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THESIS

IMMUNOGENETICS OF SALMONID SERUM PROTEINS

Submitted by

Richard A. De Long

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

October, 1973

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COLORADO STATE UNIVERSITY

October 19, 1973

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY Richard A. De Long

ENTITLED Immunogenetics of Salmonid Serum Proteins

BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy

Committee on Graduate Work

Adviser

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ABSTRACT OF THESIS

IMMUNOGENETICS OF SALMONID SERUM PROTEINS

Serum proteins were studied in the fish family Salmonidae using two-dimensional single electroimmunodiffusion (2-D Single EID) as an effective technique for electrophoretic separation and electroimmunodiffusion of complex macromolecules. Two-dimensional single EID was also an effective preparatory technique for the isolation of selected antigen-antibody precipitin loops. Use of these selected precipitin loops in immunization procedures allowed production of hyperimmune antisera that were useful in comparative immunodiffusion and electroimmunodiffusion tests.

Sera from various species were simultaneously compared two at a time using 2-D Single EID. Patterns of complete and partial identities were obtained for homologous proteins possessed by all salmonid species tested. Albumins showed complete identity patterns for all species of the western North American Salmo tested. A pattern of complete identity between albumins of coho salmon, Oncorhynchus kisutch, and Yellowstone cutthroat trout, Salmo clarki lewisi, likewise confirmed the close relationship of Oncorhynchus species with the western North American Salmo. A comparison of albumins from the European brown trout, Salmo trutta, and S. clarki lewisi resulted in a pattern of partial identity with a long spur. This supports the subgeneric designation of Parasalmo for the western North American Salmo. Albumins were also

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useful in demonstrating the degree of phylogenetic divergences between species from different subgenera and genera in the subfamilies Salmoninae and Thymallinae.

Transferrins may be of value in delineating relationships between rainbow and cutthroat trouts and intraspecific groups of each. An F_1 hybrid (S. clarki x S. gairdneri)gave a transferrin pattern of partial identity for the S. gairdneri transferrin when reacted with an S. clarki stomias antiserum.

A unique pattern of partial identity with the formation of two spurs was obtained for transferrins from golden trout, Salmo aguabonita, and S. clarki stomias. The transferrin double partial identity pattern was considered to be supporting evidence that both species were derived evolutionarily from a recent common ancestor.

Transferrin polymorphisms demonstrated by agarose gels may be further resolved by using acrylamide-agarose gels, cellulose acetate-gel media, and starch gel-agarose mixtures. Caution should be exercised in analyzing molecular variation in proteins. Genetic and environmental sources of variation are discussed as well as reasons for spurious heterogeneity.

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Notes to Chapter I

1. Hereafter referred to as the Elegantiae. Valla began composing the first book in 1435 and continued his compilation of the rest at least until the end of 1439, probably later. For a discussion of the chronology, vid. L. Barozzi, R. Sabbadini, Studi sul Panormita e sul Valla (Firenze 1891) 145-146.

2. Ibi nanque Romanum imperium est, ubicunque Romana lingua dominatur (I praef.4).

3. The need for a defense of Latin against foreign influences is expressed by Sidonius (Epist.5.5) when he upbraids Syagrius for adopting German instead of Latin. Yet I do not believe that by "Gothicisms" Valla simply means northern (e.g. German) linguistic influences upon Latin. Certainly Alexander of Villedieu and Eberhard of Bethune might be characterized as "Goths," coming from the North; but they still write in Latin, albeit not classical Latin. Further, the Italian Bartolists are said to have come under the Gothic influence of the North, in their use of Gothic script, for instance (III praef.80). As we shall see, Valla defends the Latin language against attacks from many directions: not only the northern dialecticians and the Bartolists, but also Isidore, and the Italian lexicographers who preserved many of his errors. Basically, then, lingua Gothica is any debased form of Latin, whether by dialectical grammar, by mediaeval adaptations of Roman legal terminology, by the use of a vulgar Latin word in place of a classical term, or, as with Isidore, by misinterpretations of the definitions of classical words.

4. Of the total number of citations of authority in the first five books of the Elegantiae, passages from these two authors account for approximately 47%. Cicero is regularly cited one and one-half times as often as Quintilian, although in Book II, the ratio reaches 2.3 times.

5. Cf. J. Fontaine's definition of elegantia in Isidore de Seville et la Culture classique dans l'Espagne Wisogothique, 2 vols. (Paris 1959): "l'art de choisir les mots et d'approprier strictment le vocabulaire à l'expression de la pensée" (I.38); L. R. Palmer, The Latin Language (London, 5th Impression 1966), hereafter cited as Palmer, defines elegantia as the "fastidious selectivity of classicism" (126). In the Elegantiae, Valla is concerned with "elegance" in both these senses. His desire to use words to express thought exactly is indicated by his interest in proprietas (discussed on p.3). The fastidious selectivity of which Palmer speaks refers to the avoidance of unusual or offensive words and well characterizes Valla's attitude towards "Gothicisms."

6. The distinction between solecism and barbarism is explained in Rhet. ad Her. 4.12.17, as in many ancient linguistic texts. The former refers to a faulty construction, the latter to a fault in the use of a single word.

7. De Orat. 3.37.149: paene unanata [sc. propria verba] cum rebus ipsis;

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INTRODUCTION

Nuttall (1904) demonstrated that blood serum proteins obtained from different mammals were species-specific. He believed that serum antigen-antibody precipitates indicated by the intensities of their reactions the relative degree of relationship of the different species being compared. While these studies provided interesting insight into comparative zoology, information was not obtained with respect to different serum proteins and their modes of inheritance.

Since World War II the development and perfection of sophisticated techniques have allowed analyses and syntheses of biorganic macromolecules. It is well established that the genetic code resides in the DNA polymers of the chromosomes and acts phenotypically by specifying the detailed structure of proteins. Proteins are made up of long polypeptide chains and for each different chain there is a specific cistron that specifies in terms of its linear sequence of nucleotide bases the particular sequence of amino acid residues. A correctly-assembled polypeptide chain assumes a secondary, helical structure and then a tertiary, three-dimensional conformation that is biologically active. Analyses of protein primary structures have allowed biochemists to synthesize in vitro various functional proteins such as insulin and bovine ribonuclease.

Comparisons of organisms in terms of the structural correspondence of either their proteins or their DNA polymers have provided relevant data for determining the degree of divergence among species. Protein homologues possessed by two different species may be variable in terms

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of their primary, sequential order of amino acids. This amino acid variability results from point mutations in nucleotide bases coding for particular amino acids. Natural selection may cause certain point mutations in DNA cistrons to spread through a population, and the number of such genetic code changes may increase or decrease in evolving cistrons as time passes. Thus homologous proteins from distantly-related species will have a greater number of amino acid sequence differences than will more closely-related species.

