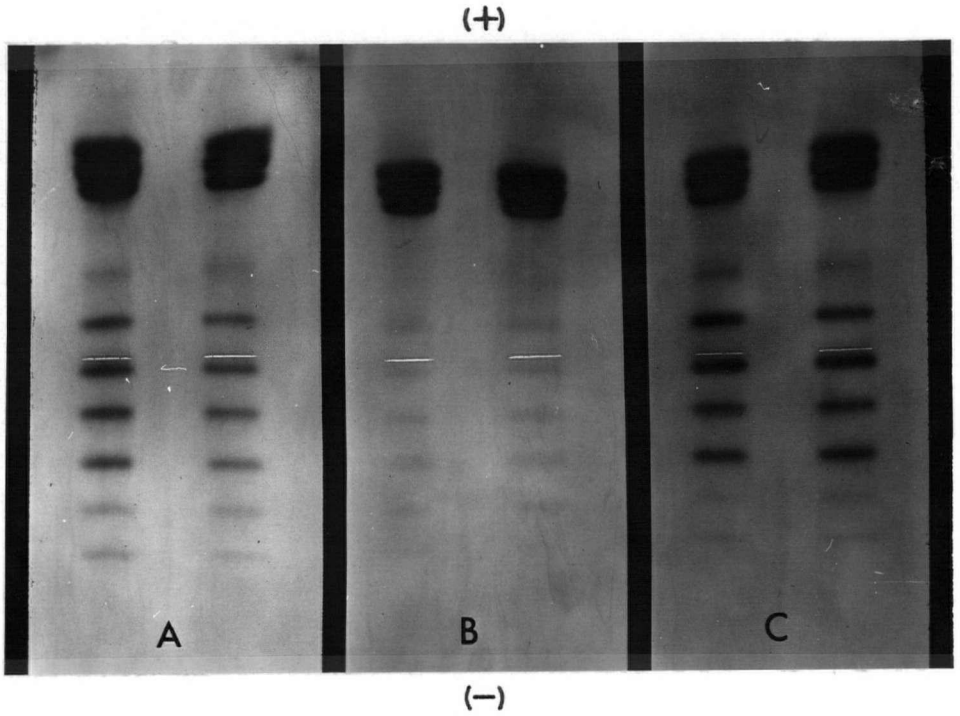

(+)
A
B
C
(−)

TABLE XI

Comparison of the size, α intercept of the weight-length relationship $W = \alpha L^{3.25}$, hematocrit and relative concentration of the hemoglobin components of the blood of presmolt coho salmon maintained in aerated (9.7 ppm O_2) and unaerated (2.2 ppm O_2) well water and of postsmolts of the same age which had been residing in seawater for approximately 1 month. Only one fish from each environment was examined.

	LENGTH (cm)	WEIGHT (g)	α INTERCEPT (mg)	HEMATOCRIT (% RBC)	RELATIVE CONCENTRATION OF HEMOGLOBIN COMPONENT (%)							
					A6-8	A3	A1	C1	C3	C4	C5	C6
Fresh water (9.7 ppm O_2)	13.5	26.4	5.59	27.0	57.9	2.6	7.1	8.6	9.3	7.8	3.7	3.1
Freshwater (2.2 ppm O_2)	9.0	7.3	5.78	54.8	81.2	2.3	2.7	4.2	3.1	3.1	2.2	1.3
Sea water (9.2 ppm O_2)	14.1	30.7	5.65	33.3	50.9	3.3	10.5	13.3	9.0	9.1	0.9	3.1

relative concentrations of the hemoglobin polymorphs. Components A6-8 comprised over 81% of the total hemoglobin which is only 4% less than the relative concentration observed at the beginning of the adaptation to the hypoxic conditions. Similar lack of change occurred in the distribution of the seven remaining hemoglobin components. These observations suggest that exposure of 11 1/2-month old presmolt coho to extremely hypoxic water resulted in a virtual cessation of growth and ontogenetic hemoglobin development as well as a marked increase in hematocrit.

DISCUSSION II

One of the major requirements in the design of experiments to determine the influence of some factor upon a particular reaction is to maintain adequate control of all the remaining factors which may influence the reaction. In biological systems, especially in cases were living animals are used, this ideal is seldom realized. In fish complex interactions between season, a variety of environmental factors, size, ration size and composition,and feeding schedule influence the observed rates of growth and development (LeBrasseur and Parker, 1964; Paloheimo and Dickie, 1966; LeBrasseur, 1969; Atherton and Aitken 1970; Brett and Higgs, 1970; Brett, 1971). When such animals are subjected to environmental manipulations it is necessary to determine whether

or not some uncontrollable factor may be limiting the response to the fish to the variable under study. This problem is expecially applicable to the present study since it encompassed two possible effects of environment upon hemoglobin polymorphism in coho salmon. First certain environmental factors could directly influence the composition of the hemoglobin polymorphs. Secondly, the environment could influence the growth of the fish and result in size-related changes in the hemoglobin pattern.If, therefore, the growth responses of the coho to variations in certain environmental factors were abnormal because of some uncontrolled variable the interpretation of any changes in hemoglobin pattern would be difficult, if not impossible. The effects of water temperature, dissolved oxygen concentration and salinity upon the patterns of growth of coho presmolts and fry observed in the present study, were generally in agreement, however, with similar information published for this and other species of salmonids.

In juvenile freshwater sockeye salmon, the rate of growth increased by 0.159%/day/degree C over the range of 5 to 15 C and is maximal at 15C (Brett, Shelbourn and Shoop, 1969). Although the coho presmolts exhibited maximum growth at 15C the effect of elevated water temperature upon the rate of growth was somewhat lower (0.112%/day/degree C).

Hermann, Warren, and Doudoroff (1962) reported that dissolved oxygen concentrations below 5.6 ppm severely curtailed growth and food consumption in juvenile freshwater coho at 20C. The present results are in general accord with

these observations since the rates of growth of presmolt coho at 5.8, 7.9, and 9.7 ppm oxygen were essentially the same, whereas at 3.1 ppm the rate was only 56% of that observed at the former concentrations. In coho fry, however, growth was reduced at 5.5 ppm oxygen and more severely at 2.2 ppm. Over 85% of the fry died at this latter concentration. Hermann *et al.* (1962) reported mortalities of 30 to 100% for fry of approximately the same age. Mortality declined to zero after July and August and this decline was correlated to a decrease in the rate of oxygen consumption as the fry progressed through their freshwater stage (*ibid.*). These observations probably explain the difference in effect of oxygen concentrations of approximately 5.5 to 5.8 ppm upon growth in presmolts and fry recorded in the present experiments.

A disparity exists between the effect of salinity upon the growth of coho presmolts reported here and fry by Otto(1971) who reported that salinities exceeding $10^{o}/oo$ inhibited growth and salinities of $30^{o}/oo$ resulted in mortalities of 30 to 50%. Growth in presmolt coho at all salinities up to $30^{o}/oo$ exceeded that in freshwater controls and was maximal at $20^{o}/oo$. The average rate of growth at $20^{o}/oo$ salinity included one value (19-day sample) which was approximately double that observed in the remaining three samples. If this value is eliminated from the calculations then the mean instantaneous rate of growth at a salinity of $10^{o}/oo$ exceeds that at $20^{o}/oo$ the respective values being

1.675 and 1.494%/day. In fry growth was approximately equal in fresh water and at salinities of 10 and 20 °/oo but reduced at 30 °/oo salinity. Although no data are available concerning the amount of potential food carried to the fish in the seawater it is probable that organisms in the sea water were a supplementary source of food to the presmolts and may account for the disparity in growth of these fish in fresh and sea water. Saunders and Henderson (1969) reported that the rate of growth of Atlantic salmon parr were the same in fresh water as in salinities of 7 and 15 °/oo, but were reduced at salinities exceeding 22 °/oo. These observations are in fairly close agreement with the present results. No satisfactory explanation can be offered for the disparity of these results with those of Otto (1971) although it is possible that the frequent anesthetization and measurement for length and weight conducted in the latter study may have adversely effected the ability of coho to adapt to higher salinities.

In general, then, there is no evidence to suggest that any abnormal responses to variations in the temperature, dissolved oxygen concentration or salinity occurred in the rates of growth of the juvenile freshwater coho salmon. A comparison of the $\propto$ intercept of the weight-length relationship $W = \propto L^{3.25}$ of presmolt coho from the artificial creek and from the eleven experimental environments after 60 days of treatment suggests that with one possible exception, little

or no acceleration of the streamlining process occurred in the treated fish. Presmolts reared at 3.1 ppm dissolved oxygen exhibited reductions in the α intercept over the experimental period but this was coupled to an extreme reduction in growth. A similar occurrence was observed in the 60-day sample of presmolts reared at 5.8 ppm dissolved oxygen. At 20 ^{o}/oo salinity α decreased to 5.57 mg which would correspond to the value in 14-month old presmolts in the artificial creek. Thus, although exposure of presmolt coho to 20 ^{o}/oo salinity for a sixty day period did accelerate the streamlining process the value of α did not decrease to that observed in normal fifteen to sixteen-month old smolts.

The foregoing considerations lead to two major conclusions based on the observation that no significant changes occurred in the hemoglobin pattern of coho fry or presmolts exposed to five water temperatures, three dissolved oxygen concentrations and three salinities. First the transition from the fry hemoglobin pattern composed of three anodic components to the presmolt pattern of five anodic and five cathodic components is not a direct result of fluctuations of water temperature and dissolved oxygen concentrations in the environment. Several major biochemical and physiological changes are known to occur during this period of transition (Baggerman, 1960; Conte *et al.*,1966; Vanstone and Markert, 1968; Giles, 1969) and it appears likely that factors influencing erythroporetic activity may be responsible for the observed shifts

in hemoglobin pattern. Since fish erythrocytes are nucleated they may be capable of increasing the relative concentration of the minor hemoglobin components A1, A3, C1, C3-6 in the red blood cells of fry which contain only components A6-8. This would make the stimulation of erythropoiesis, possibly a new cell line, totally unnecessary. Fantoni *et al.* (1969) have demonstrated that new erythroid cell lines are activated during the ontogenetic transition in synthesis of fetal to adult hemoglobin in mammals. Mammalian erythrocytes, however are non-nucleated and therefore incapable of synthetizing new proteins.

A second aspect of the present findings was that while the various environmental regimes to which presmolt coho were exposed did not result in any direct changes in the hemoglobin electrophoretic pattern, their influence upon growth did in certain instances result in fish which were physically larger than untreated postsmolts in which hemoglobin metamorphosis had begun. Presmolts reared for 60 days in freshwater at 15 C and in 10^{o}/oo salinity at approximately 9 C were equal to or larger than 16 1/2-month old postsmolts which had been in seawater for one month. In addition, certain individuals from the 20^{o}/oo salinity treatment and the 10C freshwater treatment were as large as the postsmolts. As discussed previously, the increase in size in the treated fish was not associated with a decrease in the α intercept of the weight-length relationship which is characteristic of normal smolts and postsmolts.

These observations suggest that physical size is not a significant factor in determining the timing of the presmolt to postsmolt shift in the multiple hemoglobin pattern of coho salmon. It is also significant that larger presmolts which had been reared in diluted sea water did not exhibit hemoglobin pattern changes since a combination of size and exposure to sea water may have been necessary to elicit a response. The elimination of this possibility is reinforced by the observation that coho postsmolts maintained in fresh water exhibited similar changes in hemoglobin pattern to normal seawater postsmolts of the same age.

Since environmental and size-related factors do not control the transition from the hemoglobin pattern of juvenile freshwater coho to that of seawater postsmolts it would appear that the changes are related to the age of the fish. The mechanism of this age-related control is unknown for both fish and higher vertebrates. In mammals the transition from fetal to adult hemoglobin synthesis apparently involves the production of an erythropoiesis-stimulating substance and the activation of different erythroid cell lines (Fantoni *et al.*, 1969; Jonix and Nijhof, 1969). Since a similar process is apparently operative in the change from alevin to fry hemoglobin in Atlantic salmon (Vernidub, 1966) it is probable that the production of the adult hemoglobin pattern during the early period of marine residence involves the stimulation of a separate cell line. Present information regarding the sites of production of erythrocytes and factors controlling red cell production in fish is insufficient to resolve the foregoing questions.

The ontogenetic variations in coho hemoglobin with age rather than size appears to conflict with observations made upon certain other salmonids in which such changes in hemoglobin pattern were related to length (Koch *et al.*, 1964; Wilkins and Iles, 1966; Westman, 1970). In all of the foregoing studies, however, the precise age of the fish was apparently unknown. Under normal rearing conditions the size of the fish is usually related to its age and it would seem reasonable to conclude that the apparent disparity could be resolved by transposing the length measurements to their respective ages. When the length-related changes in the hemoglobin polymorphs of sea-run and landlocked Atlantic salmon are compared (Westman, 1970) it is evident that the landlocked fish generally exhibit more rapid changes than those which migrate to sea. Since landlocked salmon grow at a slower rate it is probable that the differences in the rates of hemoglobin changes would be eliminated or at least reduced by relating the hemoglobin changes to age rather than length.

In only one instance did an environmental factor influence the hemoglobin pattern of juvenile coho salmon. Fish reared in freshwater containing 2.2 ppm dissolved oxygen throughout the entire period of smolting and early marine residence did not exhibit the changes in the hemoglobin pattern normally associated with the early postsmolt period. Although chronologically postsmolts these fish ceased growing after being acclimated to the low oxygen concentrations. The relative concentrations of the ten hemoglobin components of this

seventeen-month old fish remained essentially undistinguishable from those of eleven-month old presmolts. The hematocrit of the low-oxygen fish increased to approximately 50% suggesting that erythropoeisis was not impaired by this treatment.

Two possible explanations could account for the effect of long-term exposure to low oxygen tensions. First, since growth virtually ceased it is possible that other aspects of their physiological development were also retarded and the fish remained physiologically presmolts. Although the α intercept of the weight-length relationship did decrease to about 5.8 mg this effect was not the result of growth and probably represents an inability to satisfy the animals metabolic requirements. In this connection, it is well established that small juvenile sockeye salmon, *Oncorhynchus nerka*,may remain in freshwater for an additional year before smolting and embarking upon their seaward migration (Foerster, 1968). Although this occurrence is less frequent in coho salmon it would seem reasonable to expect these smaller fish to return to their presmolt physiological condition. Secondly, it is possible that the hypoxic conditions exerted some direct influence upon the erythropoeitic organs thereby retarding the production or erythroid cells capable of synthetizing adult hemoglobins. Such a system could involve the production of specific erythropoeitins for the selective stimulation of erythrocytes containing presmolt or adult hemoglobins. Further experimentation involving the exposure of marine coho to hypoxic conditions and the injection of postsmolt plasma into

presmolts in order to elucidate the actual mechanism involved.

No relationship between hematocrit and water temperature, dissolved oxygen concentration or salinity was observed in either coho presmolts or fry. In view of the severity of some of the treatments this result was somewhat unexpected. Grigg (1969) observed increased hematocrits in bullheads acclimated to 9 C when compared to those acclimated to 24 C. Hematocrits tended to increase with increasing water temperature in the common carp, *Cyprinus carpio* (Houston and DeWilde, 1968) and rainbow trout, *Salmo gairdneri*, (DeWilde and Houston, 1967). Cameron (1970) demonstrated that higher temperatures, hematocrits were raised in field populations of pinfish, *Lagodon rhomboides*, stripped mullet, *Mugil cephalus*, but that these increases were not observed during acclimation to temperatures of 10 and 25 C under laboratory conditions. It must be noted that in all the foregoing studies extensive overlap occurred in hematocrit values at the various acclimation temperatures and that the differences observed were generally in the order of 2-6%. It is quite likely that the small sample size and concommitant high variability may have obscured small effects of the environmental variables upon hematocrit in the present studies.

SUMMARY II

1) Coho presmolts (11-months old) and fry (3 1/2-months old) were exposed to five different water temperatures from 1 to 15 C, three dissolved oxygen concentrations from 2.2 to 8.1 ppm and three salinities from 10 to 30 °/oo. Changes in growth, hematocrit and hemoglobin pattern were recorded.

2) Growth increased with increasing water temperatures to a maximum of 15 C in both groups of fish.

