AN INVESTIGATION OF THE ELECTROPHORETIC PATTERNS OF FISH SERUM AND PLASMA PROTEINS, WITH SPECIAL REFERENCE TO THE INFLUENCE OF A DIURNAL OXYGEN PULSE ON SERUM PROTEINS

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ABSTRACT

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by Gerald Ray Bouck

Conditions for separation and evaluation of fish plasma and/or serum proteins were investigated. Specific recommendations for utilizing the Beckman/Spinco model R paper electrophoresis system are listed. Certain confidence limits for this system are described.

A survey was made of the electrophoretic serum and/or plasma protein fractions of twenty-three species of fish, one species of immature aquatic insect, and one species of freshwater crayfish. Each pattern was found to be sufficiently unique to differentiate between species and in one case, sub-species were differentiated.

Fundamental differences between the serum protein fractions of man and fish, as well as between fish species are described. As shown by electrophoretic separation, the number of protein fractions, their positions, and their conjugation with polysaccharides and lipids differ widely.

The iso-electric protein in fish serum appears to be similar to mammalian gamma globulin. A rank order of iso-electric quantities in twenty-three species of fish revealed that "pollution sensitive" fish have less of this fraction than do "pollution tolerant" fish.

Gerald Ray Bouck

Bluegills (Lepomis macrochirus), largemouth bass (Micropterus salmoides), and yellow bullheads (Ictaluris natalus) were subjected to a diurnal low-oxygen pluse in an artificial stream. Serum patterns of bluegills and bass were significantly changed while the pattern of bullheads did not change. This conforms to an empirical estimate of their pollution tolerance. Other changes noted were (1) a significant change in the distribution of serum glycoproteins in bluegills and bullheads, (2) a change in the relationship of certain protein fractions to body weight or length, (3) a change in protein composition as shown by the conjugation of protein with polysaccharides in bluegills and bullheads. During the periods of low dissolved oxygen, the fish vomited indigested food, increased ventilation activity, reduced swimming activity and lost normal body color.

Robert C Ball

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INTRODUCTION

The blood of mammals has been recognized as a sensitive indicator of their physiological condition. Although there are notable differences between the blood of fish and mammals, many similarities exist. Both contain erythrocytes, leucocytes, and platelets or thrombrocytes. These are carried in a liquid menstruum and circulate in a closed circulatory system. This menstruum contains a mixture of electrolytes, amino acids, non-cellular proteins and other basic organic materials. The fluid portion of the blood is termed plasma. If whole blood is allowed to clot, fibrinogen and other coagulation factors are transformed into a fibrin reticulum which retracts and expresses the remaining fluid portions. The remaining fluid portion is termed serum and is essentially plasma with the fibrinogen and coagulation factors removed.

The non-cellular proteins of the serum are believed to be produced by the reticulo-endothelial tissue cells. These non-cellular proteins of man's serum have been extensively investigated by many researchers and one hundred twenty-six of these studies have been summarized by Ehrmantraut (1958). Some conclusions from these studies have universal applications and are germane to this study.

That component of the plasma and/or serum which is non-mobile at pH 8.6 in barbital buffer during paper electrophoresis is termed gamma globulin and is widely believed to

possess great antibody functions. However, it has been demonstrated that antibodies exist in other fractions of the serum and/or plasma. Elevation of the quantity of this fraction and the general reduction of other protein fractions of the serum continues the "stress" pattern described by Dunn and Pearce (1961). However, the drastic alteration of any fraction is indicative of physiological change.

Some other functions of man's plasma and/or serum proteins are mentioned by Weil (1959). Conjugation and subsequent transportation of various organic and inorganic substances such as hormones, metallic ions, lipids, and polysaccharides materials to plasma and/or serum protein occurs. Conjugation with these proteins appears to greatly increase the solubility and enhances the transportation of these materials to areas of utilization. Weil (1959) also points out that blood proteins form hydrostatic pressure to force fluids into the tissues on the arterial side and maintain osmotic pressure by drawing fluid from the tissues into the blood on the venous side of the capillaries. The latter is a function which he asserts could not be accomplished by electrolytes alone.