A second mechanism of gene change, that of gene or intracistronic duplication, in combination with recurrent point mutation, is capable of vastly more sudden or dramatic effects. The newly-created cistron may code for a quite different protein with a different physiological role. Evidence suggests that cistrons coding for myoglobin and hemoglobin were derived from an ancestral cistron for globin. By a similar gene duplication process the ancestral hemoglobin cistron gave rise to cistrons coding for various hemoglobin chains.

It is often assumed that proteins with their accumulated amino acid differences constitute "evolutionary clocks" with constant rates of evolutionary change. For one particular protein, performing much the same task in a wide spectrum of species, the assumption of unchanging rates of accumulation of tolerable mutations may seem to be a valid hypothesis. However, only over long time spans does a constant, linear rate of evolutionary change have validity. For shorter time spans the rate of change in protein amino acid sequences will vary from one period to another as the selective factors on the species change. Studies by Jukes and Holmquist (1972) suggest that the "evolutionary

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clock" does not run at a constant rate for all species. They found that the rate of change in snake cytochrome c has been three or four times more rapid than in turtle cytochrome c during the time elapsed from a common ancestral origin.

During the evolution of a new protein, rapid rates of change may occur. Dickerson and Geis (1969) documented the first example of the adaptation of pre-existing structures to new uses at the molecular level. During the early evolution of mammals, through the process of gene or intracistronic duplication, the cistron for alpha lactoalbumin, a polysaccharide-synthesizing enzyme, was derived from the cistron for mammalian lysozyme, a polysaccharide splitting enzyme. Selection pressure was unusually severe on the alpha lactoalbumin cistron and changes in amino acid sequence were unusually rapid.

Some of the best evidence that amino acid sequence replacements accumulate in a protein with the passage of time is provided by cytochrome c, hemoglobin and fibrinopeptide studies. Cytochrome c, a respiratory hemoprotein, has been modified but slowly in evolutionary time and is of greatest value for comparing organisms that are distantly related. Dickerson (1973) reported that despite the accumulation of 16 to 19 amino acid differences between horse and fish cytochromes, x-ray analyses have shown that at least for the past 400 million years there has been no change in the folding of the cytochrome c molecule.

Dickerson (1973) indicated that the interaction of functional proteins with macromolecules determines the rate of evolution. Thus since fibrinopeptides do not interact with other molecules, they readily

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accumulate mutational changes and change rapidly. For these reasons fibrinopeptides are of value for comparing species with fairly recent divergences.

Augustin (1962) demonstrated that degrees of structural correspondence between proteins in different species may be determined immunologically by cross reactions with antisera. Such cross reactions measure in a relative way the similarity between proteins with respect to amino acid sequences, for the antigenic sites against which their antibodies are directed are shaped by the amino acid groups exposed at the surfaces of the folded molecules.

In order to demonstrate phylogenetic relationships at the higher taxonomic levels of family, order, class and phylum, evolutionarily stable eye lens proteins are useful. At the species level, however, eye lens proteins show wide cross reactions between a wide range of vertebrate species. Such cross reactions indicate that the compared species share many components exhibiting slow rates of evolutionary change. Blood serum proteins in contrast to eye lens proteins are more limited in their cross reactions thus indicating more rapid evolutionary changes between distantly related species. While as a group, serum proteins change more rapidly than do eye lens proteins, different serum proteins do not change uniformly since they are synthesized by different cistrons with different rates of evolutionary change. Serum proteins show immunochemical similarities within a taxonomic range mainly restricted to the same class. Therefore, serum proteins from species of class Osteichthyes do not cross react with species of class Aves or class Mammalia.

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To demonstrate intergeneric and interspecific differences both within and between the salmonid subfamilies Salmoninae, Coregoninae and Thymallinae, a conservatively-changed protein such as serum albumin is valuable. While albumin does not evolve as rapidly as fibrinopeptides, it does evolve rapidly enough to yield significant information. Using immunodiffusion tests of partial identity, the relative amount of antigenic distance between albumins from related species may be determined. The antigenic distance is estimated not as the amount of difference, but as the amount of similarity between related salmonid species.

In recent years electrophoretic studies of serum proteins and enzymes from different vertebrate species have demonstrated the species-specific nature of electrophoretic patterns. The existence of many electrophoretic variants in certain proteins has been demonstrated among individuals of a population. For those variant patterns resulting from multiple alleles at a locus, the terms "allomerism" or "polymorphism" are used. The latter term describes a situation where two or more alleles coding for a protein persist in the gene pool of the population with the rarest allele occurring in a frequency too high, i.e., usually greater than 0.01, to be accounted for by recurrent mutation alone.

For characterizing different populations or subspecies of a species, various immunoglobulins have been studied. Transferrin, an iron-binding glycoprotein, is highly polymorphic and separates electrophoretically into sharp zones. It appears that this protein is more variable and has undergone more rapid evolutionary changes than has serum albumin.

Since 1949 new and improved techniques have been developed for separating and characterizing different macromolecules from complex

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protein mixtures such as serum. Valuable immunogenetic information for a variety of species has been obtained by using electrophoretic and immunological techniques. Support media such as cellulose acetate and agar gels allow separation of proteins on the basis of differential electrophoretic charge while starch and polyacrylamide gels separate proteins by molecular size and shape in addition to electrophoretic charge. Immunological techniques rely upon proteins as antigens reacting with corresponding antibodies. Immunoelectrophoresis, a combination of electrophoresis followed by immunodiffusion in agar and agarose gels, results in greater resolution of separate serum components than may be achieved by electrophoretic methods alone. A recently-developed technique, two-dimmensional single electroimmunodiffusion, abbreviated 2-D Single EID, has been used in this study. The advantages of 2-D Single EID include superior resolution of antigen-antibody precipitates, detection of protein microheterogeneity, and a preparatory technique for making antibody precipitants to select native antigens. In addition to the above advantages, less time is required per run than for the older immunoelectrophoresis and immunodiffusion techniques.

The present study utilized and compared immunoelectrophoresis, immunodiffusion and two-dimensional single electroimmunodiffusion techniques for analyses of serum proteins from twelve species and subspecies of four genera representing two subfamilies of the family Salmonidae.