3) Growth was inhibited in fry at oxygen concentrations of 5.5 ppm and more strongly at 2.2 ppm oxygen. In presmolt coho, growth was severely reduced at 3.1 ppm oxygen but not at 5.8 ppm or higher.

4) Growth was optimal at a salinity of 20 °/oo and decreased at 30 °/oo in both groups of fish although the decrease was much more evident in the fry than in the presmolts.

5) No change in hematocrit or hemoglobin pattern occurred in response to any of the treatments in either fry or presmolts.

6) Postsmolts maintained in oxygenated freshwater developed the hemoglobin electrophoretic pattern characteristic of seawater postsmolts of the same age.

7) The hemoglobin pattern of juvenile coho reared in hypoxic freshwater (2.2 ppm dissolved oxygen) throughout the presmolt and early postsmolt period failed to exhibit the changes characteristic of normal postsmolts and remained indistinguishable from the pattern exhibited by twelve-month old presmolts.

PART THREE

OXYGEN EQUILIBRIUM CHARACTERISTICS OF THE HEMOGLOBIN AND WHOLE BLOOD OF COHO FRESHWATER ADULTS AND FRY

INTRODUCTION III

Although the oxygen equilibria of fish blood have been studied for some time, almost nothing is known of ontogenetic variations in oxygen affinity, Bohr and Root shifts and heme-heme interaction for these animals. This is especially surprising since significant differences in the oxygenation characteristics of the embryonic or fetal and the adult hemoglobins have been reported for mammals (Prosser and Brown, 1961), birds (Mission and Freeman, 1972) and amphibians (Riggs, 1951; Wood, 1971). The fetal hemoglobin of the skate, *Raja binoculata,* (Manwell, 1958) and the spiny dogfish *Squalus suckleyi,* (Manwell, 1963) exhibits a higher oxygen affinity and, for the latter species, a higher heme-heme interaction than adult hemoglobin. The possibility of ontogenetic variations in the oxygen equilibrium of the blood of free-swimming juveniles was not investigated in these species.

Somewhat more is known regarding the oxygenation characteristics of the individual components of the multiple hemoglobins of fish. Work on such diverse species as the Japanese eel, *Anguilla japonica,* (Yamaguchi *et al.*, 1962 b; Hamada *et al.*, 1964; Yoshioka *et al.*, 1968; Itada, Turitzin, and Steen, 1970), rainbow trout *Salmo irideus* (Binotti *et al.*,

1971), chum salmon *Oncorhynchus keta*, (Hashimoto, Yamaguchi and Matsuura, 1960) and loach (Yamaguchi *et al.*, 1962 a), has demonstrated that in general the electrophoretically distinguishable components of fish hemoglobin differ in one or more of their oxygenation characteristics. This diversity in the functional characteristics of the hemoglobin polymorphs lends support to the concept that each component may serve to more effectively meet the oxygen requirements of the fish under different physiological and environmental conditions.

Willmer (1938) asserted that the lack of a large Bohr shift in the blood of certain fish is an adaptation to high environmental carbon dioxide tensions irregardless of oxygen availability, whereas Krough and Leitch (1919) suggested that fish living at low environmental tensions possessed hemoglobin with a high oxygen affinity in order to saturate the blood with oxygen at the gills and a significant Bohr shift to facilitate the maintenance of a high blood:tissue oxygen gradient. The large Bohr effect observed in the blood of active fish which do not encounter low environmental oxygen tensions supposedly offsets the increase in oxygen affinity which occurs when environmental temperature is reduced, thereby maintaining a high blood:tissue oxygen gradient (Black, Kirkpatrick and Tucker, 1966a,b; Black, Tucker and Kirkpatrick, 1966a). Although the foregoing interpretations may apply to certain species of fish there are many cases in which they do not apply (Hashimoto *et al.*, 1960; Lenfant, Johansen and Grigg, 1967), thereby making generalizations somewhat difficult. The situations further complicated by the possible action of various allosteric effectors

which may modify one or more of the functional characteristics of the hemoglobin.

Since coho salmon are anadromous fish, they encounter a wide range of environmental conditions. It has been demonstrated in this thesis that the hemoglobin components of fry and postsmolts are considerably different (Part I),but that the electrophoretic hemoglobin pattern of juvenile freshwater fish is essentially unaffected by variations in environmental temperature, dissolved oxygen concentration and salinity (Part II). The following experiments were undertaken to determine whether ontogenetic changes in hemoglobin composition were associated with changes in the oxygen equilibrium characteristics of the blood and to interpret the functional significance of the ontogenetic variations in relation to the transport of oxygen by the blood under normal environmental conditions.

METHODS III

FISH EMPLOYED

Migrating adult coho were trapped at the Department of Fisheries of Canada facilities at Robertson Creek during August and September, 1971, and transported to our aquarium facilities in West Vancouver where they were maintained in 10-foot diameter circular aquaria provided with flowing aerated well water. Although these fish were used in the majority of the following experiments, a few migrating adult coho were obtained from the Big Qualicum River on Vancouver Island. No

difference in the hemoglobin pattern was observed in fish of either sex or locality.

PREPARATION OF HEMOGLOBIN SOLUTIONS

The fish were removed from the aquarium and killed by a blow on the head. Approximately 50 ml of blood was collected by aortic tail puncture into a 50 ml syringe fitted with a 1 1/2-inch 18 gauge needle and containing 1 ml of sodium heparin solution (1000 U.S.P. units/ml, Connaught Medical Research Laboratories, Toronto, Ontario). The blood was well mixed and samples removed for determination of hematocrit concentration and ATP concentration. The remaining blood was centrifuged at 3000 rpm in a Phillips-Drucker centrifuge and the plasma and buffy layer of white blood cells removed by aspiration. The packed erythrocytes were washed twice with 1.0% NaCl. After the final wash the erythocytes were cooled in an ice bath, hemolyzed with 5 times the erythocyte volume of ice cold 0.001 M tris-HCl, pH 7.5 and frozen at -40 C for 1 hour. The frozen suspension was then slowly thawed at room temperature and centrifuged at 37,000 X g for 1 hour in a refrigerated Sorvall Model RC-2 centrifuge. The supernatant was removed and subjected to ultrafiltration through a PM-30 DIAFLOW ultrafiltration membrane (Amicon Corp., Lexington, Massachusetts) at 50 p.s.i. pressure of nitrogen. This filtration resulted in a solution containing 8 to 22.7 g% hemoglobin.

In order to further reduce the phosphate concentration of the filtered adult hemoglobin solution 1.0 M NaCl in 0.001 M

tris-HCl buffer, pH 7.5, was added to yield a final NaCl concentration of 0.1 molar. Approximately 1.2 to 2.0 g of the hemoglobin in a volume of 5 to 15 ml were applied to a 2.5 X 40 cm water jacketed chromatographic column of G25-fine Sephadex (Pharmacia Canada Ltd.) equilibrated with 0.1 M NaCl in 0.001 M tris-HCl buffer, pH 7.5, maintained at 2 C (Berman, Benesch and Benesch, 1971). The hemoglobin was eluted with the equilibration buffer at a pressure head of 30 cm of water and collected in 3 to 10 ml fractions in a refrigerated fraction collector (Buchler Instruments Inc.). After concentrating the eluted hemoglobin to approximately 10 g% with the Diaflow molecular filter tris-HCl buffer (1.0 M tris-HCl containing 0.1 moles /l of NaCl), pH 7.5, was added to yield a final tris-HCl concentration of 0.1 molar. The total phosphate concentration of this solution was estimated to be approximately 0.038 μmoles /ml or 0.1 moles phosphate/mole hemoglobin employing the method of Bartlett (1959). The hemoglobin concentration was reduced to approximately 4.0 g% by dilution with 0.1 M NaCl in 0.1 M tris-HCl. The pH of this dilution buffer was previously adjusted to yield a final pH near the desired value and final pH adjustments were made with 0.1 N HCl or NaOH.

Hemoglobin components A6-8 were isolated from the original concentrated hemolyzate with a modification of the method of Binotti *et al.* (1971). Sufficient 1.0 M tris-HCl, pH 8.6, was added to the hemolyzate to yield a final buffer concentration of 0.1 molar and the pH adjusted to 8.5. A refrigerated chromatographic column (8.0 X 5.0 cm, h X d) of DEAE-Sephadex, A-50, was equilibrated at 2 C with 0.1 M tris-

HCl, pH 8.5 employing a polystaltic pump (Buchler Instruments Inc.) to maintain a constant flow rate of 90 ml/hr through the column. Up to 4.0 grams of hemoglobin in a volume of 50 ml were applied to the column and eluted overnight with 0.1 M tris-HCl buffer. Under these conditions only components A6-8 of the adult hemoglobin were retained on the exchange resin as determined by electrophoresis. These three components were then eluted with 1:1 (v:v) mixture of 0.1 M tris-HCl, pH 8.5 and 0.1 M KH_2PO_4. Solid NaCl was added to the elutant to yield a final NaCl concentration of 0.1 molar and the pH adjusted to 7.5 with 0.1 N HCl. The hemoglobin was concentrated to approximately 10 g% by pressure filtration and chromatographed on the G25-Sephadex column as described previously. Following a final concentration by pressure filtration the hemoglobin was buffered with 0.1 M tris-HCl containing 0.1 moles/1 NaCl at the appropriate pH. The total phosphate of this solution was approximately 0.2 moles/mole hemoglobin as determined by the method of Bartlett (1959).

MEASUREMENT OF INTRACELLULAR ATP CONCENTRATION

The concentration of adenosine triphosphate, (ATP), in the blood was determined by measuring the decrease in optical density at 340 mμ when diphosphopyridine nucleotide (DPN) is oxidized from its reduced form (DPNH), according to the following set of reactions; (Sigma Technical Bulletin 366-UV, 1967):

$$\text{ATP} + 3\ \text{phosphoglycerate} \underset{\text{kinase}}{\overset{\text{phosphoglycerate}}{\rightleftharpoons}} \text{ADP} + \text{1,3-diphosphoglycerate}$$

$$\text{1,3-diphosphoglycerate} + \text{DPNH} \underset{\text{phosphate dehydrogenase}}{\overset{\text{glyceraldehyde}}{\rightleftharpoons}}$$

$$\text{glyceraldehyde-3-phosphate} + \text{DPN} + P_i$$

The method is not specific for ATP but measures the total concentration of the nucleoside triphosphates. Since ATP comprises the vast majority of the organic phosphate in fish blood (Rapoport and Guest, 1949; Gillen and Riggs, 1971) the contribution of these other nucleoside triphosphates to the enzyme reaction is probably negligible. Analysis were performed on duplicate or triplicate samples (0.5 to 1.0 ml) of whole blood using the enzyme kit distributed by the Sigma Chemical Company.

MEASUREMENT OF HEMOGLOBIN CONCENTRATION

Total hemoglobin concentration was assayed by measuring the absorbance at 540 mμ after conversion of all hemoglobin to the cyanmethemoglobin form with an aqueous alkaline solution containing ferricyanide and cyanide reagent (Uni-Tech Chemical Manufacturing Co.). Duplicate or triplicate 20 ml samples of whole blood or hemoglobin solution were added to the 5 ml of cyanide-ferricyanide reagent and the absorbance measured using the reagent as a blank. The absorbances were related to hemoglobin concentration by comparison to a low range Hemochrome-

Fe standard (Uni-Tech Chemical Manufacturing Co.) treated in the same manner.

Methemoglobin concentration was determined on duplicate samples of blood or hemoglobin solutions by measuring the decrease in absorbance at 635 mμ upon the addition of sodium cyanide to a hemoglobin solution (Evelyn and Malloy, 1938).

COLLECTION OF WHOLE BLOOD FOR MEASUREMENT OF OXYGEN EQUILIBRIA:

Blood was removed from the tail as described previously (Methods I). The blood was well mixed to ensure uniform distribution of erythrocytes and aliquots delivered into 25 or 50 ml pear-shaped boiling flasks which contained a few grains of sodium heparin (160,000 U.S.P. units/gram).

Coho fry were anesthetized with 2-phenoxyethanol and blood collected in lightly heparinized capillary tubes from the severed caudal peduncle. The blood was expelled into 5 ml culture tubes containing a few grains of sodium heparin and immersed in an ice bath. A few additional drops of sodium heparin solution were added to the culture tubes during the collection period. The blood from a total of 113 fry was collected on January 17, 1972. This required about 75 minutes and yielded 11.5 ml of blood. One-half ml of 1.0% NaCl was added to bring the volume to 12.0 ml. The blood was well mixed and divided into aliquots to be used in the determination of oxygen equilibria.

DETERMINATION OF OXYGEN EQUILIBRIA

Oxygen equilibria of whole blood and hemoglobin solutions were determined by a modification of the method of Edwards and Martin (1966). The blood or hemoglobin solution to be studied was divided into 2 aliquots and placed in 25 or 50 ml pear-shaped boiling flasks which served as tonometers. The flasks were clamped to a variable speed wrist-action shaker and partially submerged in a water bath at the appropriate equilibration temperature. Air and nitrogen gas were saturated with water vapor by bubbling through a series of flasks which were half filled with water and maintained at the desired equilibration temperature by submersion in the water bath. Each gas was then delivered to one of the tonometers at the rate of 200 ml/minute and the blood or hemoglobin solution shaken until the tonometer receiving nitrogen had an oxygen tension of less than 0.5 mm Hg and the tonometer receiving air had an oxygen tension exceeding 120 mm Hg.

In order to determine the effect of carbon dioxide upon the oxygen equilibrium of whole blood, gas mixtures (certified grade Matheson of Canada Ltd.) containing 0.447 ± .009 % (v:v) CO_2:balance N_2 and 0.452 ± .009% CO_2:balance air were substituted for the pure nitrogen or air in the equilibration procedures. Since such gas mixtures may become stratified after standing for some time the cylinders were rolled back and forth along the floor for 10 minutes prior to use.

After equilibration a portion of the oxygenated blood was drawn into a 1.0 ml disposable plastic syringe marked in divisions of 0.01 ml and fitted with a 1 1/2-inch long

21 gauge needle (Becton-Dickenson Ltd.). The air was expelled from the syringe and the volume of oxygenated blood recorded. An appropriate volume of deoxygenated blood was drawn into the syringe to yield the desired percentage of oxygenated blood. Approximately 0.05 ml of mercury was then drawn into the syringe anaeobically and the needle sealed by inserting it into a rubber stopper. The mercury was run back and forth in the syringe to thoroughly mix the blood for 2 to 3 minutes. The partial pressure of the blood in the syringe was then measured using a water-jacketed Radiometer PO_2 electrode, type E5046, in conjunction with a Radiometer acid-base analyzer, type PHM 71b or PHM 72b. The circulating coolant in the water jacket was the same temperature as the blood equilibration temperature. Two oxygen equilibrium curves could be determined within a 4-5 hour period.

The oxygen content of the hemoglobin at various mixing ratios was calculated from the following relationship:

$$\text{Content (ml } O_2\text{/100 ml solution)} = S(C_{Hb}) + C_b\left(S - \frac{PO_2}{P_s}\right)$$

where S is the proportion of oxyhemoglobin in the mixture, C_{Hb} is the oxygen capacity of the hemoglobin in ml O_2/100 ml solution or whole blood and is calculated by multiplying the hemoglobin concentration in g% by the oxygen capacity in ml O_2/g hemoglobin, C_b is the oxygen content of the buffer or plasma in ml O_2/100 ml buffer or plasma at air saturation, P_s is the partial pressure of oxygen in mm Hg at air saturation and PO_2 is the measured partial pressure of oxygen in the mixture. The percent saturation of the hemoglobin was then calculated as the ratio of oxygen content to oxygen capacity of the

hemoglobin in vol % multiplied by a factor of 100.

The solubility of oxygen in plasma was taken as 0.79 ml O_2/100 ml plasma at 10 C (Stevens, 1968) and the solubility in the 0.1 M tris-HCl buffer was measured as 0.75 vol % at the same temperature. No correction was made in this latter value for the decrease in oxygen solubility caused by the dissolved hemoglobin since the maximum hemoglobin concentration was only 0.64 m molar which would have a negligible effect on the oxygen content.