Laurell (1961) points out that many metallic cations are chelated by these proteins, thus preventing their loss in the bile or urine. In example, transferrin (= siderophilin) chelates iron and may serve as a supply of iron for hemoglobin production during anemia and other stress periods.

Prosser and Brown (1961) have discussed the ability of blood proteins to act as a buffer against pH changes and this aspect is too well recognized to bear further discussion here.

Whether these relationships are true for the blood of inframammalian vertebrates and invertebrates is being studied by many investigators at this time. Some evidence indicates that in general these relationships are valid for fish.

Brachet (1957) and Anker (1961) have reported that RNA (ribose nucleic acids) has a high ability to synthesize proteins. A reduction of RNA would decrease the quantity of proteins produced by that organism and this change in protein bio-synthesis might be reflected in the serum protein fractions. Fujiya (1961b) has shown that kraft pulp mill waste causes serious reduction of RNA in the cells of the biliary duct system, hepatic and pancreatic systems of Sparus macrocephalus.

Neuhold and Sigler (1960) have demonstrated that fluoride intoxication causes significant changes in the electrophoretic patterns of carp (Cyprinus carpio). Fujiya (1961a) also has demonstrated that exposure to sublethal concentrations of copper sulfite, lead acetate, hydroxide ions, and hydrogen ions causes drastic changes in the electrophoretic serum protein patterns of carp and other fish. These changes correspond well to the "stress" pattern of Dunn and Pearce (op. cit.) and appear to be due to changes in protein metabolism.

Fine and Drilhon (1960, 1961) have demonstrated that an antibody function is present in the serum of certain fish.

From the previous statements one can infer that any significant change in the composition of the serum proteins is an event indicative of serious physiological ramifications.

The increased emphasis on pollution control demands the continual search for better methods of detecting and evaluating pollution. One of the greatest needs in this area is a method for evaluating the influence of chronic, sub-lethal pollution conditions. Use of the electrophoretic technique on serum proteins promises to be useful in this area and as such constitutes the basis for evaluation of stress due to low oxygen-tension in this study.

Low oxygen-tension stress represents one of the most common conditions associated with polluted water. In its most subtle form it appears as a diurnal pulse beginning a reduction late in the afternoon and reaching its lowest level prior to sunrise. During daylight hours, such water may show normal oxygen tensions and under certain conditions may even become super-saturated.

Tarzwell (1957) has listed a numerical criterion for oxygen content of water as follows: not lower than 3 ppm at any time and not lower than 5 ppm for more than 8 hours in any 24 hour period. If one interprets this criterion for oxygen content of water literally, it is possible to allow a diurnal oxygen pulse which drops rapidly to 3 ppm for eight hours and then returns to normal.

It was decided to simulate such conditions in an artificial stream and determine if these conditions produced changes in the serum proteins of fish. It was believed that the use of warm-water fish would produce information of greatest interest to pollution biologists.

Other objectives of this study were to refine and evaluate the techniques of paper electrophoresis and survey the electrophoretic patterns of several species of fish.

ELECTROPHORETIC METHODS

Electrophoretic Migration of Protein on Paper

Proteins are colloids and as such have an electrically charged surface which by mutual repulsion prevents their coalescense. Proteins are also amphiproctic and can be made to vary their external charge by varying the pH of their medium. By placing proteins in a buffered solution of proper pH and molar concentration, one can induce molecular migration by subjecting them to an electrical field. These protein molecules migrate according to their electro-chemical properties and will separate into groups or zones of electo-chemically similar molecules.

Paper electrophoresis consists of soaking filter paper strips in such a buffered solution, applying a protein sample near the center of the strip, and then applying sufficient current to maintain an electrical field on it. After migration and subsequent separation of the various protein fractions, a protein denaturing agent is used to irreversably afix the fractions in their position on the paper. Location reagents are then applied to the paper strip to locate the protein fractions, and other fractions which have been separated.

The literature contains many specific instructions for the electrophoretic separation and evaluation of human plasma and/or serum. However, these instructions are only of limited

value for studies of fish since they were designed for a single species with samples containing high quantities of albumin and because they may apply only to one particular electrophoresis cell, type of buffer, and/or type of substrate.