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REVIEW OF LITERATURE

The first application of immunoelectrophoretic analysis (IEA) to the study of fish was by Bargetzi (1958) who demonstrated differences in serum proteins between two species of whitefish, Coregonus fera and Coregonus macrophthalmus in Lake Neuchatel, France. An IEA study of sockeye or red salmon, Oncorhynchus nerka, by Krauel and Ridgway (1963) gave evidence of protein polymorphism for components in different electrophoretic zones. Variations in the number of components in each category were 0-2 in prealbumins, 0-4 in alpha -1 globulins, 1-6 in alpha -2 globulins, 1-5 in single-arced beta globulins, 0-4 in double-arced beta globulins, 1-2 in single-arced gamma globulins, and 0-1 in double-arced gamma globulins. As many as 25 components could be detected although no individual serum was found which possessed all of them. Female fish were identified by the presence of a serum "vitellin" lipoprotein. Similar lipoprotein components were demonstrated in female Atlantic salmon, Salmo salar, by Drilhon and Fine (1963) and in female chinook salmon, Oncorhynchus tshawytscha, by Armour (1969). Chellevold (1970) reported that mature females of the subfamily Coregoninae of the family Salmonidae could be identified by the presence of a specific serum protein not found in males. This specific serum protein was probably characteristic of all female salmonids since antisera serum could also distinguish females from males in two salmonid species, rainbow trout, Salmo gairdneri, and lake trout, Salvelinus namaycush. Another study by Dufour and Barrette (1967) demonstrated lipoprotein and glycoprotein

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polymorphisms in brown trout, Salmo trutta, rainbow trout, Salmo gairdneri, and brook trout, Salvelinus fontinalis.

In studies by Chellevold (1970), serum albumins from species of various coregonine genera, Coregonus, Leucichthys, Prosopium, and Stenodus gave the immunological reaction of complete identity. This was evidence that the albumin fractions from the four whitefish groups were unchanged since divergence from a common progenitor. Chellevold concluded that various levels of systematic study can be undertaken using protein properties. The eye lens proteins are diagnostic on the family level, the rapidly migrating proteins are diagnostic on the genus level, while the slower migrating proteins such as transferrins can probably be utilized for species and population studies.

In salmonid fishes serum transferrin polymorphisms have been identified in Arctic char, Salvelinus alpinus, by Nyman (1965). Other salmonid species showing transferrin variants included rainbow trout, Salmo gairdneri, brown trout, Salmo trutta, and brook trout, Salvelinus fontinalis as demonstrated in studies by Dufour and Barrette (1967). Moeller (1970) detected transferrin variants in Atlantic salmon, Salmo salar, and Payne, Child and Forrest (1971) reported geographical variation in S. salar transferrins from European and North American populations. Utter, Ames and Hodgins (1970) obtained significant differences for transferrin variants from smolts of coho salmon, Oncorhynchus kisutch, taken from different streams entering Puget Sound and tributaries of the lower Columbia River. Similar transferrin variant differences for four stocks of rainbow trout, S. gairdneri, were reported by Utter and Hodgins (1971).

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Very little information on protein polymorphisms is available for cutthroat trout, Salmo clarki, a wide ranging polytypic species. A study by Liebelt (1968) suggested that there may be differences among different populations of cutthroat trout segregating in different spawning streams tributary to Yellowstone Lake. However, no polymorphisms for specific protein components were established.

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METHODS AND MATERIALS

During the summer of 1969 work was begun on salmonid serum proteins. Early efforts were with the technique of immunoelectrophoresis in an attempt to resolve serum protein mixtures. This technique proved to be of limite value for resolving electrophoretic variants and demonstrating identity of serum components. Separation of serum components for use as specific antigens was not accomplished. Only small quantities of trout sera were collected and equipment adequate for separation of small fractions was not available for use. The development of agarose gel as used by Laurell (1965) in a two-dimensional immunoelectrophoretic technique offered some promise of better resolution, but it was a macro test and required relatively large amounts of antiserum. Several more summers of research resulted in little progress.

In July, 1971, a visit was made to the laboratories of Dr. Alfred J. Crowle in Denver, Colorado. During the previous six months he had succeeded in miniaturizing the Laurell technique using a cover of agarose gel on 75 mm x 50 mm glass slides. This new micro test required only 0.25 to 0.30 ml of antiserum per slide. Because of its superior capabilities, Crowle's new 2-D Single EID technique was utilized and significant progress was made during the balance of the summer.

Reagents - For 2-D Single EID a barbital buffer of pH 8.2 was found to be superior to many others tried. One of ionic strength 0.066 was used in the electrode chambers; the other of half this ionic strength was was used in the agarose gel. 7.37 gms (0.04 M) barbital (diethylbarbituric acid), 2.34 gms (0.04 M) NaCl and 1.04 gms (0.026 M) NaOH were

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dissolved in distilled H_2O, and brought to a volume of 1 liter to make the first; and then diluted 1:1 with distilled water to make the second. Subsequent tests indicated that the buffer with an ionic strength of 0.033 could be used in both the electrode chambers and agarose gels.

Sea-Kem agarose was used to prepare the gels.

For staining washed electroimmunodiffusograms a solution of 0.5 gm thiazine red R, 0.5 gm $HgCl_2$ and 100 ml of 2% acetic acid was used. Crowle (1973) reported that addition of 0.5 gm of light green SF and 0.5 gm of Buffalo black NBR to the preceding solution will give a triple stain with maximum sensitivity for agarose gels. A 2% acetic acid solution was used to destain the slides.

Collection of blood - Blood was collected from various salmonid species both from hatchery stocks and field populations. Plastic syringes (2½ cc) and size 18, 19, 20, 21 and 22 syringe needles were used in bleeding fish. Both cardiac and caudal artery punctures were used with the latter route being the easiest and doing the least amount of damage to the fish. After drawing the blood, needles were removed from syringes and blood ejected into 5 ml Vacutainer tubes. After the blood clotted, the clear, yellowish-colored serum was withdrawn with micropipettes, placed in Beckman 0.5 ml microcentrifuge tubes and centrifuged for several minutes. Serum was withdrawn with micropipettes, placed in clean microcentrifuge tubes and frozen at -20° C.

Preparation of antisera - For preparing antisera, blood serums were obtained from a cutthroat population in the Thompson River located in Forest Canyon in Rocky Mountain National Park, Colorado. According to

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Wernsman (1973 MS) this population is essentially the native greenback cutthroat trout, Salmo clarki stomias, but with a slight introgression of the introduced Yellowstone cutthroat trout genotype, Salmo clarki lewisi, into the population. This antiserum will be referred to hereafter as FCC (Forest Canyon Cutthroat) antiserum. Blood serums from golden trout, Salmo aguabonita, from Bear Lake, Wyoming, were also used for antiserum production. Antisera were produced in three domestic rabbits by using the following procedure:

1. Initial immunization was made subcutaneously under the foreleg using 0.1 ml of pooled trout sera in ½ ml of Freund's complete adjuvant.
2. One month later three booster immunizations were made on a daily basis using alternate sides of the rabbit. These boosters were:
   a. 0.1 ml pooled trout sera in 1 ml adjuvant
   b. 1.0 ml pooled trout sera in 1 ml adjuvant
   c. 5.0 ml pooled trout sera in 1 ml adjuvant
3. Six days after the last booster immunization the rabbits were bled by cardiac puncture.

Blood from three rabbits was placed in 50 ml capped glass tubes and allowed to clot. Serums were removed, centrifuged and placed in 5 ml Vacutainer tubes and frozen at -20° C.