DETERMINATION OF OXYGEN CAPACITY

The method of Tucker (1967) was employed to determine the oxygen content of air-saturated blood and hemoglobin solutions. A water-jacketed cuvette of about 2 ml capacity containing a magnetic stirring bar was fitted with a Radiometer PO_2 electrode. A partially degassed solution of 6 g of potassium ferricyanide and 3 g of saponin in a litre of water was delivered into the cuvette and the oxygen tension measured. A 50 µl sample of the oxygenated blood was delivered into the cuvette anaerobically. The hemoglobin was converted to cyanmethemoglobin thereby effecting the release of the bound oxygen into the ferricyanide reagent. The increase in PO_2 of the ferricyanide solution was measured and the oxygen content of the hemoglobin calculated by the following formula:

$$\text{Oxygen Content (vol \%)} = \frac{\Delta PO_2 \times 100V \times \alpha}{760 \times v}$$

ΔPO_2 is the increase in PO_2 of the ferricyanide reagent in mmHg recorded after the addition of v µl of blood or hemoglobin, V is the volume of the cuvette in µl and α is the solubility coefficient of oxygen in the ferricyanide reagent in ml O_2 dissolved per ml ferricyanide reagent at an oxygen partial pressure of 760 mm Hg.

In most cases the oxygen capacity was related to the ml O_2 bound by one gram of oxyhemoglobin. Hemoglobin, and methemoglobin concentrations and hematocrits where appropriate were measured before and after the determination of the oxygen equilibria.

CALCULATION OF P_{50} AND n

Since the foregoing transformation of the mixing ratios of oxygenated and deoxygenated hemoglobin to percent saturation of the hemoglobin based on the ratio of oxygen content to oxygen capacity of the hemoglobin at various oxygen partial pressures seldom resulted in measurements of the PO_2 at half-saturation of hemoglobin (P_{50}), this value was calculated from the Hill approximation, $\frac{Y}{100-Y} = kP^n$, where Y is the percent saturation, k is a constant, P is the oxygen partial pressure and n is an estimate of the heme-heme interaction (Manwell, 1960; Prosser and Brown, 1961). The value of n is determined from the least squares fit of a double logarithmic plot of the various values of $\frac{Y}{100-Y}$ and P. The value of P_{50} is then calculated from this estimate of n when log

$(\frac{Y}{100-Y}) = 0.$

Hill's approximation frequently fails to describe the oxygen equilibrium curve at the high or low percentage saturations (Wyman,1948; Antonini *et al.*, 1964; Wyman, 1964) which could lead to significant errors in the calculated values of P_{50} and n. For this reason, estimates of P_{50} and n for each equilibrium curve were calculated using all the points from 1 to 95% saturation and also from 20 to 80% saturation of the hemoglobin with oxygen. These data along with the equilibration conditions and hemoglobin concentration are provided in Table XII, for each oxygen equilibrium curve constructed in this study. The estimates of P_{50} and n calculated over the 20 to 80% range in oxygen saturation were utilized during the discussion of results since they are probably the more accurate of the two sets of estimates.

RESULTS III

ELECTROPHORETIC IDENTITY OF THE HEMOGLOBIN SOLUTIONS

The electropherograms of the solutions of the entire complement of adult coho hemoglobins and of the anion exchange fraction eluted with a 1:1 (v:v) mixture of 0.1 M tris-HCl, pH 8.5, and 0.1 M KH_2PO_4 are presented in Plate 10. Both hemoglobin solutions were approximately 60 hours old from the time the adult salmon were killed and were representative of the solutions employed in the oxygen equilibria studies.

TABLE XII

The equilibration conditions, hemoglobin concentrations and calculated values of oxygen capacity, oxygen affinity and heme-heme interactions for all the oxygen equilibria measured in this thesis. The latter two parameters were calculated over two ranges of saturation of hemoglobin with oxygen to demonstrate the effect of including very high or low saturation values in the calculation of heme-heme interaction.

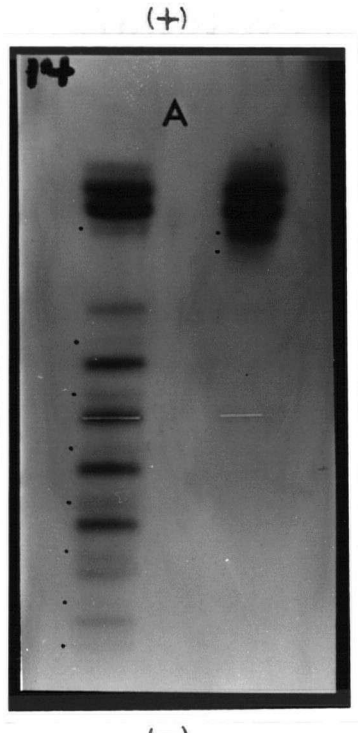
	Equilibration Temperature (C)	pH	[Hb] (%g)	MetHb (% of Hb)	Hb Oxygen Capacity (ml O_2/g Hb)	20-80% Saturation P_{50} (mm Hg)	n	1-95% Saturation P_{50} (mm Hg)	n
1 Fry-Hb	9.8	6.82	3.60	7.0	0.78	34.3	1.99	34.2	2.04
2 Fry-Hb	9.8	7.08	3.72	5.7	0.71	31.8	2.17	31.6	2.17
3 Fry-Hb	9.8	7.44	3.50	6.5	0.94	8.4	1.69	8.6	1.44
4 Fry-Hb	9.8	7.50	4.04	8.0	0.92	6.0	1.86	6.2	1.98
5 Fry-Hb	9.8	7.90	3.50	6.5	0.90	4.9	1.62	4.8	1.54
6 Fry-Hb	9.8	8.50	3.72	5.7	0.80	3.9	1.75	4.0	1.67
7 Fry-Hb	15.2	7.38	4.20	8.6	0.85	17.0	2.17	17.1	2.12
8 Fry-Hb	15.2	7.90	4.20	7.7	0.77	9.9	2.12	10.2	1.80
9 Fry-WB	9.3	*	6.79	0.0	1.23	5.5	1.38	5.5	1.52
10 Fry-WB	10.0	*	6.05	--	1.23	4.4	1.64	4.2	1.90
11 Fry-WB	9.3	**	6.63	0.0	1.23	12.5	1.62	12.6	1.58
12 Adult-Hb	9.8	6.95	4.10	3.3	1.12	23.9	2.67	24.0	2.37
13 Adult-Hb	9.8	7.43	4.10	5.3	1.09	17.9	1.98	17.7	1.99
14 Adult-Hb	9.8	8.20	4.10	4.4	1.08	14.0	1.40	13.8	1.54
15 Adult-Hb	10.0	8.00	3.70	4.8		15.6	1.44	15.6	1.44
16 Adult-Hb	10.0	7.48	3.70	5.9	1.17	17.6	1.51	17.4	1.57
17 Adult-Hb	10.0	7.48	3.70	5.9	1.12	18.8	1.63	18.0	1.56
18 Adult-Hb	10.0	7.48	3.70	5.9	1.15	16.6	1.63	16.7	1.65
19 Adult-Hb	10.0	7.48	3.70	5.9	1.11	19.7	2.03	19.7	1.94
20 Adult-WB	9.3	**	12.10	2.4	1.14	15.6	1.75	15.0	1.64
21 Adult-WB	9.3	*	11.60	2.4	1.16	10.7	1.26	10.3	1.34
22 Adult-WB	5.0	*	12.33	0.0	1.17	9.8	1.39	9.6	1.51
23 Adult-WB	10.0	*	8.95	0.0	1.19	12.5	1.60	12.4	1.74
24 Adult-WB	14.8	*	12.38	0.0	1.05	14.8	1.79	14.6	1.74
25 Adult-WB	9.9	*	12.46	0.0	1.13	12.8	1.68	12.4	1.64
Adult WB-SW	11.2	*	4.60	--	1.20	11.4	1.38	11.1	1.71

*Equilibrated with air and pure nitrogen

**Equilibrated with air and nitrogen containing 0.45% carbon dioxide by volume.

PLATE 10:

A comparison of the electrophoretic mobilities of the hemolyzates of the blood of freshwater adult coho salmon (left) and the hemoglobin components A6-8 (right) prepared from the adult hemolyzates by ion-exchange chromatography. The protein bands adjacent to the black dots are tentatively identified as the met-derivatives of the hemoglobin component immediately anodic to their positions.


(+)
14
A
(−)

All ten components normally present in adult hemoglobin were observed in the purified adult hemolyzates. Eight additional hemoglobin bands were also observed and are marked with a black dot (electropherogram A1, Plate 10). These additional components were all approximately equidistant from the component immediately anodic from their positions. Since these additional components were not observed in fresh hemoglobin samples (Part I), and oxidation of the ferrous iron to the ferric form would increase the positive charge on the hemoglobin which would therefore tend to be more cationic the eight bands marked with a dot in the adult hemoglobin electropherogram were designated as methemoglobin.

The second fraction eluted from the anion exchanger was identified as hemoglobin components A6-8 (electropherogram A2, Plate 10). No traces of components A1, A3, C1, C3, C4, C5, or C6 were present. Two methemoglobin bands were observed and are marked with black dots. The slower migrating of these latter bands was not present in electropherogram A1, of Plate 10, indicating that oxidation of the hemoglobin components A6, 7 and 8 was more extensive following isolation by ion exchange. Since the electropherograms of the multiple hemoglobins of fry blood (Part I), were essentially identical to the electropherogram A2 (Plate 10) this latter fraction was termed fry hemoglobin in the presentation of the following results. Recovery of this "fry hemoglobin fraction" from the hemolyzates of freshwater adult coho blood was estimated

to exceed 94% on the basis that components A6-8 accounted for 50% of the hemoglobin of the adult coho (Part I).

BLOOD PARAMETERS AND ERYTHROCYTE ATP CONCENTRATION OF ADULT FRESHWATER COHO SALMON

Table XIII presents the hematocrit, hemoglobin concentration, percent methemoglobin, red cell ATP concentration and molar ratio of ATP to hemoglobin of the blood of several of the adult freshwater coho sacrificed in this study. A molecular weight of 66,000 was assumed for hemoglobin in determining this latter ratio.

Individual hematocrits of the adult coho ranged from 23.0 to 50.4% while total hemoglobin concentration varied from 6.2 to 12.4 g%, with the respective means being 39.3% and 10.6 g%. Methemoglobin levels ranged from approximately 2.0 to 7.5% of the total hemoglobin in adult freshwater coho during the period of October, 1971 to January, 1972, as determined by the method of Evelyn and Malloy (1938). Although the fish were becoming extremely ragged and covered with fungus by December, the data do not indicate a trend of increasing methemoglobin levels with length of freshwater residence.

ATP concentrations in the blood tended to decrease with the age of the adult coho during their period of freshwater residence (Table XIII) although considerable variability was observed among the data. The concentration of ATP in whole blood generally exceeded 1.0 μmoles/ml and was as high

TABLE XIII

Hematological parameters and ATP concentrations of the blood of some of the freshwater adult coho used in the preparation of hemoglobin components A6-8 and adult hemolyzates.

DATE	SEX	HEMATOCRIT (% RBC)	HEMOBLOGIN (g%)	METHEMOGLOBIN (% of total)	ATP (μmoles/cc blood)	ATP:Hb (moles/mole)
15/10/71	U*	32.9	9.3	7.5	1.50	1.26†
19/10/71	F**	23.0	6.2	4.7	1.03	1.06
24/10/71	F	43.3	11.7	1.9	1.70	0.96
24/10/71	F	38.9	11.2	4.8	1.58	0.94
29/10/71	F	40.3	11.3	--	1.73	1.01
11/11/71	F	43.7	12.38	--	--	--
30/11/71	F	50.4	12.1	--	1.43	0.78
30/11/71	M††	42.9	11.8		0.97	0.54
29/12/71	F	33.9	8.6	7.1	1.11	0.86
29/12/71	F	43.4	11.0	6.9	1.39	0.84

*sex unknown.

**female.

†assuming a molecular weight of hemoglobin of 66,000.

††male.

as 1.73 µmoles/ml. No ATP was detected in any of the samples of plasma examined and the whole blood ATP concentrations are therefore a direct estimate of red cell ATP concentrations. The molar ratio of ATP to hemoglobin declined from 1.26 to approximately 0.85 during the period of mid-October to late-December. No inferences can be made regarding the possibility of differences in erythrocyte ATP concentration between adult male and female coho since only 1 male was sacrificed in this study.

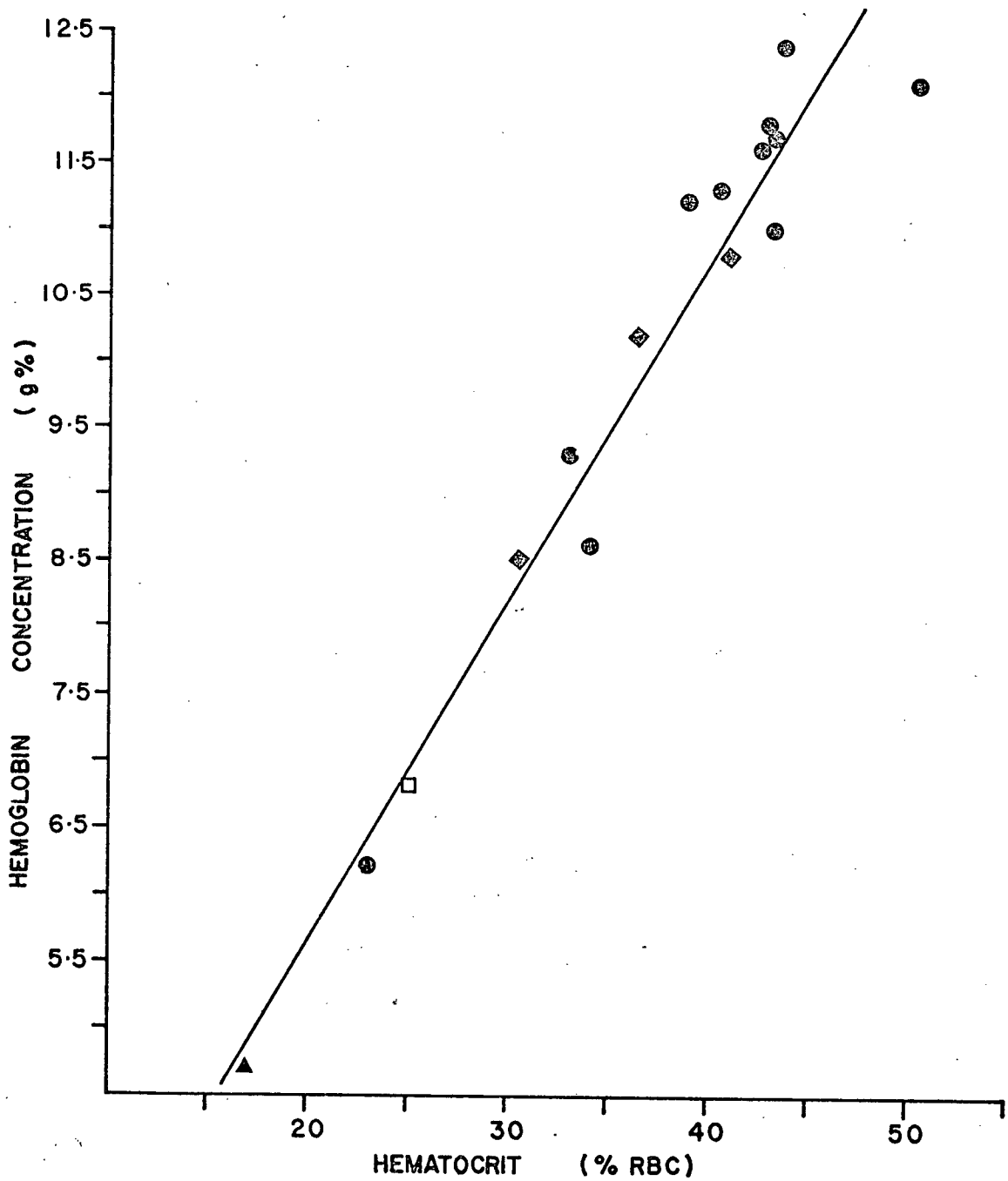
A linear relationship was observed between hemoglobin concentration and hematocrit (Figure 3) described by the equation:

$$Hb = 0.514 + 0.255\ Hct$$

for which r = 0.97, n = 13 and hemoglobin concentration is in grams per 100 ml blood. The solid triangle of Figure 3 represents the pooled blood of 113 coho fry anesthetized with 2-phenoxyethanol prior to sampling and was included in the calculation of the foregoing equation. The solid diamond-shaped points representing the blood of 2-year-old seawater grilse provided by Dr. J. C. Davis were not included in the calculation of the hemoglobin-hematocrit relationship. It was evident, however, that little, if any change occurred in this relationship through the free-swimming stage of the coho salmon life cycle.