Preparation of hyperimmune, monospecific antisera - The availability of the 2-D Single EID technique has recently been used by Crowle, Revis and Jarrett (1972) as a preparatory technique for making precipitins to selected antigens. Using their technique, electroimmunodiffusion

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produced loops of albumin and transferrin precipitates were sliced with a scalpel from the gels and used for preparation of immunizing antigens. The isolated precipitates were mixed with 0.5 ml of a phosphate buffer with an ionicity of 0.05 and pH of 7.4. The resultant mixture was frozen and then thawed to break up the gel structure and then emulsified with 0.5 ml of incomplete Freund's adjuvant. Antisera were produced according to the following schedule:

1. Initial immunization was made subcutaneously under the foreleg using 1 ml of the antigen-phosphate-incomplete Freund's adjuvant mixture.
2. One week later the same procedure above was used under the other foreleg.
3. Two weeks following the secondary stimulation 1 ml of a crude whole serum mixture was injected subcutaneously under the foreleg.
4. One week after the final inoculation, the rabbits were bled from the ear, blood collected and sera handled as previously.

Strong titers of antibodies to albumin and transferrin were obtained by this technique. Two monospecific antisera were produced, an albumin antiserum to a Yellowstone River outlet population of S. clarki lewisi, and a transferrin antiserum to an S. clarki lewisi population from Little Thumb Creek, a tributary stream of Yellowstone Lake.

Technique for 2-D Single EID - Crowle (1973).

Equipment - Experiments were performed on 75 mm x 50 mm microscope slides. Polyethylene spacers 3 mm square and 0.67 mm thick were used

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to support cover slides while casting the agarose gels. Support slides for antiserum application were prepared by applying two layers of water-proof tape spacers (2 mm wide and 4 cm long) along the 50 mm edges of 75 x 50 mm slides. Other small items of equipment included dialysis tubing (for protecting the anodic electrophoresis sponge and buffer against antiserum contamination during secondary electrophoresis), a Hamilton 10 microliter syringe with cut off tip (to charge origin well with sample), screw-capped test tubes (for dissolving agarose with heat under mild pressure), 10 ml and 1 ml pipettes, wooden applicator sticks, and Petri dishes for developing chambers for electroimmunodiffusograms.

A Gelman Deluxe electrophoresis chamber with two foam rubber sponges, one in each electrode compartment, was used for 2-D Single EID. Agarose covered slides were laid upside down with the gel edges resting on the sponges. The chamber was kept in a refrigerator at 4^{o} C. during electrophoresis with a power cord connected to a power supply kept outside of the refrigerator.

Procedures -

Preparation of glass slides - Slides were thoroughly cleaned with a mildly abrasize window-cleaning soap (Bon-Ami) followed by rinsings with tap water and then with distilled water, draining, and drying at room temperature.

Slides intended to support the agarose were pre-coated with hot 0.2% agarose in distilled water by spraying with a Pre-Val paint sprayer atomizer. Slides were air dried or placed in a drying oven at 60^{o} C.

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Casting the agarose gel - This procedure provided a thin, flat and uniform layer of gel within which 2-D Single EID was performed. In a 50 ml screwed-capped test tube 0.3 gm of agarose granules were mixed in 30 ml of pH 8.2 barbital buffer of 0.033 ionic strength, and the cap tightened. The suspension was heated in a boiling water bath for 5 to 15 minutes until the agarose granules were completely dissolved. While the agarose was dissolving, agarose-coated and dried slides were placed on two applicator stick supports on the workbench. This prevented the hot agarose solution from being drawn over the edge onto the benchtop by capillary attraction. A 3 mm square 0.67 mm thick spacer was placed at each corner of this slide and an uncoated clean slide laid on these spacers offset along the 75 mm edge by about 2 mm to provide a lip for receiving the hot 1% agarose solution.

With a 10 ml pipette more than 3 ml of boiling hot 1% agarose solution were drawn up, the tip of the pipette brought to the lip of the lower slide and the agarose quickly run between the two slides. About 2.7 ml was required to create a slight bulge of liquid around the edges of the slides. The top slide was nudged back to make a sandwich, the gel allowed to set for a few minutes. The sandwich was placed on sticks in a Petri dish with water saturated filter paper, and refrigerated at 4^{o} C. After 10 minutes the upper uncoated glass slide was removed from the lower agarose covered slide by slipping the cover slide off gently and evenly.

Primary electrophoresis - A 1 mm diameter round well was cut in the gel at the lower right corner of the slide 1 cm from each edge. With a Hamilton microsyringe this well was filled just level with the surface

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of the gel with serum diluted 1:1 with 0.033 ionic strength buffer. In order to observe the movement of albumin, this mixture was labeled with Evans blue by means of an applicator stick dipped very lightly in the stain powder.

The prepared slide was inverted and rested on about a 3 mm width of agarose gel at each 50 mm side on cathodic and anodic sponges of the electrophoresis apparatus. The origin well was close to the cathodic sponge.

The inverted slide was electrophoresed with 75 volts at 4° C. until the Evans blue-tagged albumin had moved 4.0 cm from the origin (usually about 1 hour). This slide was removed and antiserum applied.

Application of antiserum - The purpose of this step was to permeate a sharply defined area of the primary electropherogram evenly with antiserum which would react with, help separate, and detect the various antigens in the sample during the secondary electrophoresis.

During primary electrophoresis a clean, uncoated glass slide with plastic tape supports was laid on two applicator sticks. Undiluted, dialyzed antiserum was applied. No more than 2.5 to 3.0 ml were required. The inverted agarose coated slide was carefully lowered as you would a microslide cover slip until the antiserum flowed up to but not into the primary electrophoresis zone. This sandwich was allowed to stand in a humid environment supported by applicator sticks at room temperature for 45 minutes. The slides were separated by gently pulling them apart. Residual antiserum was rinsed from the slide lightly with 0.033 ionic strength buffer and the excess drained away. Secondary electrophoresis then followed.

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Secondary electrophoresis - The polarity switch on the electrophoresis chamber was reversed to minimize pH changes in the buffer chambers. The antiserum-charged slide, still upside down, was placed on the sponges, the antiserum-charged 75 mm edge resting on the dialyzing membrane-protected anodic sponge and the opposite primary electropherogram 75 mm edge on the cathode sponge. This slide was electrophoresed with 30 volts at 4° C. until the Evans blue-tagged albumin had migrated to within about 1 cm of the anodic edge of the slide (about 2 hours).

Development of the precipitin pattern - Following secondary electrophoresis many faint precipitin loops were visible by indirect lighting. These can be intensified and other loops made visible by giving the antigen-antibody systems more time to precipitate. The slide was placed right side up in a humidified Petri dish for overnight development at 4° C.

Washing and staining - Precipitin loops were still further developed and intensified by the following procedures of washing and staining.

The electroimmunodiffusogram, after its overnight incubation at 4° C., was washed for 6 hours at room temperature with gently circulating, slowly flowing tap water (e.g., as in washing photographic prints). Most city tap waters have sufficient dissolved solids and ions to wash away unreacted antiserum and enhance the precipitin loop development. However, some cities like Fort Collins, Colorado, have water supplies that are low in dissolved solids and ions and the agarose gels will often appear milky white, indicative of incomplete removal of unreacted antiserum. In order to remove these residual antisera, the

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slide was washed for several hours in a 0.86% physiological saline solution buffered with phosphates at pH 7.4 (8.6 gms NaCl, 0.17 gms dibasic sodium phosphate, and 0.093 gms dihydrogen potassium phosphate made up to a volume of 1 liter with distilled H_2O) until the milky white color disappeared. Then the slide was soaked in distilled water with agitation for 1 hour.

Without drying the slide, the washed gel was stained in 0.5% thiazine red R for 10 minutes at room temperature. The slide was then transferred to 2% acetic acid and soaked in this solution with several changes and intermittant agitation until the background gel could not be further destained. The slide was dried at room temperature in a cabinet protected against dust settling on the surface. The finished slide was then stored as a finished record.

Identification of transferrin - Transferrin, an iron-binding glycoprotein with beta -1 mobility was identified by staining with bathophenanthroline (4,7 - diphenyl -1,10 phenanthroline), a sensitive reagent for iron. The following unpublished results of Uriel and Chuilon were outlined by Williams and Chase (1971):

Reagents

a. Bathophenanthroline: 0.01% solution of bathophenanthroline, sulfonated, sodium salt. 2 mg of the iron-reagent were dissolved in 2 ml of distilled H_2O. 18 ml of 0.02 M sodium acetate were added and the solution was used immediately.

b. Thioglycolic acid (Mercaptoacetic acid).

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Procedure

a. Slides were immersed for 2 hours in the bathopenanthroline reagent.

b. 0.1 ml of thioglycolic acid was added.

c. After several hours the iron-reagent was removed and the slides were washed with several baths of 2% acetic acid.

Remarks

The iron-binding antigen-antibody complexes stained red. High quality, iron-free, chemicals and solvents must be used in all operations, beginning with the washing in buffered saline of the immunodiffusion and 2-D Single EID slides. Very thin, undried gels seemed to stain best.

Comparison of two serum patterns - In the older techniques of immunodiffusion and immunoelectrophoresis the resolution of various serum components in a whole serum mixture was often poor. With the 2-D Single EID technique resolution was superior and serum mixtures were simultaneously compared on the same slide. Wells were cut in the agarose layer approximately 6 mm apart, and each well filled with a different species serum mixture. Primary electrophoresis was carried as before, but with the sample in the right side well trailing the sample from the left well. Antiserum was applied and secondary electrophoresis carried out. Upon development of the electroimmunodiffusograms various patterns of complete, partial, and non-identity were obtained between homologous proteins from each serum mixture.

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2-D EID AND COMPARATIVE 2-D EID TECHNIQUES

When only small quantities of fish sera are available, separate fractionation procedures are needed for obtaining purified protein components for double diffusion tests. However, the use of two-dimensional electroimmunodiffusion (2-D EID) permits the separation of whole sera by primary electrophoresis (Figure 1), and their subsequent reactions with antisera by means of secondary electrophoresis (Figure 2).

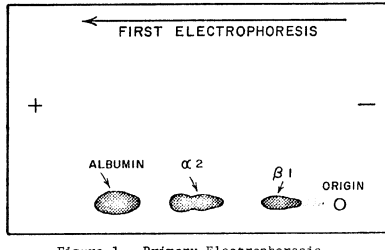

Figure 1. Primary Electrophoresis.

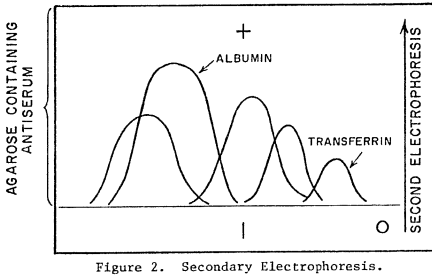

Figure 2. Secondary Electrophoresis.

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Formation of precipitin arcs results from electroimmunodiffusion involving immunodiffusion of the various serum components simultaneously with their secondary electrophoresis into antiserum impregnated agarose (Figure 3).

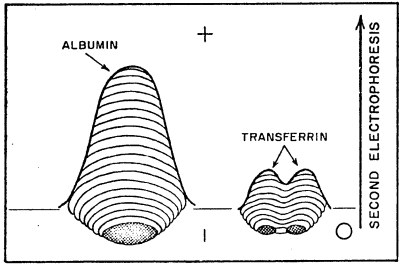

Figure 3. Electroimmunodiffusion of albumin and transferrin with precipitates formed with anti-albumin and antitransferrin antibodies.

Immunologic sieving during secondary electrophoresis distinguishes between two variants of the same antigen such as transferrin having different electrophoretic mobilities. This occurs because the concentration of these two variants is less than at either center, therefore, the marginal concentrations of antigens cannot advance electrophoretically against antibodies in the antiserum as rapidly as it could from the two centers (Figure 3).

When one wishes to compare two different sera by 2-D Single EID, wells are punched in the agarose gel as in Figures 4a, 4b, 4c, and 4d. Primary electrophoresis is carried out, antisera added and secondary

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electrophoresis performed. Precipitin loops between the two compared components will usually fit into one of the four categories illustrated in Figures 4a, 4b, 4c, and 4d.

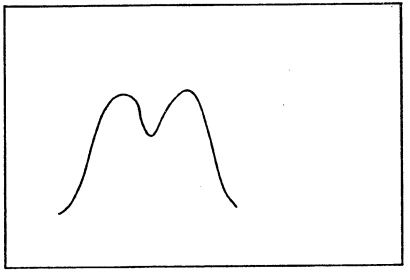

Figure 4a. Pattern of complete identity. When two proteins are completely identical serologically their precipitin loops will completely fuse.

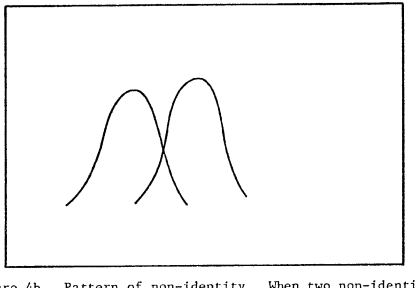

Figure 4b. Pattern of non-identity. When two non-identical antigens are compared using an antiserum with antibodies against both, their precipitin loops will intersect each other.