FIGURE 3

The relationship between blood hematocrit and hemoglobin concentration in coho salmon. The solid circles represent fresh-water adult coho; the open squares , the pooled blood of 113 coho fry; the solid, triangle the blood of a 2-year-old seawater grilse and the solid diamonds, the blood of 2-year-old seawater grilse measured by Dr. J. C. Davis. All the points with the exception of the latter grilse were used to calculate least squares fit of the regression line. $Hb = 0.514 + 0.255\ Hct$ for which $r = 0.97$ and $n = 13$.


12·5
11·5
10·5
9·5
8·5
7·5
6·5
5·5
HEMOGLOBIN CONCENTRATION (g%)
20
30
40
50
HEMATOCRIT (% RBC)

INFLUENCE OF ATP UPON THE OXYGEN EQUILIBRIUM OF ADULT COHO HEMOGLOBIN

The oxygen equilibrium characteristics of adult coho hemoglobin were determined at four concentrations of adenosine triphosphate. The equilibration temperature and pH were 10 C and 7.48, respectively. ATP concentrations of 0.0, 0.08, and 0.76 moles/mole hemoglobin resulted in essentially identical oxygen equilibria (Figure 4) in which P50 range from 16.6 to 18.8 mm Hg and heme-heme interaction from 1.51 to 1.63 (Table XIV). Increasing the ATP concentration to 7.56 moles/mole hemoglobin resulted in a slight decrease in oxygen affinity (P_{50} = 19.7 mm Hg) and a relatively large increase in heme-heme interaction (n = 2.03). The equilibrium curves recorded at all four ATP concentrations coincided at oxygen saturations exceeding 70%. The increase in heme-heme interaction observed at 7.56 moles ATP/mole hemoglobin was therefore a reflection of the shift to the right of the lower portion of the equilibrium curve.

No change in oxygen capacity was observed in relation to ATP concentrations. Oxygen capacity averaged 1.14 ml O_2/g hemoglobin which is somewhat less than the theoretical capacity of 1.3 ml O_2/g hemoglobin. Since the only effect of ATP upon the oxygen equilibria of solutions of adult hemoglobin occurred at ATP concentrations which were over five times the maximum concentration observed in whole blood it was concluded that ATP did not substantially alter the oxygen equilibria. ATP, therefore, was not included in the buffer

FIGURE 4

The effect of ATP upon the oxygen equilibrium curve of solutions of adult freshwater coho hemoglobin. The hemoglobin solutions contained 3.70 G% hemoglobins, and 5.9% methemoglobin and were equilibrated at pH 7.48 and 10C. The molar ratio of ATP to hemoglobin employed were 0 (open circles), 0.076 (crosses), 0.76 (solid circles), and 7.6 (open squares).

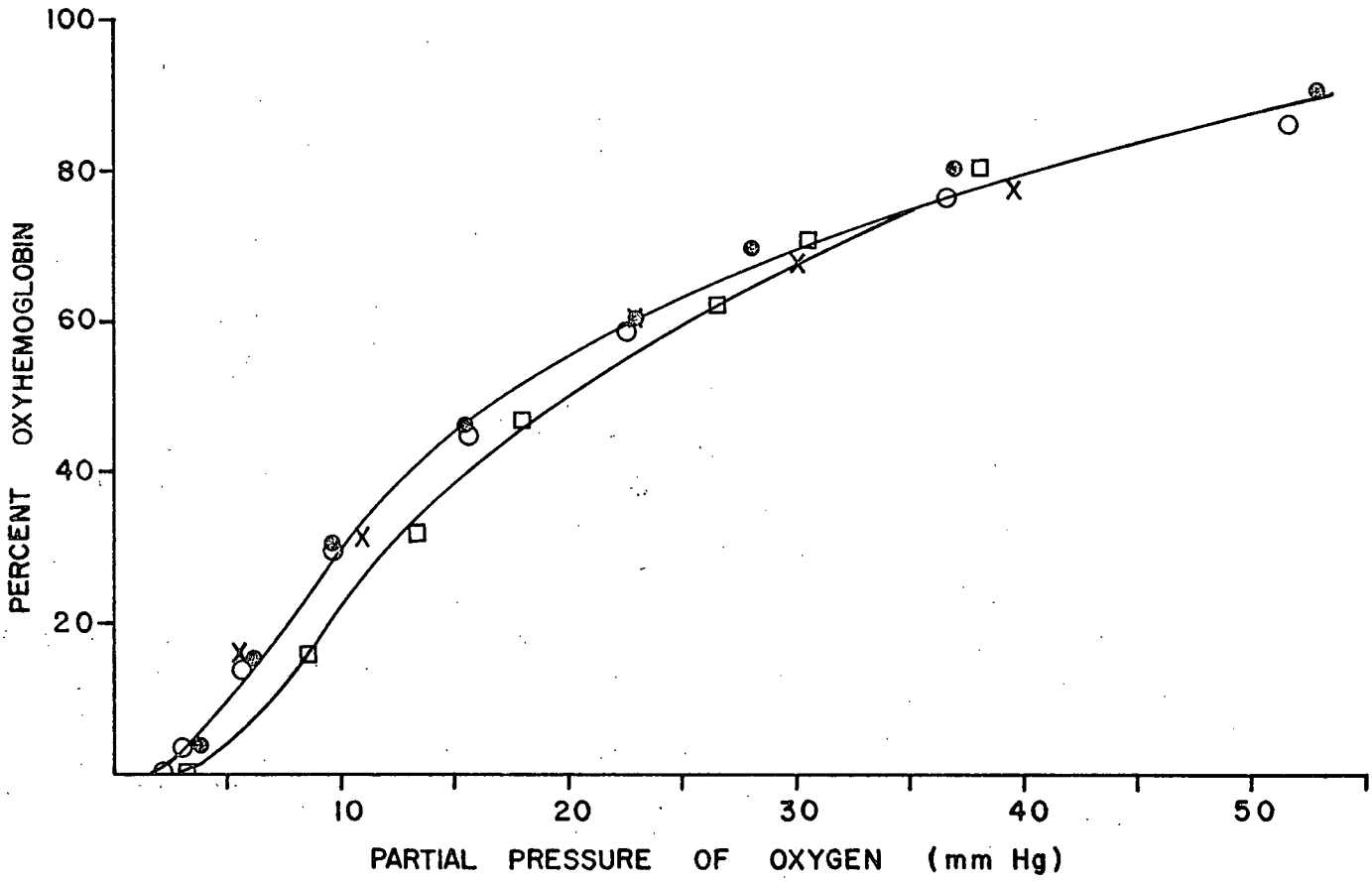

100
80
60
40
20
PERCENT OXYHEMOGLOBIN
10
20
30
40
50
PARTIAL PRESSURE OF OXYGEN (mm Hg)

TABLE XIV

The influence of various concentrations of adenosine triphosphate upon the oxygen capacity, oxygen affinity and heme-heme interaction of hemolyzates from freshwater adult coho salmon.

ATP CONCENTRATION (mole/mole Hb)	OXYGEN CAPACITY (ml O_2/g Hb)	P_{50} (mm Hg)	n
0	1.17	17.6	1.51
0.08	1.12	18.8	1.63
0.76	1.15	16.6	1.63
7.56	1.11	19.7	2.03

solution in the remaining experiments.

EFFECT OF EQUILIBRATION TEMPERATURE UPON OXYGEN EQUILIBRIA

The oxygen equilibrium curves of solutions of fry hemoglobin equilibrated at 9.8 and 15.2 C and pH of approximately 7.4 and 7.9 are presented in Figure 5, while the oxygen capacity and calculated values of P_{50} and n for each set of equilibration conditions are presented in Table XV. At pH 7.90 an increase in temperature from 9.8 to 15.2 shifted the curve to the right and P_{50} increased by 5.0 mm Hg from 4.9 to 9.9 mm Hg. At pH of approximately 7.4, however, the same increase in temperature resulted in an increase in P_{50} of 8.6 mm Hg.

These latter equilibrium curves were determined at equilibration temperatures of 9.8 and 15.2 C and a pH of 7.44 and 7.38 respectively. If the former curve is corrected to pH 7.38 (see Bohr effects, following section), the P_{50} would be 9.6 mm Hg, which would then result in a change in P_{50} of 7.4 mm Hg between fry hemoglobin solutions equilibrated at 9.8 and 15.2 C. Thus the same temperature increase of 5.4 C resulted in a much larger reduction in oxygen affinity at pH 7.38 than at pH 7.90.

The oxygen capacity of the fry hemoglobin appeared to decrease at higher temperatures (Table XV) but these data must be viewed with caution since different hemoglobin preparations were employed at the two equilibration temperatures.

TABLE XV

The effect of different equilibration temperatures upon the oxygen capacity, oxygen affinity and heme-heme interaction of fry hemoglobin solutions (components A6-8) at different values of pH and of fry and adult whole blood equilibrated with air and pure nitrogen.

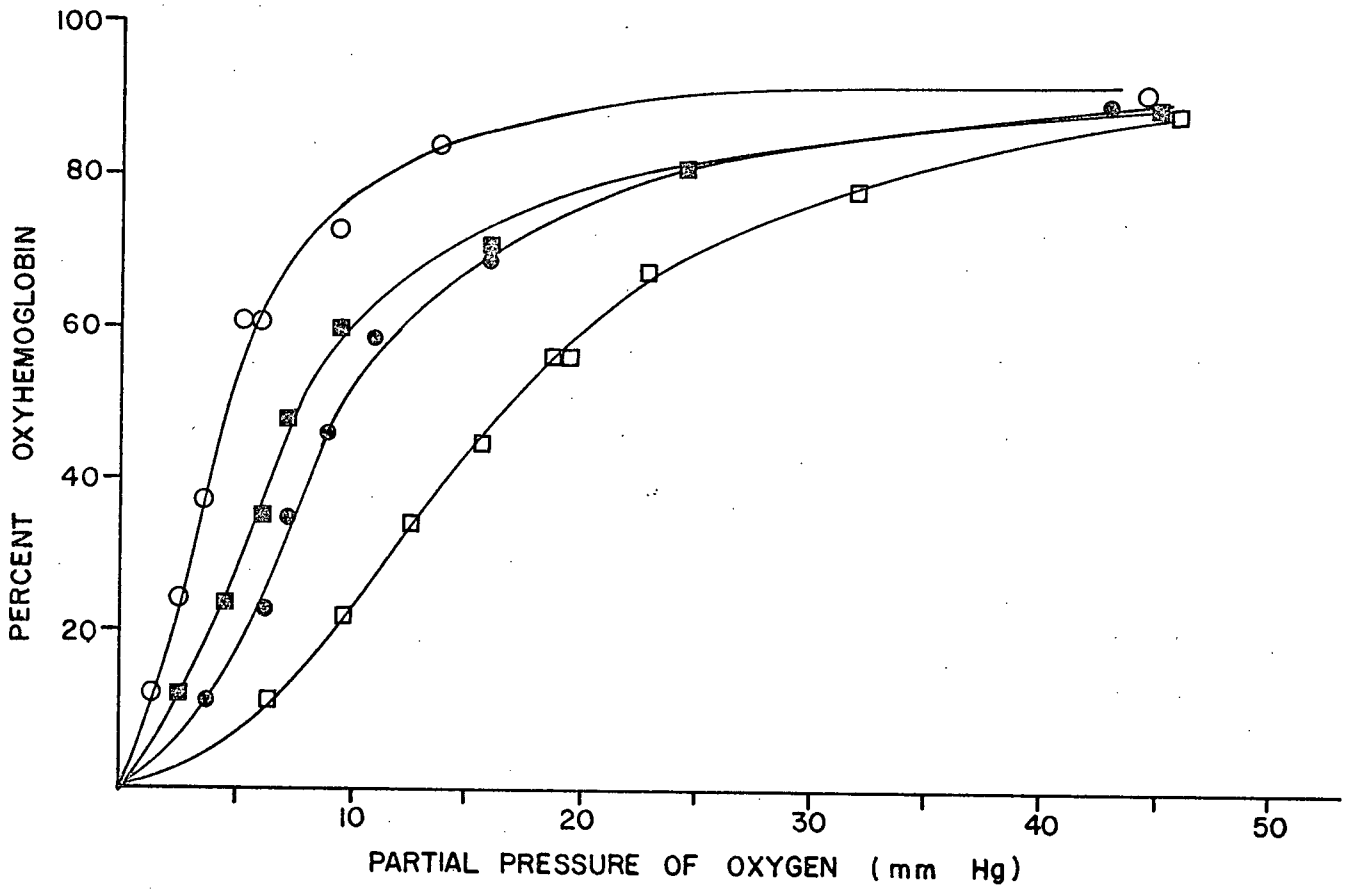
	pH	EQUILIBRATION TEMPERATURE (C)	OXYGEN CAPACITY (ml O_2/g Hb)	P_{50} (mm Hg)	n
Fry Hemoglobin*	7.44	9.8	0.94	8.4	1.69
Fry Hemoglobin	7.38	15.2	0.85	17.0	2.17
Fry Hemoglobin	7.90	9.8	0.90	4.9	1.62
Fry Hemoglobin	7.90	15.2	0.77	9.9	2.12
Fry Blood**	--	10.0	1.23	4.4	1.64
Adult Blood	--	5.0	1.17	9.8	1.39
Adult Blood	--	10.0	1.19	12.5	1.60
Adult Blood	--	14.8	1.05	14.8	1.79

* Fry refers to hemoglobin solutions in tris-HCl buffer

** Oxygen equilibria determined using whole blood.

FIGURE 5

The effect of equilibration temperature and pH upon the oxygen equilibrium of solutions of hemoglobin components A6-8 (fry hemoglobin). The oxygen equilibria were measured at pH 7.90 and 9.8 C (open circles), pH 7.44 and 9.8 C (solid squares), pH 7.90 and 15.2 C (solid circles), and pH 7.38 and 15.2 C (open squares). Hemoglobin concentration and percentage methemoglobin were 3.50 g % and 6.5% at the equilibration temperature of 9.8 C. At 15.2 C the respective values were 4.20 g% and 8.6% at pH 7.38 and 4.20 g% and 7.7% at pH 7.90.