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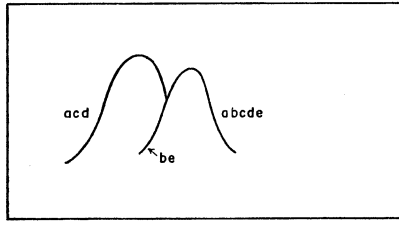

Figure 4c. Pattern of partial identity. When the two proteins have a number of antigenic groups in common (besides other groups which are different), and if the antiserum is capable of reacting with the common groups as well as with the groups specific for one of the two proteins, a pattern with a spur is obtained. The antigenic determinants common to both proteins (a, c, and d) are responsible for the continuous loops, whereas those specific for only one of the proteins (b, and e) cause the formation of the spur at the point of confluence.

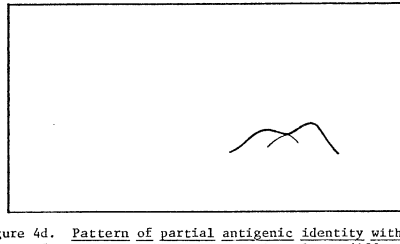

Figure 4d. Pattern of partial antigenic identity with formation of two spurs. When two proteins electrodiffuse against an antiserum containing antibodies against antigenic determinants specific for each of them as well as against their common determinants, this pattern is obtained. The spurs are fainter and shorter than in the non-identity pattern.

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RESULTS AND DISCUSSION

A 2-D Single EID pattern of coho salmon, Oncorhynchus kisutch, serum from the Bellvue Hatchery near Fort Collins, Colorado, and a serum of Yellowstone cutthroat, Salmo clarki lewisi, from Trail Creek, a tributary of Yellowstone Lake, gave a pattern of complete identity (Figure 5) for albumins when reacted with anti-albumin antibodies contained in the rabbit FCC antiserum. However, this does not mean that albumins from the two species were completely identical as the particular antiserum may not detect all antigenic determinants on the globular albumin surfaces. Because of allotypy, a variable response by

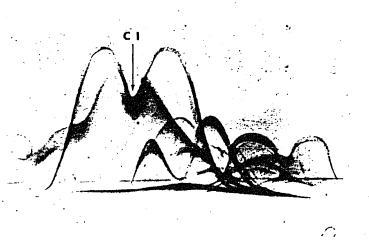

Figure 5. 2-D EID pattern between O. kisutch and S. clarki lewisi. Note a complete identity (CI) pattern for albumins when reacted with FCC antiserum.

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different rabbits to the same antigenic mixture, several antisera should be used as well as antisera to the other species being compared, in this case, O. kisutch and S. clarki lewisi. If similar complete identity patterns are obtained with all antisera, this would be additional evidence supporting the close phylogenetic relationship between the North American salmonid genus Oncorhynchus and the subgenus Parasalmo of the genus Salmo as proposed by Behnke (1968) based on morphological comparisons (Figure 7).

When sera of a brown trout, S. trutta, from the Drake Hatchery, Drake, Colorado, and a cutthroat trout, S. clarki lewisi, from Trail Creek, a Yellowstone Lake tributary, were compared, the pattern of partial identity between homologous albumins was obtained (Figure 6).

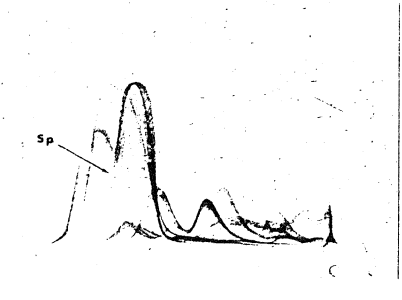

Figure 6. 2-D Single EID pattern between S. trutta and S. clarki lewisi showing the formation of a spur(Sp) in a partial identity pattern for albumins when reacted with FCC antiserum.

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The precipitin spur of partial identity suggested that the S. trutta albumin lacked some antigenic determinants possessed by S. clarki lewisi. According to Crowle (1973), in a serologically balanced system, the length and the intensity of a spur are inversely proportional to the degree of antigenic determinant similarity between the two antigens. That is, the shorter the spur and the more intense the spur precipitate, the greater is the similarity of antigenic determinants. A longer spur and more diffuse spur precipitate would indicate a lesser similarity of antigenic determinants. Since S. trutta, an European species of the subgenus Salmo, has been separated zoogeographically for a long period from North American Salmo progenitors, albumins from S. trutta have undergone more mutational changes and diverged more antigenically from albumins of the North American Salmo species, classified in the subgenus Parasalmo.

Efforts have been made to quantify serum protein spur lengths for use in immunological systematics of primates. Albumin spur lengths have been quantified and computerized by Goodman and Moore (1970) and compared with data from micro complement fixation and quantitative precipitation values. These comparisons revealed concordances as well as discordances among the three techniques for albumin analyses. Sarich (1970) indicated that considerable antigenic differences are possible in a protein-like primate albumin with approximately 600 amino acid residues.

When the amino acid sequences of salmonid albumins are determined and their primary, secondary and tertiary structures known, the relationships between their amino acid differences may reveal a more accurate picture of evolutionary relationships.

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In the more recently evolved North American Salmo species, patterns of complete identity were obtained for albumins from four different species, S. aguabonita, S. apache, S. clarki lewisi and S. gairdneri. While there were amino acid differences in the albumins as indicated by differential electrophoretic positions, these amino acid differences did not cause detectable antigenic differences. Thus the complete identity patterns support the phylogeny of Salmoninae depicted in figure 7 reproduced from Behnke (1968).

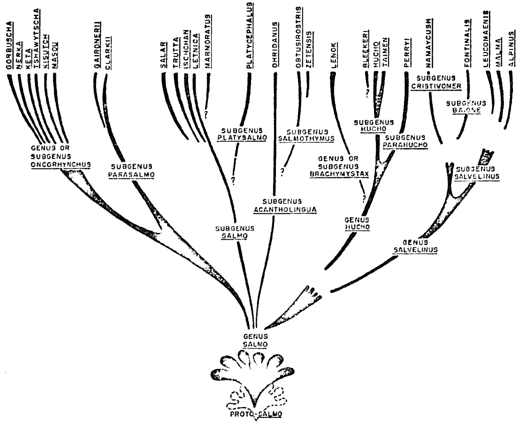

Figure 7. A suggested phylogeny of the subfamily Salmoninae.

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Intergeneric comparisons of albumins from two species, brook trout, Salvelinus fontinalis, and S. clarki stomias population from Island Lake watershed, Boulder, Colorado, gave a pattern of partial identity (Figure 8). The length of the spur would suggest a long evolutionary separation between the subgenera Parasalmo of the western North American Salmo and Salvelinus (chars) in the subfamily Salmoninae A similar pattern of partial identity for albumins from Arctic grayling, T. articus, from Grebe Lake, Yellowstone National Park, and Yellowstone cutthroat, S. clarki lewisi, from Trail Creek, also suggested a long evolutionary separation between the Thymallinae (graylings) and the Salmoninae (trout and chars) (Figure 9). Behnke (1972) suggested that

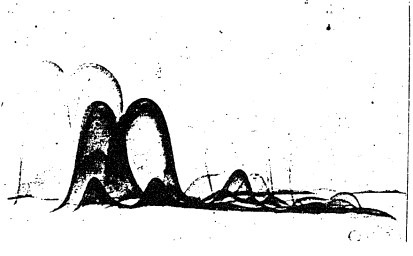

Figure 8. 2-D Single EID pattern between S. salvelinus and S. clarki lewisi x S. clarki stomias showing a pattern of partial identity for albumins when reacted with FCC antiserum.