100
80
60
40
20
PERCENT OXYHEMOGLOBIN
10
20
30
40
50
PARTIAL PRESSURE OF OXYGEN (mm Hg)

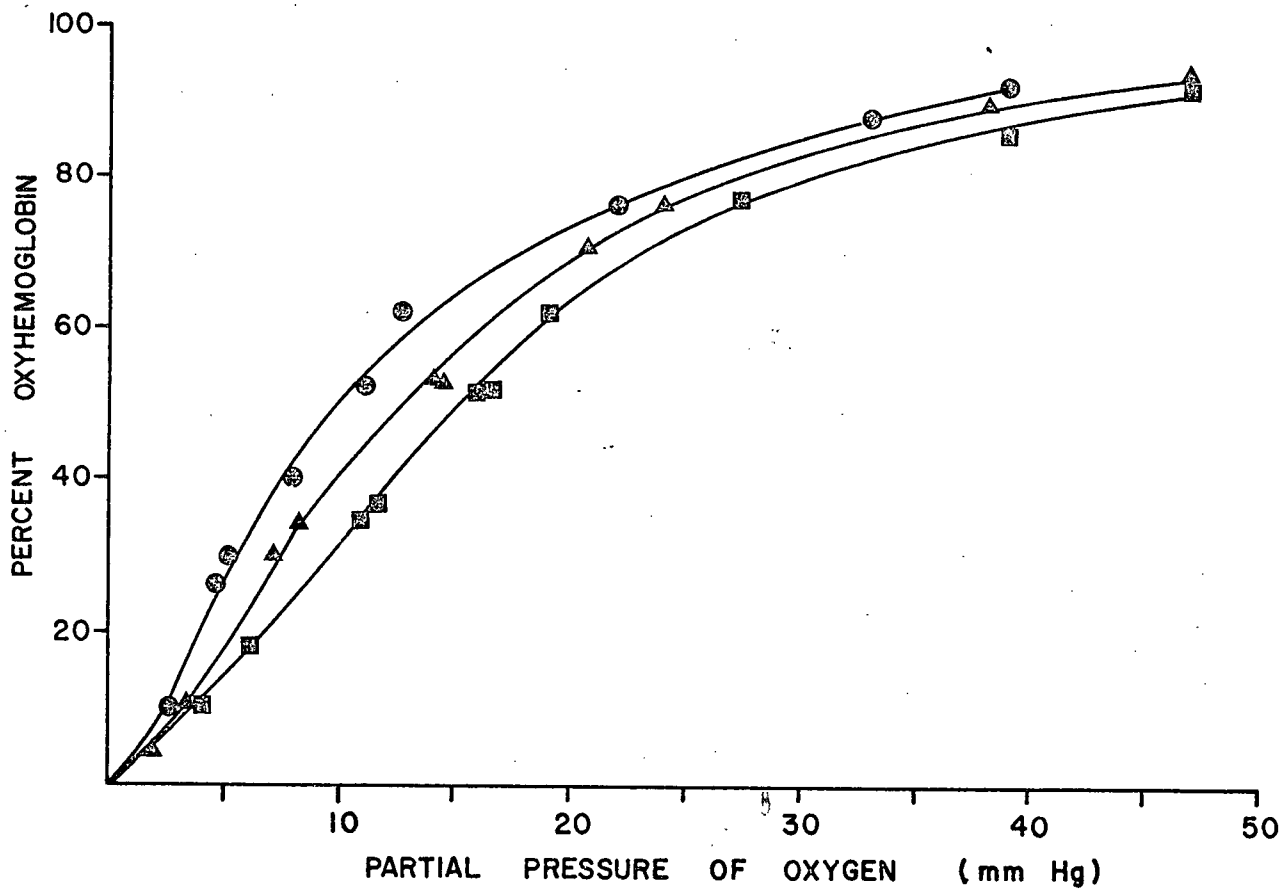
The estimate of heme-heme interaction, n, increased at 15.2 C relative to 9.8 C and was independent of pH (Table XV). At 9.8 C, n, was 1.69 and 1.62 at pH 7.44 and 7.90, respectively, while at 15.2 C, n was 2.17 and 2.12 at pH 7.38 and 7.90, respectively.

The oxygen equilibria of whole blood from adult coho living at 10 C were determined at 5.0, 10.0 and 14.8 C. The blood was equilibrated with air and pure nitrogen. The oxygen affinity of this blood was relatively unaffected by changes in the equilibration temperature since the calculated values of P_{50} at these temperatures were 9.8, 12.5, and 14.8 mm Hg, respectively (Table XV). Thus P_{50} increased approximately 2.5 mm Hg for each 5 degrees rise in temperature.

Heme-heme interaction increased as the equilibration temperature was elevated such that n was 1.39, 1.60 and 1.79 at 5.0, 10.0 and 14.8 C, respectively (Table XV). The result of this increase in subunit cooperativity was that with increasing temperature the equilibration curves were shifted to the right to a greater extent at intermediate oxygen saturations of 25 to 65% than at high saturations (Figure 6). Thus at a PO_2 of 25 mm Hg, the oxygen saturation at 5.0 and 14.8 C differed by 6%, whereas at 15 mm Hg, this difference increased to 25%. Since the oxygen capacities recorded at the three equilibration temperatures were similar, the amount of oxygen bound to the hemoglobin of adult coho salmon at oxygen partial pressures exceeding 25 mm Hg was essentially unrelated to temperature over the range of 5.0 to 14.8 C.

FIGURE 6

The effect of equilibration temperature upon the oxygen equilibrium of the whole blood of freshwater adult coho salmon. The blood was equilibrated with air and pure nitrogen at 5.0 C (solid circles), 10.0 C (solid triangles), and 14.8 C (solid squares), for which the hemoglobin concentrations were 12.3, 9.0 and 12.4 g% respectively. Methemoglobin was below detectable limits.


100
80
60
40
20
PERCENT OXYHEMOGLOBIN
10
20
30
40
50
PARTIAL PRESSURE OF OXYGEN (mm Hg)

Page 105 does not exist

EFFECTS OF pH AND CARBON DIOXIDE UPON OXYGEN EQUILIBRIA

The oxygen equilibria of fry hemoglobin over the pH range of 6.82 to 8.50 are presented (Figure 7) along with the respective estimates of oxygen capacity, P_{50} and heme-heme interaction (Table XVI). All experiments were performed at an equilibration temperature of 9.8 C. The oxygen capacity of fry hemoglobin was not decreased over the pH range of 7.44 to 8.50 but an apparent reduction in capacity was observed below this range. Oxygen affinity, however, was strongly pH-dependent and the estimate of P_{50} increased from 3.9 to 34.3 mm Hg as the pH decreased from 8.50 to 6.82. The heme-heme interaction was relatively constant at 1.62 to 1.85 in the pH range of 7.44 to 8.50 but increased to 2.17 and 1.99 at pH 7.08 and 6.82, respectively (Table XVI).

The oxygen equilibria of adult hemoglobin at pH 6.95, 7.43 and 8.20 are illustrated in Figure 8 and the estimates of oxygen capacity, oxygen affinity and heme-heme interaction are presented in Table XVI for equilibration temperatures of 9.8 to 10.0 C. The oxygen capacity of adult hemoglobin was unaffected by variations in pH and remained at approximately 85% of the theoretical capacity of 1.3 ml O_2/g Hb. Heme-heme interaction, however increased from approximately 1.4 at pH 8.00 to 8.20 to 2.67 at pH 6.95. Thus in both fry and adult hemoglobin subunit cooperativity increased as the pH of the buffer was lowered. The oxygen affinity of adult hemoglobin was only slightly reduced as pH was lowered

TABLE XVI

The oxygen capacity, oxygen affinity and heme-heme interaction of solutions of fry hemoglobin (components A6-8) and adult hemoglobin at various pH and of fry and adult whole blood at two tensions of carbon dioxide. The abbreviations Hb and WB refer to hemoglobin solutions and whole blood, respectively.

	pH	EQUILIBRATION TEMPERATURE (C)	PCO_2 (mm Hg)	OXYGEN CAPACITY (ml O_2/g/Hb)	P_{50} (mm Hg)	n
Fry-Hb	8.50	9.8	0.23	0.80	3.9	1.75
Fry-Hb	7.90	9.8	0.23	0.90	4.9	1.62
Fry-Hb	7.50	9.8	0.23	0.92	6.0	1.86
Fry-Hb	7.44	9.8	0.23	0.94	8.4	1.69
Fry-Hb	7.08	9.8	0.23	0.71	31.8	2.17
Fry-Hb	6.82	9.8	0.23	0.78	34.3	1.99
Adult-Hb	8.20	9.8	0.23	1.08	14.0	1.40
Adult-Hb	8.00	10.0	0.23	1.13	15.6	1.44
Adult-Hb	7.48	10.0	0.23	1.17	17.6	1.51
Adult-Hb	7.43	9.8	0.23	1.09	17.9	1.98
Adult-Hb	6.95	9.8	0.23	1.12	23.9	2.67
Fry-WB	--	9.3	0.23	1.23	5.5	1.38
Fry-WB	--	9.3	3.35	1.23	12.5	1.62
Adult-WB	--	9.3	0.23	1.18	10.7	1.26
Adult-WB	--	9.3	3.35	1.14	15.6	1.75

FIGURE 7:

The effect of pH upon the oxygen equilibrium of solutions of hemoglobin components A6-8 (fry hemoglobin). The equilibration temperature was 9.8 C and the hemoglobin concentration and levels of methemoglobin ranged from 3.5 to 4.0 g % and 5.7 to 8.0 % respectively. The oxygen equilibria were measured at pH 8.5 (open circles), 7,90 (open triangles), 7.50 (small solid circles), 7.44 (open squares), 7.08 (large solid circles) and 6.82 (solid triangles).

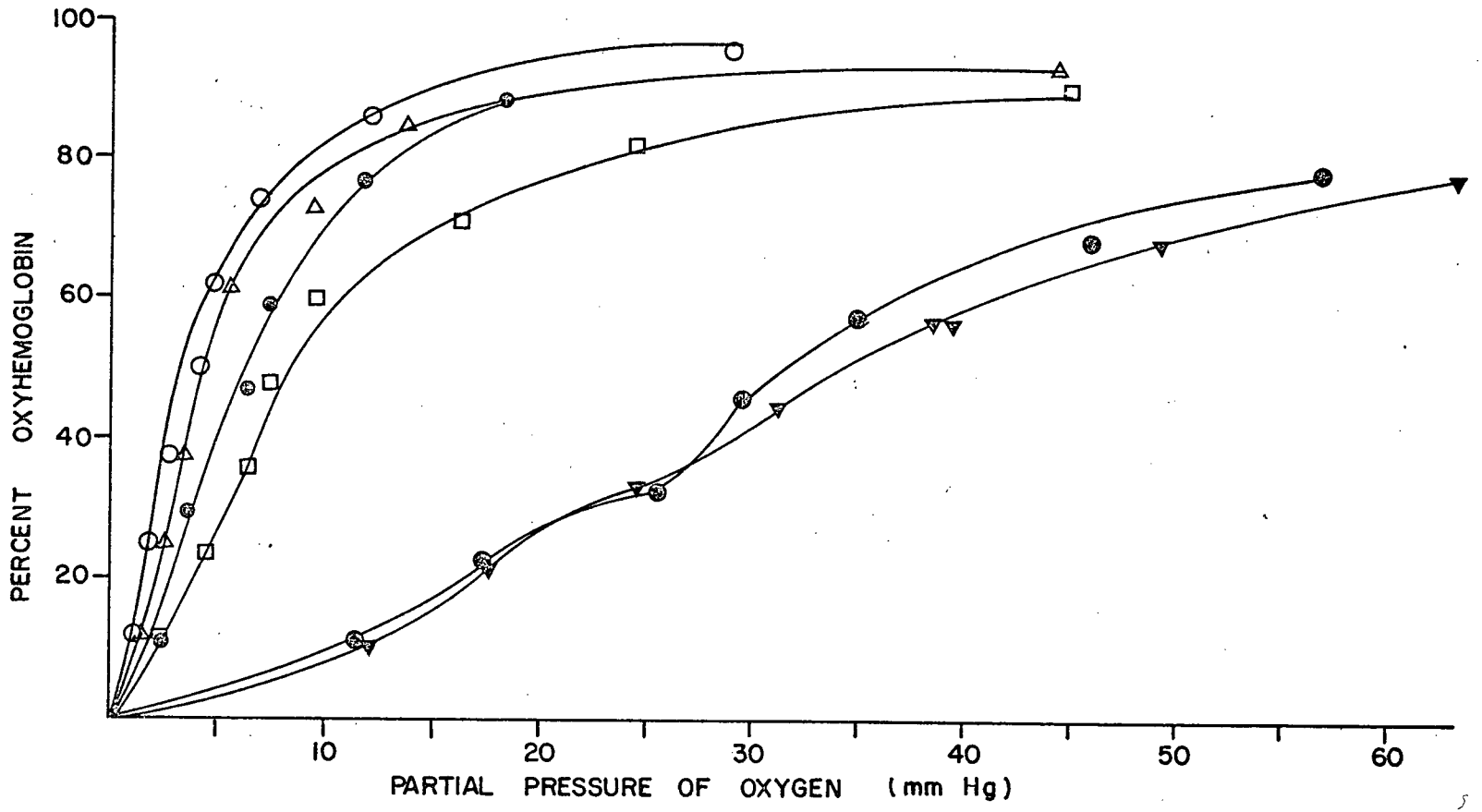
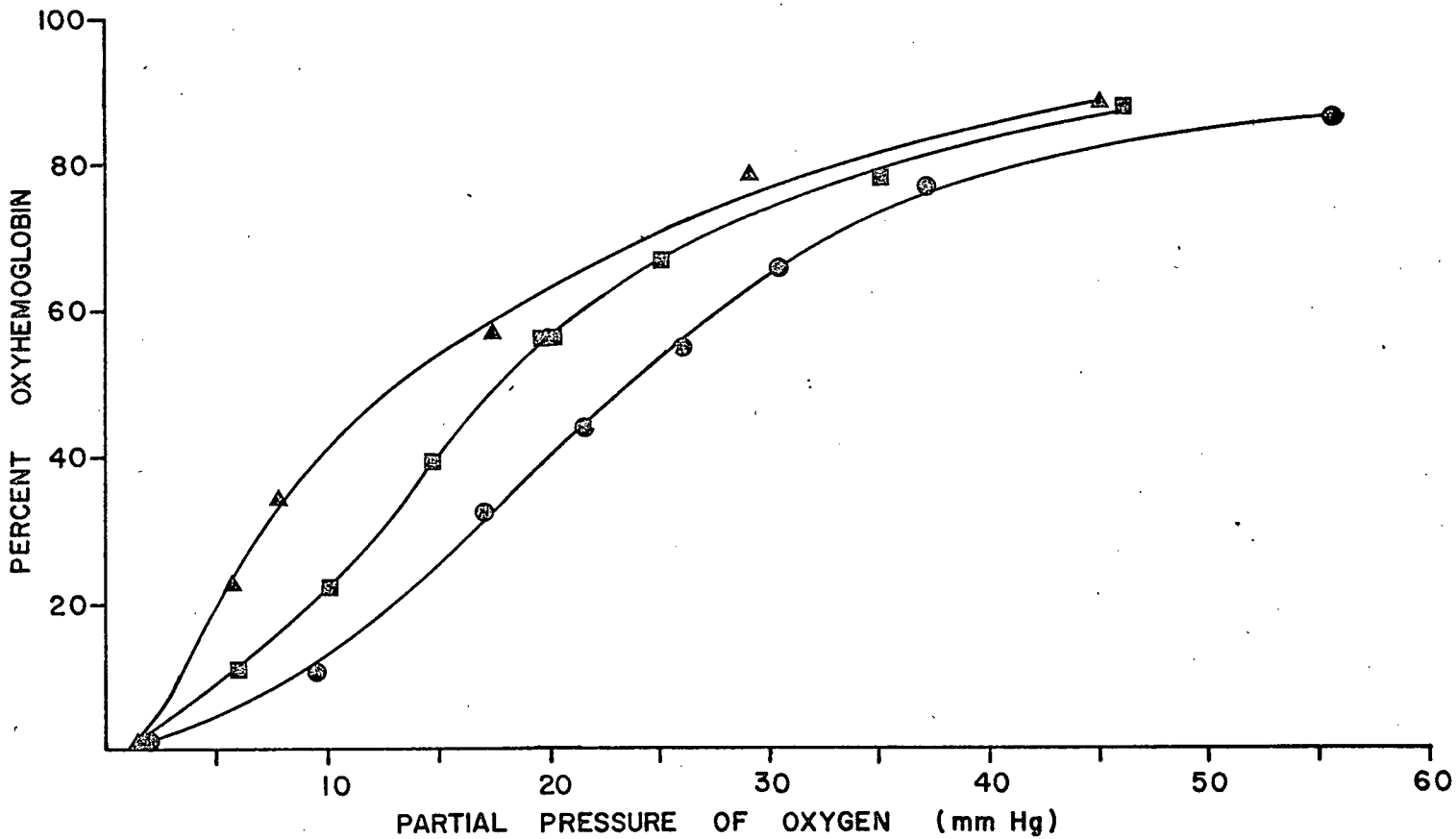

100
80
60
40
20
PERCENT OXYHEMOGLOBIN
10
20
30
40
50
60
PARTIAL PRESSURE OF OXYGEN (mm Hg)

FIGURE 8

The effect of pH upon the oxygen equilibrium of solutions of freshwater adult coho hemoglobin equilibrated at 9.8 C. The oxygen equilibria determined at pH 6.95 (solid triangles), 7.43 (solid squares) and 8.20 (solid circles). Hemoglobin concentration was 4.1 g % and methemoglobin ranged from 3.3 to 5.3 %.