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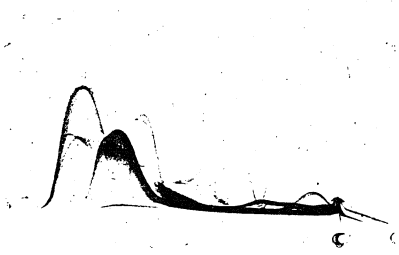

Figure 9. 2-D Single EID pattern between T. arcticus and S. clarki lewisi showing a pattern of partial identity for albumins when reacted with FCC antiserum.

the separation between the three salmonid subfamilies, Salmoninae, Thymallinae, and Coregoninae took place no later than the Miocene.

One class of plasma proteins, the transferrins, were examined for polymorphisms. Manwell and Baker (1970) reported that transferrins were the most useful serum proteins for population or subspecies studies. An interesting 2-D Single EID pattern was obtained from an F_1 hybrid cross of Salmo clarki lewisi x Salmo gairdneri made by Mr. William Klein of the Colorado Division of Wildlife. In this F_1 hybrid a single albumin precipitin loop occurred (Figure 10). This indicated that the albumin molecules resulted from F_1 homozygous, allelic cistrons coding at a single locus for a single polypeptide chain. Further

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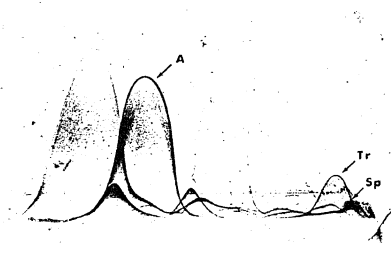

Figure 10. 2-D Single EID pattern of F_1 hybrid S. clarki lewisi x S. gairdneri serum reacted with FCC antiserum. One albumin loop (A) and the formation of a spur (Sp) in a pattern of partial identity for transferrin (Tr) were apparent.

confirmation for homozygous, allelic, cistronic control of albumin synthesis was obtained from a complete identity pattern between albumins from individuals of the two trout species. Studies of various intergeneric, salmonid, F_1 hybrids may give evidence for heterozygous genotypes giving patterns of partial identity for albumins and other serum proteins.

In the beta -1 region of the same F_1 hybrid (Figure 10) a partial identity reaction for transferrins was observed. Since transferrin is a rapidly modifiable protein, the following explanation is offered.

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The transferrin precipitin loop on the right was due to transferrin from S. gairdneri cross reacting with FCC antiserum while the transferrin loop on the left was due to S. clarki lewisi transferrin. The spur of partial identity indicated that the two transferrins were antigenetically different probably as a result of amino acid sequence differences. Thus in one F_1 hybrid, 2-D Single EID pattern, the more conservative nature of albumin and the more rapidly modifiable nature of transferrin were illustrated.

Blood samples were collected from eleven golden trout, S. aguabonita, from Bear Lake, Wyoming. The source of golden trout in Wyoming came from California about 1920. Behnke (personal communication) reported that the present S. aguabonita stock used for propagation in Wyoming is identical to S. aguabonita from the South Fork of the Kern River in California, and has not been hybridized with S. gairdneri or S. clarki. When sera from S. aguabonita and S. clarki stomias from Island Lake were simultaneously reacted with FCC antiserum (Figure 11) patterns of complete identity for albumins and partial identity for transferrins were obtained. The transferrins showed an unusual genuine pattern of partial identity with formation of two spurs. According to Schultze and Heremans (1966) this two spur partial identity pattern will be obtained if the antiserum which diffuses against two related proteins contains antigenic determinants specific for each of them as well as for their common determinants. This may be added evidence for the hypothesis that the golden trout is closely related to and probably derived from a widely distributed group of goldenlike trout with morphological similarities to the inland cutthroat trout as suggested by Schreck and Behnke (1971).

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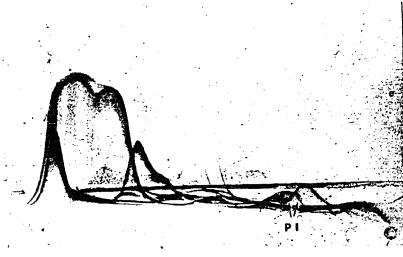

Figure 11. 2-D Single EID pattern between S. aguabonita and S. clarki lewisi x S. clarki stomias from Island Lake showing patterns of complete identity for albumins and a two spur partial identity (PI) pattern for transferrins when reacted with FCC antiserum.

In one S. aguabonita serum a transferrin polymorphism was detected (Figure 12). Double humped precipitin loops showing complete identity patterns were indicative of codominant allelic variants giving a summation pattern. While these transferrin molecules differed in some amino acid residues and thus had different electrophoretic mobilities, their antigenic determinants were apparently the same as evidenced by coalescing loops.

In another S. aguabonita serum the transferrins showed a pattern of partial identity (Figure 13) when a sodium barbital-barbital buffer of

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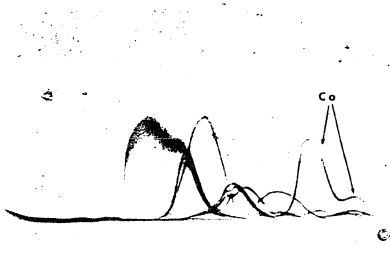

Figure 12. 2-D Single EID pattern of S. aguabonita showing coalescing (Co) transferrin loops suggesting complete identity between the two variants when reacted with antiserum to S. aguabonita.

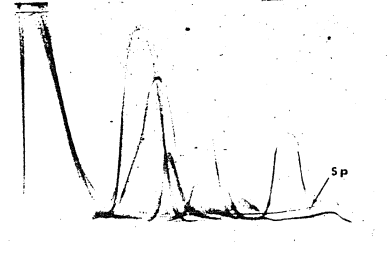

Figure 13. 2-D Single EID pattern of S. aguabonita showing coalescing transferrin loops with a partial identity spur (Sp) when reacted with S. aguabonita antiserum in a sodium barbital-barbital buffer at pH 8.6.

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pH 8.6 was used. Whether this partial identity transferrin spur was genuine or spurious was open to question since precipitation was not as complete at pH 8.6 as it was at pH 8.2.