100
80
60
40
20
PERCENT OXYHEMOGLOBIN
10
20
30
40
50
60
PARTIAL PRESSURE OF OXYGEN (mm Hg)

and the P_{50} increased by approximately 10 mm Hg over the pH range of 8.20 to 6.95.

The Bohr shift ($\emptyset = \Delta \log P_{50}/\Delta$ pH) was determined for fry and adult hemoglobin (Figure 9). The Bohr effect of adult hemoglobin was approximately linear over the pH range of 6.95 to 8.20 ($\emptyset = 0.172$). Although this relationship in fry hemoglobin was always negative over the pH range of 6.82 to 8.50, it was strongly non-linear so that the calculated values of $\emptyset$ were -0.033, -1.729, and -0.182 in the pH ranges 6.82 to 7.08, 7.08 to 7.50 and 7.50 to 8.50, respectively.

The oxygen equilibria of the whole blood of fresh-water adult coho and the pooled blood of 113 eleven-month-old fry at 9.3 C and carbon dioxide tensions of 0.2 and 3.4 mm Hg are presented in Figure 10. With the foregoing increase in PCO_2 the oxygen affinities of adult and fry blood were decreased by 4.9 and 7.0 mm Hg respectively although no changes were observed in the respective oxygen capacities (Table XVI) which were 90 to 95% of the maximum theoretical capacity. Both groups of blood exhibited increases in the value of n as the PCO_2 was raised. The pH values of Figure 9 corresponding to the log P_{50} of adult blood at carbon dioxide tensions of 0.2 and 3.4 were estimated to be 9.0 and 7.85, respectively. Similar calculations yielded the respective pH values of 7.7 and 7.4 for fry blood.

FIGURE 9

The Bohr effect of solutions of hemoglobin components A6-8 (fry hemoglobin), (solid circles) and freshwater adult hemoglobin (solid squares) at equilibration temperatures of 9.8 to 10.0 C.

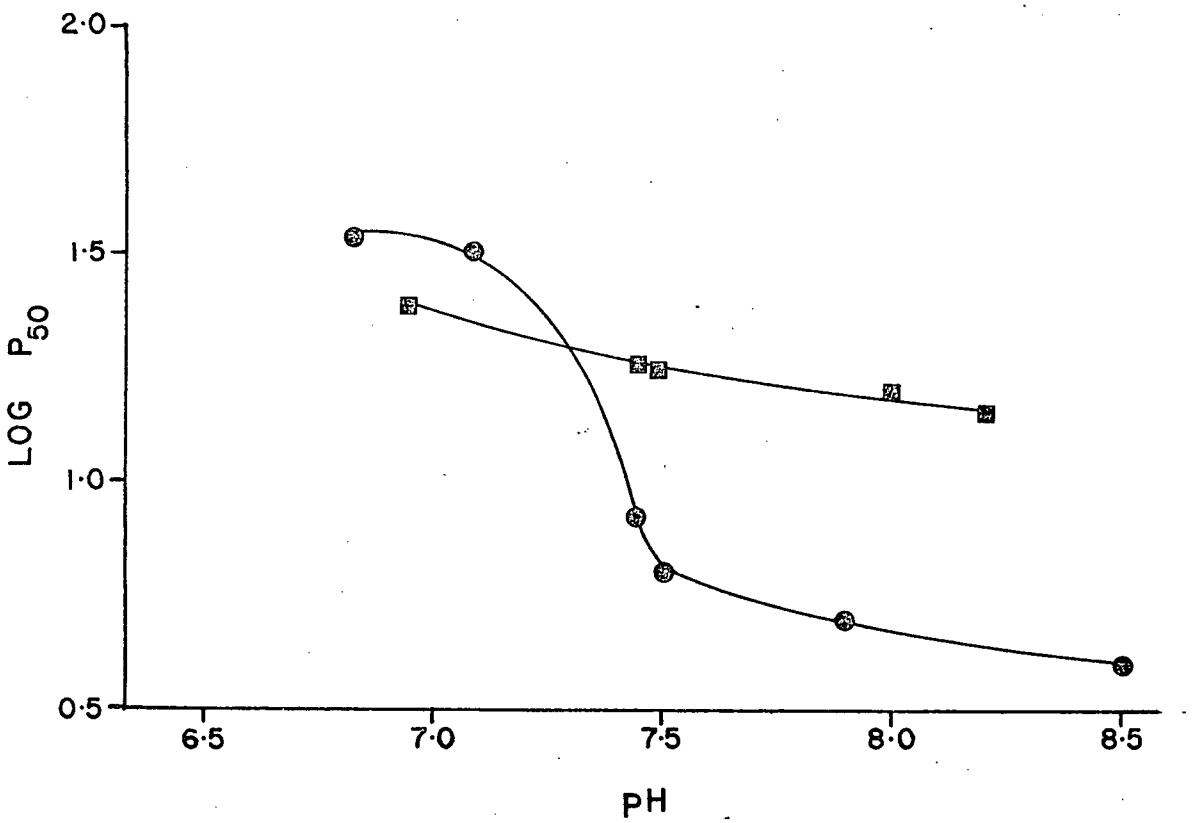
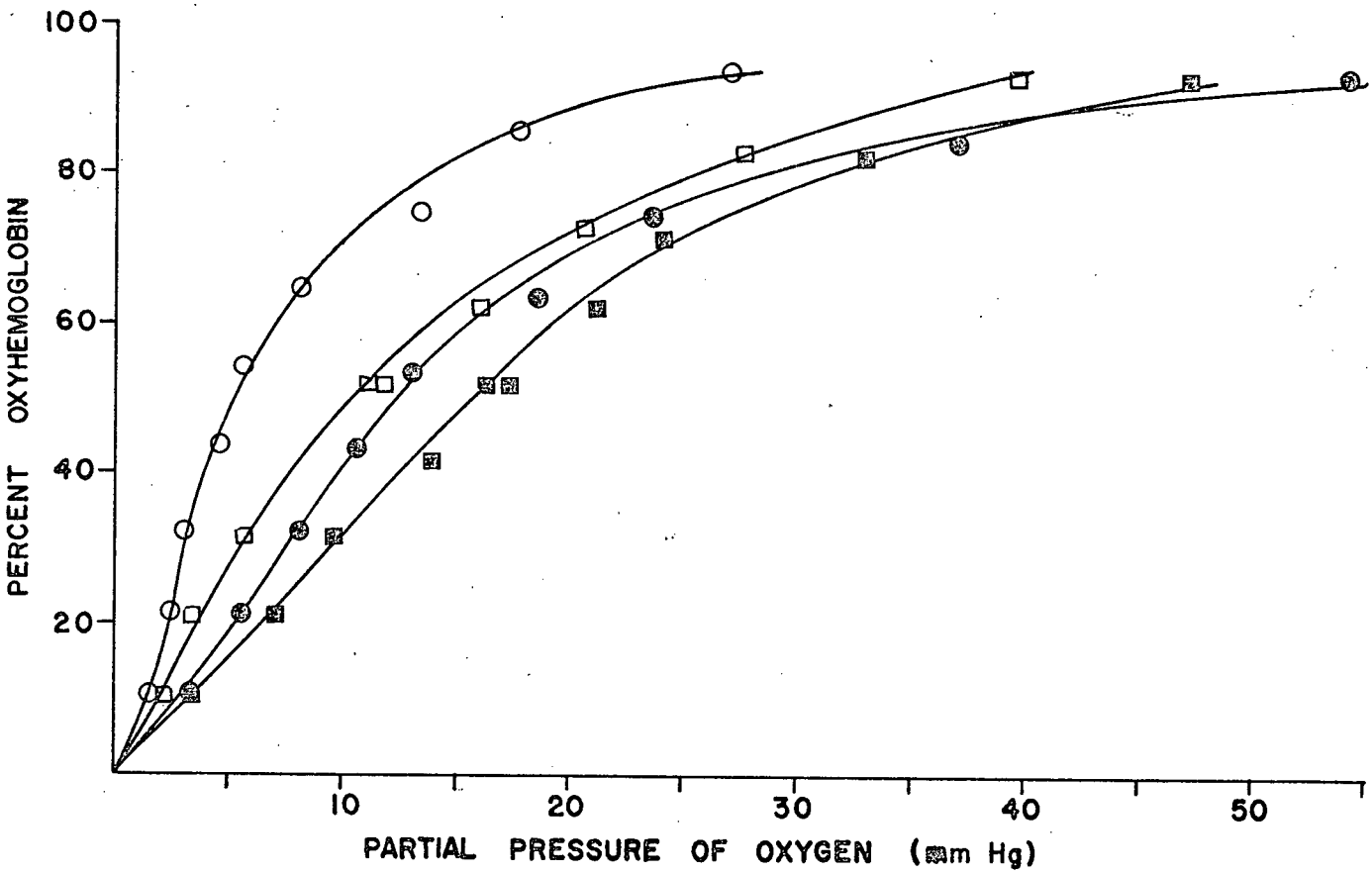

2·0
1·5
1·0
0·5
LOG P50
6·5
7·0
7·5
8·0
8·5
pH

FIGURE 10

The influence of carbon dioxide upon the oxygen equilibria of the blood of coho fry (circles) and freshwater adults (squares). The blood was equilibrated with air and pure nitrogen (open squares and circles) or with air and nitrogen containing 0.45 % carbon dioxide by volume (solid squares and circles)at 9.3 C. Hemoglobin concentration was 6.6 to 6.8 g% for fry blood and 11.6 to 12.1 g% for adult blood. No methemoglobin was formed during these experiments.


100
80
60
40
20
PERCENT OXYHEMOGLOBIN
10
20
30
40
50
PARTIAL PRESSURE OF OXYGEN (mm Hg)

DISCUSSION III

The recovery of hemoglobin components A6-8 from hemolyzates of adult coho blood was estimated to exceed 90%. The majority of the loss occurred during the concentration of these components with the Amicon molecular filter following their elution from the ion-exchange column. About 0.2 and 0.4 ml of the concentrated hemoglobin was retained in the filter. Since washing the filter would have defeated the purpose of the procedure, this volume of hemoglobin solution was discarded. These losses were not specific for any particular component, therefore, no change in the relative proportions of the three components occurred. Under no circumstances were components A1, A3, C1,C3,C4,C5 or C6 present in any of the preparations of components A6-8.

It may be somewhat questionable whether the relative proportions of the three components A6-8 are identical in the blood of fry and adult coho. Some variation in the relative proportions of A6:A7:A8 were visible in electropherograms of hemoglobin from coho of all ages although this variation did not exhibit a consistent pattern with age. Such individual variation in the relative concentration of hemoglobin components has been observed in other salmonids (Hashimoto and Matsuura, 1960b; Westmann, 1970) and is generally much larger than that observed for coho blood. This difficulty is further complicated by the formation of methemoglobin during the isolation of components A6-8. From the observed changes in electrophoretic behaviour of the cathodic and slower

migrating anodic hemoglobin components, it would appear likely that the oxidized form of component A8 would migrate to the same position as unoxidized component A6 during electrophoresis. This would account for the apparent increase in the relative concentration of component A6, following isolation of the fry hemoglobin components from adult hemolyzates.

Efficiency of acid-soluble phosphate reduction from the hemolyzates by the combined procedures of pressure filtration and column chromatography was estimated to be 91-96%. This estimate was based on the percentage reduction in total phosphate bound as ATP alone. Since it has been demonstrated that approximately 55-80% of the acid soluble phosphate of erythrocytes is in the form of ATP (Rapoport and Guest, 1941; Gillen and Riggs, 1971), the estimate of the efficiency of phosphate removal is probably too low. In absolute terms, 0.1 and 0.2 moles of phosphate per mole of hemoglobin were retained in the final preparations of adult and fry hemoglobin, respectively. The higher phosphate concentration in the fry hemoglobin is a reflection of the 0.05 M phosphate concentration in the buffer employed to elute hemoglobin components A6-8 from the ion exchange column.

There can be no doubt that the ability of the fry hemoglobin to combine with oxygen was impaired following ion exchange chromatography. Whether or not this decrease in oxygen capacity in these preparations influenced the characteristics of oxygenation of the functional hemoglobin. is questionable. A comparison of the oxygen equilibrium

curves of solutions of fry hemoglobin prepared by ion-exchange chromatography with those of the whole blood of fry under similar conditions may provide some insight into this problem. At 10 C fry whole blood equilibrated with air and pure nitrogen exhibited a P_{50} of 4.4 mm Hg and n of 1.64. Assuming that the pH of the plasma is similar to that of trout blood under similar conditions of equilibration (Cameron and Randall, 1972) the pH of the plasma would be approximately 8.2. Siggaard-Andersen (1963) presents equations relating intra-erythrocyte pH to plasma pH which if applied to the present data yield an intra-erythrocyte pH of 7.6 to 7.8. The estimates of P_{50} and n of fry hemoglobin solutions at equilibration conditions were 4.0 to 4.9 and 1.6 to 1.8, respectively. Thus although the oxygen capacity of fry hemoglobin in solution was much less than that in whole blood, 0.7 to 0.9 and 1.23 ml O_2/g Hb respectively, it would appear that both had similar oxygenation characteristics when subjected to similar equilibration conditions.

The most probable explanation for the foregoing disparity lies in the methods employed in the estimation of methemoglobin and total hemoglobin. In the method of Evelyn and Malloy (1939) methemoglobin concentration is estimated by the decrease in absorption at 635 mμ upon the addition of sodium cyanide to a sample of blood or hemoglobin in phosphate buffer at pH 6.7. Total hemoglobin, however was determined spectrophotometrically as the cyanmet-derivative in an alkaline solution. Since the small amounts of brown precipitate

which formed in the fry hemoglobin solutions following ion-exchange and subsequent adjustment of pH to values from 6.8 to 8.5 were partially redissolved at a higher pH, it is likely that the estimates of methemoglobin may have been too low and the estimates of total dissolved hemoglobin too high. This would result in reduced estimates of oxygen capacity. Since hemoglobin has a tetrameric structure from 0 to 4 of the iron atoms per molecule of hemoglobin could be oxidized to the ferric form, resulting in five electrophoretically distinguishable bands for each component. The electropherograms of both adult and fry hemoglobin solutions employed in this study suggest that only one met-derivative of each hemoglobin component was formed during the preparative procedures. Since oxidation of some one to three of the heme groups of the four incorporated in the hemoglobin may alter the oxygen affinity of the remaining non-oxidized groups (Riggs, 1970) high levels of methemoglobin are to be avoided. The methemoglobin levels recorded in this study are similar to those reported for other studies on fish hemoglobins (Aggarwal and Riggs, 1969; Gillen and Riggs, 1971).

The oxygen equilibrium curve of adult coho hemoglobin was only slightly modified by the presence of ATP and these effects were only observed at ATP concentrations which were 5 to 10 times those observed in the adult erythrocytes. ATP has been found to be the most common acid-soluble organic phosphate in teleost erythrocytes and accounts for well over half of this group of compounds in these cells (Rapoport and Guest, 1941; Gillen and Riggs, 1971). Virtually no information is available

regarding ontogenetic changes in ATP in fish erythrocytes although decreases in the concentration of this compound in coho salmon during the period of freshwater residence prior to spawning were observed in the present study. During the latter part of this period the molar ratio of ATP to hemoglobin was approximately 0.8 which is similar to the ratio of 0.76 observed in the Rio Grande cichlid (Gillen and Riggs, 1971) and of 0.65 observed in 2-year old sockeye grilse (Giles, unpublished observations).

Certain organic phosphates are known to modify the oxygen equilibria of a wide variety of animals. In humans, 2, 3-diphosphoglycerate sharply decreases the oxygen affinity of adult hemoglobin but not of foetal hemoglobin. Inositol hexaphosphate (IHP) similarly influences the hemoglobin of developing chicks and of certain turtles (Benesch and Benesch, 1969). Wood (1971) observed that the oxygen affinities of both larval and adult hemoglobins of the salamander, *Decamptodon ensatus*, were reduced by ATP and that the difference in oxygen affinity of the blood at these two life stages was accounted for by higher erythrocyte ATP concentrations in the adult. Gillen and Riggs (1971) demonstrated that the oxygenation characteristics of the hemoglobin of *Cichlasoma cyanoguttatum* were strongly influenced by ATP which generally lowered the oxygen affinity, increased the Bohr effect and caused variations in the heme-heme interaction. In addition, these effects were pH dependent. The log P_{50} of the hemoglobin at a molar ratio of ATP to hemoglobin of approximately 10 was increased by 0.75 at pH 6.7 and by 0.47 at pH 7.2 in comparison to phosphate-free

hemoglobin solutions.