Heremans (1970) mentioned three possible reasons for transferrin heterogeneity besides primary, sequential variation. The first was prosthetic variation of side chain groups like carbohydrates caused by enzymes operating on completed chains. The enzymes, in turn, are influenced by genetic and non-genetic factors. A second reason for molecular heterogeneity was configurational isomerism resulting from the pH, ionic strength, temperature and pressure to which the molecule was exposed. A third reason for spurious electrophoretic heterogeneity in transferrin may be the possibility of interaction with components of the buffer. To quote Heremans: ". . .it may be well to remember that all that glitters is not gold. . . nor is all heterogeneity molecular variation."

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FUTURE CONSIDERATIONS

What are the potentials of serum proteins for delineating evolutionary affinities and estimating the time of divergence between various salmonid species? From albumin immunological tests of identity included in this thesis, I believe that albumins are useful for estimation of immunological distance at the family, subfamily, genus and subgenus levels. The only exception observed was a pattern of complete identity of fusion between albumins from O. kisutch and S. clarki lewisi. This complete identity pattern, however, is not too suprising in light of the rather close relationship between the Oncorhynchus species and the Salmo clarki-S. gairdneri group. In one immunoelectrophoretic test, serum from a Yellowstone cutthroat, S. clarki lewisi, from Clear Creek, a tributary of Yellowstone Lake, failed to cross react with a commercially prepared rabbit anti-carp serum. Upon closer examination there was a very faint precipitin albumin arc indicating that the anti-carp albumin antibodies had reacted with at least some albumin antigenic determinants shared by the cutthroat trout. Based upon comparative albumin tests in this thesis, I believe that further identity tests of salmonid albumins with polyvalent anti-albumin sera to one species as well as to the other species may elucidate a more complete picture of the various salmonid lines depicted in Figure 7 from Behnke (1968).

One of the problems facing evolutionary biologists and fish zoogeographers is understanding the formation of sympatric sibling species. The problem of the origin of sympatric populations of char,

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Salvelinus alpinus, in the Swedish Gulf of Bothnia region has been reviewed by Svardson (1961) and Behnke (1972). Svardson believes that sympatric pairs of Swedish chars resulted from the invasion of several lakes by two ancestral "species." The sympatric pairs are morphologically similar, but Svardson believes these similarities are the result of introgression leading to convergence. Behnke, however, believes that the ancestral stocks hybridized in varying degrees in the Gulf of Bothnia tributaries in postglacial times to produce a highly heterogeneous genotype. Subsequent waves of invasions into headwater lakes established sympatric populations after the original invading stock evolved slight differences in spawning behavior from succeeding invaders. If this had been the case, then the slightest differences apparent today are the result of divergence from essentially a single common ancestor within the past 10,000 years in each lake and not convergence from distinct species. I believe that an examination of serum transferrin and other rapidly evolving proteins for polymorphic variants between several sympatric stocks and allopatric populations will reveal differences in proportion to the duration of genetic isolation. Comparisons of the differences should indicate the degree of differentiation and patterns of affinities derived from common ancestors. From such evaluations, the number of monophyletic lines involved in producing the present diversity could be estimated and also their time of separation from each other.

Efforts to establish a better understanding of speciation among species of western North American Salmo would be greatly aided by analyzing transferrin polymorphic variants as well as transferrin

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patterns of complete and partial identity. Behnke (1972) has observed that two forms of cutthroat trout occur in the upper Snake River, Wyoming; a fine-spotted form and a large-spotted "Yellowstone" form. It would be interesting to analyze transferrin variants as well as perform transferrin identity tests in order to examine the nature of this apparent isolation of two distinct groups of cutthroat trout in a continuous environment.

In this thesis a pattern of double partial identity was obtained for transferrins from S. aguabonita and an S. clarki stomias population. This pattern was reproducible and was possibly further evidence of the derivation of the golden trout complex from an interior cutthroat type progenitor as suggested by Schreck and Behnke (1971). Other members of the golden trout complex with dubious affinities, the Mexican golden trout, S. chrysogaster, the Gila trout, S. gilae, and the Apache trout, S. apache, may be studied using electroimmunodiffusion and immunodiffusion tests of their serum transferrins. Schreck and Behnke (1971) also mention the need to further elucidate the nature of a red-banded trout indigenous to desiccating basins in southern Oregon and to some tributaries of the Upper Pit and McCloud rivers of the Sacramento River drainage in northern California. This red-banded trout is assignable neither to S. gairdneri nor S. clarki. Transferrin identity tests could possibly aid in determining the affinities of this trout of uncertain taxonomic status.

According to Crowle (1973), "electroimmunodiffusion currently is at a stage in development similar to that of immunoelectrophoresis in 1954." Since electroimmunodiffusion techniques are quite new, interpretations of test results are yet to be perfected.

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Increased information for salmonid serum protein immunogenetics may be obtained using various new media now available for electro-immunodiffusion. Among these are a cellulose acetate gel support called Cellogel that is marketed by Reeve Angel, 9 Bridewell Place, Clifton, New Jersey 07014. Smaller pore size of Cellogel permits testing of smaller test samples, sharper separations of fractions and slower diffusion of low mobility gamma fractions. Considerable time may be saved by the use of rehydrated agarose coated sheets available in 5" x 7" size from Bioware, Inc., P. O. Box 8132, Wichita, Kansas 67208. The sheets may be cut to 75 mm x 50 mm size, rehydrated with water and then equilibrated with buffer prior to use. Another new support medium is dehydrated acrylamide agarose. This material is called Induboise AcA and is marketed by Gallard-Schlesinger Chemical Manufacturing Corporation, 584 Mineola Avenue, Carle Place, New York 11514. Easily rehydrated with electrophoresis buffer prior to use, this acrylamide-agarose gel acts as molecular sieve resulting in better resolution and very thin bands, comparable to those given by acrylamide gels. Another medium mentioned by Crowle (1973) is a mixture of 6% starch gel and 1% agarose in the primary electrophoretic zone. Starch gels like polyacrylamide gels allow molecular sieving of macromolecules in addition to their separations based upon differences in electrophoretic charges. This is important in demonstrating polymorphic genetic variants of haptoglobins and transferrins. Secondary electrophoresis of these separated variant molecules into an antiserum-charged agarose layer results in coalescing precipitin loops. These coalescing loops indicate the presence of two or more electrophoretically different but antigenically related populations of molecules

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(e.g., isomers, degradation products, electrophoretically different molecules complexed with a common antigen). Skewed loops indicate electrophoretically gradually varying heterogeneous antigen molecules.

The utilization of preparatory electroimmunodiffusion will allow the isolation of specific proteins for use as immunizing antigens. Resulting antisera may be used to more effectively study individual salmonid serum proteins as well as associations between several serum proteins.

Finally a more complete immunological perspective among salmonid species may be achieved through utilizing antisera produced in chickens and goats in addition to rabbits.

Additional proteins such as haptoglobins, LDH isozymes and other enzymes should be examined for possible taxonomic value. The most significant information will come from amino acid sequence data of albumins, transferrins and other protein families. This will reveal a more complete picture of salmonid phylogenetic relationships and species affinities.

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