Complex interactions between blood pH, state of oxygenation and 2,3-diphosphoglycerate (2,3-DPG) have been reported for human hemoglobin (Brewer and Eaton, 1971). Since increases in red cell pH have an antagonistic effect upon oxygen affinity to increases in red cell 2,3-DPG concentration it is possible that they serve as part of a regulatory mechanism in the unloading of oxygen in the capillary beds. Such a mechanism implies that the hemoglobin exhibits a significant Bohr effect. The small Bohr effect observed in adult coho hemoglobin may therefore account in part to the lack of sensitivity to ATP. Such arguments must be considered with caution, however, since 50% of the adult hemoglobin is composed of components which when isolated exhibit a very marked Bohr shift in the pH range of 7.1 to 7.5. Also evidence is accumulating to suggest that organic phosphates may not be involved in the regulation of hemoglobin oxygen affinity in certain mammals and birds. Thus the decrease in P_{50} of 17 mm Hg observed in chicks just after hatching does not correspond to variations in the levels of erythrocyte ATP or inositol hexaphosphate (IHP) both of which may modify oxygen affinity of the hemoglobin of unhatched chicks (Mission and Freeman, 1972). Although the foetal and adult hemoglobin of sheep exhibit a Bohr shift and lamb erythrocytes have a much greater 2,3-DPG concentration than adult red cells this organic phosphate does not have a significant effect upon the oxygen affinity of sheep of either age (Baumann, Bauer, and Rathschlag-Schaefer, 1972). It would appear therefore that in vertebrates modification of

the oxygen equilibrium by organic phosphate compounds is a common but not universal phenomenum.

The oxygen equilibria of hemoglobin components A6-8 were significantly different from those of the whole hemolyzate of adult coho blood. The oxygen affinity of adult blood was only slightly decreased by increasing temperature ($\frac{\Delta \log P_{50}}{\Delta T}$ = 0.016 - 0.021) whereas much larger decreases were observed in solutions of hemoglobin components A6-8 ($\frac{\Delta \log P_{50}}{\Delta T}$ = 0.056 at pH 7.90). In addition the Bohr effect observed in adult hemoglobin was approximately linear from pH 6.95 to 8.20 and quite small, $\emptyset$ = 0.172, whereas that of components A6-8 was non-linear so that $\emptyset$ = 0.033, -1.729, and -0.182 in the pH ranges of 6.82 to 7.50, 7.08 to 7.50, and 7.50 to 8.50, respectively. These differences in Bohr shift resulted in components A6-8 possessing a higher affinity for oxygen above pH 7.3, but a lower affinity below this pH than the complete hemolyzate of adult blood at 9.8 to 10 C. The heme-heme interaction of both hemoglobin solutions was generally less than 2.0 and exhibited similar increases as the pH was lowered or the equilibration temperature raised.

Although only components A6-8 could be successfully isolated from hemolyzates of freshwater adult blood the data indicated that the hemoglobin components of adult blood must be divisible into at least two groups based on differences in oxygen affinity and Bohr effects.

Binotti *et al.* (1971) reported that at 20 C component I of trout, *Salmo irideus*, hemoglobin had a P_{50}

of approximately 17.0 mm Hg and no Bohr effect whereas component IV had a large non-linear Bohr effect and a P_{50} at pH 7.4 of approximately 42 mm Hg. At pH 7.9, the oxygen affinity of both components was equal. In addition, component IV demonstrated a decrease in oxygen capacity (Root shift), below pH 7.0, whereas no such effect was observed in component I over the pH range of 6.8 to 7.6. The heme-heme interaction of component I decreased as pH was raised, whereas that of component IV increased as pH was raised from 6.12 to 7.1 and thereafter remained relatively constant. Hashimoto *et al.* (1960) observed that hemoglobin component S of the blood of adult chum salmon had a higher oxygen affinity below pH 7.8 than the second component (F) and was relatively unaffected by variation in equilibration temperature ($\frac{\Delta \log P_{50}}{\Delta T}$ = 0.006 for component S and 0.017 for component F), salt and buffer concentration and pH when compared to component F. The Bohr effect of component F was linear from pH 7.0 to 7.8 and very large, ϕ = -2.35. In addition, the oxygenation characteristics of the whole hemolyzate were generally compatible with those expected if no interaction occurred between the two hemoglobin components. A Root effect was observed in component F below pH 7.26 but not in component S. Similar differences in oxygenation characteristics have been observed in the multiple hemoglobins of such diverse species as the Japanese eel, *Anguilla japonica* (Yamaguchi, *et al.*, 1962; Hamada *et al.*, 1964; Yoshioka *et al.*, 1968; Itada *et al.*, 1971), and the loach (Yamaguchi *et al*, 1963).

To date no satisfactory explanation for the wide

diversity in oxygenation characteristics of the multiple hemoglobins of fish has been made. The problem of interpretation is made even more complex since it appears that in some fish the oxygen equilibria of whole hemolyzates are not simply the average of the equilibria of the individual components (Yamaguchi *et al.*, 1963). There has been some speculation that the hemoglobin polymorphs may represent a molecular adaptation to satisfy different physiological requirements (Hashimoto *et al.*, 1960; Binotti *et al.*, 1971). In the instances where a Root shift is observed in one hemoglobin component but is lacking in other components this speculation may be justifiable since the Root shift appears to be an important factor in the functioning of the swim bladder in certain fish. It has not, however, been established that the other functional characteristics of the various hemoglobin polymorphs which have of necessity been studied in hemoglobin solutions rather than in intact cells are actually representative of the oxygenation characteristics of the whole blood. Similarly it has not been demonstrated that all hemoglobin polymorphs occur within the same erythrocyte in fish blood although human erythrocytes do contain both hemoglobin A1 and A2 (Matioli and Neiwisch, 1965). Such information is vital to the discussion of possible molecular interactions between the various hemoglobin polymorphs and may explain the observations of Yamaguchi *et al.* (1963).

From data of Cameron and Randall (1972) for trout blood, the pH of the plasma of coho salmon at 9.3 C was calculated to be 8.27 at a PCO_2 of 0.2 mm Hg (air

equilibration) and 7.872 at a PCO_2 of 3.4 mm Hg. Applying the equations relating erythrocyte pH to plasma pH for human blood (Siggaard-Andersen, 1963) the respective values of intra-erythrocyte pH at these two CO_2 partial pressures would be approximately 7.81 and 7.52.

Relating the measured increases in log P_{50} to these calculated values of intracellular pH yields Bohr shift estimates of -1.228 and -0.569 for the blood of fry and adult coho salmon, respectively, when the PCO_2 is raised from 0.2 to 3.4 mm Hg.

It is evident that the Bohr shift estimates calculated from fry and adult hemoglobin solutions, while being qualitatively similar were quantitatively much different from those observed in whole blood. In the case of coho fry the difference can be resolved by assuming that the estimates of intracellular pH at 0.2 and 3.4 mm Hg were both 0.15 units too high. The estimates of red cell pH were based upon equations developed for human blood and it is quite possible that such relations do not hold for fish blood.

The disparity in Bohr shift estimates for adult coho hemoglobin solutions and whole blood are, however, much greater and more difficult to resolve. The P_{50} recorded in whole blood at 0.2 mm Hg PCO_2 would correspond to the value occurring at pH 9.0 in hemoglobin solutions. Such a pH is physiologically unrealistic and it must be concluded that the oxygenation characteristics of adult hemoglobin in intact red cells are extremely different from those observed in

hemolyzates. Although increases in oxygen affinity are often observed following hemolysis (Black, Tucker and Kirkpatrick, 1966b) and can often be explained on the basis of changes in the concentrations of allosteric effector substances or increases in pH decreases in oxygen affinity are much more difficult to interpret. In this connection Hashimoto *et al.* (1960) demonstrated that the type and concentration of buffering substance as well as the presence of various salts may profoundly influence the oxygenation characteristics of hemolyzates and that these effects may be different in the various hemoglobin polymorphs. Additionally, oxygen-linked NH_2 groups may be involved in the formation of carbamino complexes with hemoglobin (Albers, 1970; Riggs, 1970) which would result in an additional factor in the Bohr shift of whole blood but not hemolyzates.

The present data do not provide any insight into the causes of the variations in the oxygen equilibrium characteristics observed in hemoglobin solutions. In any case the qualitative relationships observed in these characteristics between fry and adult hemoglobin appear to be maintained in whole blood. Estimates of heme-heme interaction in fry blood and hemolyzates were similar and the qualitative change in n with changes in pH were similar for both adult and fry hemoglobin solutions and whole blood.

A brief summary of the oxygen affinities and effects of carbon dioxide reported in the literature for the whole blood of various salmonids along with the relevant observations from the present study is presented in Table XVII. Although

TABLE XVII

A summary of the oxygen affinity of the blood of various salmonids recorded at different equilibration temperatures and carbon dioxide tensions.

FISH	EQUILIBRATION CONDITIONS P_{50} (mm Hg)	PCO_2 (mm Hg)	TEMPERATURE (C)	REFERENCE
Rainbow trout, *Salmo gairdneri*	9.0	0.3	6	Eddy & Hughes (1970)
	24.0	3.0	6	
" "	9.0	0.3	10	Cameron (1971)
	18.5	3.0	10	
	14.0	0.3	15	
	20.0	3.0	15	
Trout*	11.0	0.3	15	Krough & Leitch (1919)
	19.0	4.5	15	
Brook trout, *Salvelinus fontinalis*	5.0	0.3	5	Black, Kirkpatrick, and Tucker (1966,a)
	26.0	10.0	5	
	12.0	0.3	15	
	43.0	10.0	15	
Landlocked salmon, *Salmo salar sebago*	8.0	0.3	5	Black, Kirkpatrick, and Tucker (1966,b)
	19.0	0.3	25	
	31.0	10.0	5	
	27.0	10.0	25	
Atlantic salmon, *Salmo salar*	7.5	0.3	5	Black, Tucker and Kirkpatrick (1966,a)
	27.0	10.0	5	
	10.0	0.3	15	
	36.0	10.0	15	
Coho salmon, *Oncorhynchus kisutch*				
11-month-old fry	5.5	0.2	9.3	Present study
	12.5	3.4	9.3	
2-year-old grilse	12.0	0.2	11.2	Davis (unpublished observations)
	20.0	3.4	11.2	
spawning adults	10.7	0.2	9.3	Present study
	15.6	3.4	9.3	
	9.8	0.2	5.0	
	12.5	0.2	10.0	
	14.8	0.2	14.8	

* Species not reported.

the age of the fish was normally not given it is probable that all except the coho fry, were over two years of age.

It is immediately apparent that at PCO_2 of 0 to 1 mm Hg the oxygen affinity of coho fry blood is much higher than that of other salmonids. The large Bohr shift of fry blood calculated previously is partially a reflection of this high affinity at a PCO_2 of 0.4 mm Hg since increasing PCO_2 to 3.4 mm Hg only increased P_{50} by 7 mm Hg. This is not an especially large decrease in oxygen affinity when compared to similar observations for other salmonids (Table XVII).

The oxygenation characteristics of the blood of coho postsmolts (seawater grilse and freshwater adults) are similar to those of other salmonids. The Bohr shift of -0.569 observed in adult coho salmon at 9.3 C is only slightly different from that of -0.53 reported for rainbow trout over the temperature range of 6 to 20 C (Eddy, 1971). The decrease in the oxygen affinity of adult coho blood at elevated temperatures is comparable to that observed in Atlantic salmon, rainbow trout and brook trout (Table XVII). Although an apparent disparity exists between the P_{50} recorded at 9.3 and 10.0 C for spawning adult blood equilibrated at 0.2 mm Hg PCO_2 the former estimate of oxygen affinity was obtained from whole blood containing 2.4% methemoglobin whereas the latter contained no detectable methemoglobin. Additionally since the seawater grilse had been reared at higher temperatures than the spawning adults, the oxygen affinities of the two groups of blood are not strictly comparable. In this connection Grigg (1969) demonstrated that thermal history influences the oxygen equilibrium

in the brown bullhead, *Ictalurus nebulosus* and that this change in oxygen affinity did not involve a change in the composition of the seven hemoglobin polymorphs. Differences in the physiological state of the blood may also have been responsible for the disparities since spawning salmon are undergoing progressive degeneration during their period of freshwater residence. In either case the disparities in P_{50} were generally less than 2 mm Hg.

It should be pointed out that a Root shift was not observed in either hemoglobin solutions or whole blood of coho salmon at any developmental stage examined. This observation was surprising since the blood of rainbow trout (Eddy, 1971), brook Trout (Black, Kirkpatrick and Tucker, 1966a), and Atlantic salmon (Black, Tucker and Kirkpatrick, 1966a) all exhibit a substantial decrease in oxygen capacity with increasing tensions of carbon dioxide. Hashimoto *et al.* (1960a) observed a Root effect in hemolyzates of chum salmon blood and demonstrated that it was component F and not component S which exhibited the Root shift.

Although the Root shift is thought to function in the maintenance of hydrostatic pressure in the swim bladder of certain physoclist teleosts this function has not been demonstrated in physostomous fish without a well developed gas gland (Steen, 1969). Similarly the role of the Root shift in the transport of gas to the tissue capillary beds of fish has not been identified although it could permit the establishment of very high blood:tissue oxygen gradients. The Bohr shift also

increases the tension of oxygen unloading by hemoglobin but cannot, under physiological conditions, produce blood:tissue oxygen gradients of the magnitude theoretically possible with a Root shift. The presence of a Root effect in the blood of fish could result in difficulties in achieving complete oxygenation at the gills. Thus at physiological dorsal aortic tensions of carbon dioxide (Holeton and Randall, 1967; Stevens and Randall, 1967) the Root shift calculated by Eddy (1971) would indicate that only 90% oxygen saturation would be acheived during passage of the blood through the gills. Until information is available relating PO_2, PCO_2 and oxygen content of the blood and tissues at the capillary level, the function of the Root effect in salmonids must remain in question.

It has been concluded that the increased demand for oxygen during exercise in trout is met by increased cardiac output and that only minor increases (about 9%) in the amount of oxygen released from each hemoglobin molecule occur when the fish are exercised (Stevens and Randall, 1967). Although PCO_2 of ventral aortic blood increased from 5.7 to about 8 mm Hg during exercise the dorsal aortic PCO_2 remained unchanged at 2.7 mm Hg (*ibid.*) . Only 60% of the hemoglobin bound oxygen was delivered to the tissues in a resting fish and this value increased to 70% during exercise (*ibid.*). Holeton and Randall (1967) demonstrated that only when environmental PO_2 was reduced to about 30 mm Hg did the ventral aortic PO_2 decrease to about 0 mm Hg. Under these conditions the dorsal aortic blood was only 37% saturated with oxygen. In both the lungfish

Neoceratodus forsteri (Lenfant, Johansen and Grigg, 1966) and rainbow trout (Cameron, 1971) ventral aortic oxygen saturations exceed 50% in non-exercised fish.

Although the interpretation of the foregoing observations is complicated by the fact that the blood of the ventral aorta represents the mixed venous return from the various organs and muscles which could vary substantially in their extraction of oxygen from the blood, it is clear that a portion of the hemoglobin-bound oxygen is not released during its circuit through the body. Furthermore, increased activity does not result in a significant reduction in this "stored oxygen". This suggests that the *in vivo* blood oxygenation characteristics of active fish serve to maintain a relatively constant blood-to-tissue oxygen gradient. It also illustrates the possible error in arbitrarily designating P_{50} as an indication of oxygen affinity since this may be more of a biochemical than a physiological parameter. It is also evident that variations in heme-heme interaction at a constant P_{50} may be more physiologically important than previously supposed since such variations would substantially alter the release of oxygen in the upper portions of the equilibrium curve.

Interpreting the oxygen equilibrium characteristics of adult coho blood in relation to the foregoing observations it is evident that they would serve to maintain a relatively constant blood-to-tissue O_2 gradient under a wide variety of environmental conditions which these fish would encounter during the extensive and frequently quite strenuous

migrations in the sea and in freshwater when returning to their spawning grounds. Since these fish seldom encounter hypoxic conditions it is probable that the dorsal aortic PO_2 is maintained above the 60 mm Hg required to achieve complete oxygen saturation of the blood. Salmon do not normally reside in water containing high carbon dioxide tensions and the reduced Bohr effect is probably not an adaptation to continual high environmental PCO_2 as has been suggested for the blood of certain tropical fish which inhibit sluggist backwaters (Willmer, 1934). Over a 10 to 20 hour period, PCO_2 of surface water in coastal areas can vary from 0.1 mm Hg during intense phytoplankton blooms to 0.5 mm Hg during intense upwelling in the northern Pacific Ocean (L. S. Gordon, personal communication). Additionally up to 0.9 mm Hg carbon dioxide have been observed near the ocean bottom (depth of 50 m) in areas of 10 to 20 km offshore during the upwelling season (*ibid.*). Such variations in PCO_2 could result in blood pH changes of 0.04 to 0.09 units if the blood pH versus PCO_2 relationships published for rainbow trout blood (Cameron and Randall, 1972) are applied. It is probable that coho postsmolts would encounter these fluctuations in carbon dioxide tension. Since the Bohr effect is not very great these fish would not experience aberrations in the oxygen carrying characteristics of the blood. The absence of a Root shift would also prevent the reduction in oxygen capacity which could occur under such conditions.

Although daily and seasonal fluctuations in Ocean water temperature are relatively small adult coho often encounter

large diel and seasonal temperature fluctuations during the freshwater spawning migration. This is also a period when the metabolic demands of migratory and spawning activity are severe. Under such conditions a large temperature-dependence of the hemoglobin oxygen affinity would be disadvantageous to the maintenance of efficient oxygen transport. Although some fish are capable of adjusting the oxygen affinity of their blood in response to environmental temperature changes (Grigg, 1969) such adaptations probably require some time to accomplish and would not be responsive to diel temperature fluctuations. It would seem probable, therefore, that the small influence of temperature upon the oxygen equilibrium of adult coho blood would serve to maintain a relatively uniform pattern of gas transport during large and rapid changes in environmental temperature.

It is much more difficult to account for the oxygenation characteristics of fry blood. Blood with a high oxygen affinity is normally found in fish residing in hypoxic environments and the Bohr shift functions to oxygen-unloading tensions of hemoglobin when fish are exposed to low environmental temperatures (Krough and Leitch, 1919; Black, 1940; Black, Kirkpatrick and Tucker, 1966 a,b; Black Tucker and Kirkpatrick, 1966a). It is obvious that the oxygenation characteristics of the blood must satisfy two sets of requirements. First, the oxygen affinity at the gills must be sufficiently high to attain near saturation of the hemoglobin at ambient oxygen tensions. If environmental

PCO_2 is high then the presence of a large Bohr shift would interfere with the saturation of the hemoglobin with oxygen. Secondly, oxygen should be released to the tissues at a sufficiently high PO_2 to maintain an adequate oxygen gradient between the blood and the tissues. The tissue PO_2 is not known for fish but is probably less than 15 mm Hg (Randall, 1969). At the tissue level the presence of a Bohr effect in the blood would increase the oxygen tensions at which oxygen is released from the hemoglobin.

Considering the high oxygen affinity of fry blood at 0.2 mm PCO_2 and the sharp increase in affinity associated with decreases in equilibration temperature it is probable that the large Bohr shift functions in part at least, to elevate blood:tissue oxygen gradients during the near-freezing temperatures encountered during the winter. This interpretation is somewhat complicated, however, since the Bohr shift occurring in fry hemoglobin solutions between pH 7.4 and 7.9 is less at 9.8 C than at 15.2 C. Although part of this conflict is resolved by the small difference in the lower pH value at 9.8 and 15.2 C (the exact pH values were 7.44 and 7.38, respectively) a full explanation must await further investigation. Considering, however, the high metabolic rate of fry and its dependence upon environmental temperature the increase in the magnitude of the Bohr shift at elevated temperature would tend to increase the tension and thereby the rate at which oxygen is delivered in the capillary beds.

Although the Bohr shift is available to decrease the oxygen affinity of fry hemoglobin the question arises as

to why this hemoglobin exhibits such a high affinity for oxygen. High oxygen affinity in fish blood has been associated with hypoxic environments (Krough and Leitch, 1919; Black, 1940) whereas active coho fry generally occupy well aerated streams and rivers in which the PCO_2 seldom exceeds 0.2 mm Hg. During extreme winter conditions, however, these fish often occupy crevasses in the stream bed probably to conserve energy when their food sources have been eliminated. Under such conditions it is likely that the local PO_2 of the water would be reduced especially in areas containing large amounts of organic material. Under such conditions the high affinity of the hemoglobin would insure adequate saturation of the blood with oxygen. Although the environmental PCO_2 would also increase under such conditions the magnitude of the Bohr shift may be decreased and interference with oxygenation of the blood would be minimized.

Although the foregoing functional interpretations of the oxygen equilibrium characteristics would seem to satisfy the requirements of oxygen transport in coho salmon during different portions of the life cycle the *in vivo* pattern of gas transport may be altogether different. Although ATP does not appear to modify the oxygen equilibrium of coho hemoglobin and of a wide variety of metabolites present in fish erythrocytes could perform a regulatory function similar to that of 2,3-DPG in human red cells. Since environmental factors may exert some selective influence upon erythrocyte metabolism the possibility of allosteric effector substances modifying oxygenation of the hemoglobin molecule in response

to certain environmental changes cannot be eliminated. These possibilities emphasize the need for *in vivo* physiological experimentation in conjunction with *in vitro* biochemical investigations into questions of this kind although in most cases there exist considerable overlap between the two approaches. Specifically measurements of tissue PO_2 and PCO_2, rates of carbonic anhydrase activity and patterns of blood flow under different environmental and physiological conditions are required in addition to present knowledge in order to understand the finer details of this vital process in fish.

SUMMARY III

1. The hemoglobin concentration of the blood of coho salmon in freshwater was directly related to the hematocrit by the least squares regression Hb = 0.514 + 0.255 Hct. The blood of seawater grilse also exhibited this relationship.
2. The concentration of ATP in the erythrocytes of adult coho decreased from 1.0 to 1.2 μmoles/mole hemoglobin in fish which had just recently returned to freshwater, to about 0.8 μmoles/mole Hb near the end of the spawning period.
3. At physiological concentrations ATP did not influence the oxygen equilibrium curve of hemoglobin solutions from adult coho. The P_{50} only decreased by 1 or 2 mm at ATP concentrations which were about 5 times those occurring in intact erythrocytes.
4. Hemoglobin components A6-8, prepared by ion-exchange chromatography from hemolyzates of adult coho blood, exhibited a high oxygen affinity at a pH above 7.5 (P_{50} < 6.2 mm Hg), a large temperature dependence which varied with pH and a non-linear Bohr effect which was highest in the pH range of 7.08 to 7.50. Since these hemoglobin components account for virtually all the hemoglobin of the blood in coho fry, these oxygen equilibrium characteristics were considered to be representative of fry hemoglobin.
5. The hemolyzates of freshwater adult coho blood had a lower oxygen affinity (P_{50} = 17.9 at pH 7.43 and 9.8 C) and a small, almost linear, Bohr effect (ϕ = 0.172). the oxygen affinity of the whole blood of adult coho exhibited a small

temperature dependence.

6. The oxygen equilibria of the pooled whole blood of 113 coho fry and of freshwater adult coho were determined at a PCO_2 of 0.37 mm Hg (air equilibration), and at a PCO_2 of 3.4 mm Hg. The oxygen affinity of the fry blood was higher than that of adult blood and a larger Bohr effect was observed in the juvenile fish confirming the observations made on the hemoglobin solutions. The effect of elevated CO_2 tensions was upon the decrease in oxygen affinity was greater in adult blood than that expected from the observed Bohr effect in adult hemolyzates which indicated that only qualitative comparisons could be made between the oxygen equilibria observed in whole blood and hemoglobin solutions.

7. The implications of the different oxygen equilibrium characteristics of the hemoglobin of fry and adult coho salmon are discussed.

MAJOR FINDINGS OF THIS THESIS

As stated in the introduction, the purpose of this thesis was to determine in some detail, the ontogeny of the multiple hemoglobins of coho salmon, discover whether or not environmental factors influenced the expression of the hemoglobin patterns and to provide some information as to whether the changes in the multiple hemoglobin pattern resulted in changes in the oxygenation characteristics of the blood. From these observations it might then be possible to draw certain conclusions regarding the functional significance of the multiple hemoglobins of the blood of coho salmon.

Initially it was determined that the expression of twelve to thirteen anodic and five to six cathodic hemoglobin components can be observed in the blood of coho salmon when the hemolyzates were subjected to electrophoresis on starch-gel in borate buffer at pH 8.5. All of these components did not occur at any one developmental stage but various combinations of the polymorphs were associated with distinct ontogenetic stages of the fish. Thus coho embryos and alevins exhibited an electrophoretic hemoglobin pattern of thirteen anodic and one cathodic components while fry hemoglobin contained only three anodic components all of which were also present in alevin blood. Postsmolt coho exhibited a hemoglobin pattern comprised of five anodic and five cathodic components four to five of which were not present in alevin or fry blood or, if present, only in trace amounts. Very little change occurred in the multiple hemoglobin pattern

during subsequent growth and sexual development after the fish had been in seawater for more than two months.

Since the transition from one hemoglobin pattern to another was associated with a change in behaviour and habitat of the fish it appeared reasonable to suspect that the environment may have been directly or indirectly responsible for the particular composition of the multiple hemoglobins observed in coho at various developmental stages. Experimental exposure of very young fry and older presmolt coho to water of various temperatures, oxygen concentrations and salinities for periods of forty-nine to sixty days did not result in any changes in the hemoglobin composition of the blood. These observations effectively eliminated the possibility that the ontogenetic variations in hemoglobin pattern were the result of variations in environmental factors. In addition maintaining postsmolts in freshwater during the normal period of early marine residence did not inhibit the expression of the postsmolt hemoglobin pattern. These observations are in agreement with the observation that both landlocked and sea-run Atlantic salmon exhibit similar ontogenetic changes in hemoglobin pattern (Westmann, 1971).

Although ontogenetic changes in the hemoglobin pattern have been correlated with the size rather than age of certain salmonids (Kock *et al.*, 1964; Wilkins and Iles, 1966; Westman, 1970) this does not appear to occur in coho salmon. Presmolts which had been exposed to elevated temperatures in freshwater or at 10 C in dilute seawater for 60 days were larger than either normal smolts or postsmolts in

seawater for thirty days, but still retained the "presmolt" hemoglobin pattern. In this connection, it is interesting to note that α intercept of the weight-length relationship only decreased slightly in these accelerated presmolts suggesting that the increase in growth was not accompanied by the normal streamlining of the fish as observed in smolts.

All of the foregoing observations suggest that in salmon the genetic control of the expression of the multiple hemoglobins is itself tightly controlled by some factor or factors associated with the ontogenetic development of the fish. Such a control mechanism could operate by regulating different erythropoietic organs which could produce erythrocytes capable of synthetizing only certain hemoglobin polymorphs. Such a substitution of one erythroid cell line for another which synthetizes a different hemoglobin molecule has been observed in mice (Fantoni *et al.*, 1969). There is also evidence that erythropoietic factors in the plasma may selectively stimulate the synthesis of one or another of the hemoglobin components of calf erythrocytes (Jonxis and Nijhof, 1969). In this connection the similarity in timing of the switch over from the blood islands to the kidney as the major erythropoietic organ in Atlantic salmon alevins (Vernidub, 1966) and the change in hemoglobin pattern in coho alevins may support the contention that the transformation from the alevin to the fry hemoglobin pattern involves the substitution of a new erythroid cell line. At the present time, however, the control mechanism regulating the transformation of one hemoglobin pattern to another remains obscure.

Whatever the control mechanism it is evident that environmental factors play an insignificant role in the regulation of the selective synthesis of the multiple hemoglobins of coho salmon.

The functional significance of the various combinations of hemoglobin polymorphs observed in coho salmon was investigated by determining some of the oxygenation characteristics of hemoglobin solutions containing the hemoglobin components characteristic of the fry and adult stages and relating these observations to the oxygen equilibria of fry and adult whole blood measured at two tensions of carbon dioxide. It was observed that fry hemoglobin had a higher oxygen affinity at pH greater then 7.3, a larger Bohr shift and a larger decrease in oxygen affinity with increased temperature than adult hemoglobin. In addition, the Bohr effect was non-linear in fry hemoglobin and rose sharply in the pH range of 7.1 to 7.5, whereas the Bohr effect of adult hemoglobin was approximately linear over the pH range of 6.95 to 8.20. In general then the oxygenation characteristics of adult hemoglobin were relatively insensitive to variations in pH and temperature, while the oxygen equilibrium of fry hemoglobin was greatly influenced by such factors.

When the oxygen equilibria of whole blood of fry and adult coho salmon were measured at a PCO_2 of 0.2 and 3.4 mm Hg. the qualitative relationships observed in the hemoglobin solutions were evident although quantitative comparisons of the observations on whole blood and hemolyzates

were not possible. The significant observation, however, was that the oxygen carrying characteristics of the blood of coho fry and adults are different and that it is the different hemoglobin components present in the erythrocytes of these two age groups that account for these oxygenation characteristics.

The foregoing studies demonstrate the necessity of confirming the identity of the multiple hemoglobin pattern during comparisons of the *in vivo* patterns of gas transport in fish especially when such comparisons are made on a seasonal basis. It is also evident that the use of blood equilibrium curves of a species of fish from one area may not represent those of the same species in another locality, especially if the rates of development are different in the two areas. Since micro starch-gel electrophoresis is a simple yet powerful method for the separation of polymorphic hemoglobins, it would appear advisable to include an electrophoretic "map" of the hemoglobin components in the various physiological investigations currently being conducted on fish blood and gas transport, thereby permitting a more meaningful comparison of the data from different studies.

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APPENDIX A

Calculation of the relationship between the relative amount of hemoglobin applied to micro-starch-gels and the estimation of the relative concentrations of the hemoglobin components of pre-smolt coho salmon.

TABLE XVIII

Spearman Rank Correlation Coefficient test of significance of the relationship between the relative amount of hemoglobin (density index) applied to the starch-gel and the estimation of the combined relative concentration of hemoglobin components A 6-8 of pre-smolt coho salmon.

SOURCE (date)	DENSITY INDEX Observed	Rank	RELATIVE CONCENTRATION COMPONENTS A6-8 Observed	Rank	CORRELATION COEFFICIENT
Creek (16/2/71)	131.3	2	79.3	1	
	123.1	1	88.4	6	
	145.8	4	87.2	5	
	184.6	6	85.0	3	
	149.8	5	86.3	4	
	133.5	3	83.0	2	-0.086 n.s.
Creek (2/3/71)	334.2	6	86.5	3	
	292.1	4	91.3	6	
	283.7	3	88.0	5	
	334.1	5	87.6	4	
	229.1	2	80.7	1	
	170.2	1	85.9	2	+0.429 n.s.
Creek (16/3/71)	338.2	6	76.1	1	
	327.5	5	84.1	6	
	254.0	4	83.0	5	
	235.8	2	77.3	2	
	237.4	3	79.0	3	
	206.7	1	82.6	4	-0.029 n.s.
Combined Controls and 10.2 C treatment (16/2/71)	129.8	1	88.5	3	
	159.0	2	90.3	4	
	159.5	3	85.5	1	
	163.9	4	91.2	5	
	178.7	5	88.0	2	-0.056 n.s.
(2/3/71)	187.0	1	88.1	4	
	222.7	2	86.9	3	
	283.3	3	88.7	6	
	332.9	4	88.6	5	
	368.6	5	86.8	2	
	404.1	6	83.5	1	-0.543 n.s.
(16/3/71)	196.3	1	82.1	1	
	224.0	2	87.1	6	
	238.2	3	83.6	2	
	256.0	4	83.9	3	
	303.1	5	85.1	4	
	313.0	6	86.5	5	-0.905 (.01>P>.05)