The general lack of a large quantity of protein in the fastest moving fraction of fish plasma or serum is a definite disadvantage. This fraction experiences the greatest amount of chromatographic capture by the paper and the farther it is caused to migrate, the more it will be absorbed. This difficulty appears to be a "saturation" phenomenon. Once the paper is saturated by the first fraction, it does not appear to capture proteins from the following fractions. This reduced quantity in the first-migrating fraction can be considerably lower than the original quantity. Therefore, it is best to use procedures which produce maximum zone separation with minimum total migration. Samples may be migrated either over night or for short periods, provided the above conditions are fulfilled.

The conditions which achieved very good separation of fish plasma and/or serum protein in the Spinco/Beckman "Durram-type" electrophoresis cell are as follows:

1. Use Schleicher and Schuell #2043a paper strips, eight per electrophoretic run.
2. Barbital buffer (Vernol buffer) is used to wet the strips. It consists of 1.66 g barbituric acid (crystals) 12.76 g sodium diethyl barbitone

(powder) and sufficient distilled water to make one liter (Smith, 1960).

3. Use constant current at 0.5 MA/cm width of strip or 12.0 MA for eight strips.
4. Migration time: eight hours.

The use of these procedures will yield consistently better results than the conditions listed in Beckman Technical Bulletin No. 6095A (1961). In some cases it may be more convenient to migrate the samples over night. To do this, the above procedures apply but the current is changed to 0.104 MA/cm width of strip or 2.5 MA for eight strips. A migration time of 14 hours gave best results for these conditions.

To obtain maximum reproducibility of migration distance for each fraction, constant voltage must be used. However, the use of constant voltage requires refrigeration to offset the constantly increasing current and its heat production. If refrigeration is not available, it appears to be necessary to operate constant-voltage separations at voltages below 150 volts. Otherwise the heat produced will cause considerable evaporation off the strips and result in streaked or blurred patterns. Temperature greatly influences conductivity. At low temperatures, the conductivity will be low and result in lower current requirements to produce a given voltage. At higher temperatures, the conductivity will be higher and higher current will be required to produce the same voltage.

Four different buffer solutions were tried and it was found that the previously listed buffer solution best met the needs of this investigation. Borate, phosphate, and barbital-acetate buffers yield poor separation of fractions (resolution) and different appearing patterns with generally fewer fractions than Smith's (op. cit.) buffer. With this in mind, one type of buffer should be used on all strips.

Although commercially prepared buffer powder is available and needs only the addition of distilled water to ready it for use, generally poorer results were obtained when it was utilized. This product was found to yield widely different conductivities and in some cases, various pH values. It is believed that these differences were associated with the generally poor resolution and fraction separation obtained when this buffer was used to separate serum proteins of fish. The protein fractions of fish serum with intermediate migration-speed are particularly difficult to separate when this buffer is used. Therefore, it appears necessary that each investigator weigh and mix the ingredients for each buffer solution.

A considerable saving of the buffer solution can be realized if it is used in the following manner: (1) Make two liters of buffer solution, place in cells and on the strips in the usual manner. (2) Make a third liter of buffer solution and wet the paper strips with this solution on successive electrophoretic runs. It will not be necessary to add further buffer solution to the cell compartments for several days. The easiest way to wet these successive strips

is to place them on the folding rack of the electrophoresis cell, place the rack in a dishpan, and add buffer solution to each strip in a gentle stream from a pipette. Allow the excess to drain off the strips or blot the strips gently, before replacing the folding-rack and its paper strips in the electrophoresis cell.

Approximately one hour should be allowed for the moisture content of the strips to equilibrate with the air in the cell. During this time, the level of buffer within the anode and cathode buffer compartments should be allowed to equilibrate. If the buffer level is higher in one compartment than in the other compartment, capillary flowage in the paper will occur. Such flowage will smear and distort the fractions and the strip will be ruined.

Continual re-use of the buffer solution on the strips is not recommended. However, the re-use of the buffer solution in the cells is allowable provided the conductivity of the buffer solution is not greatly altered by evaporation. Evaporation can be greatly reduced by keeping the cells covered at all times. It should be noted that the buffer solution is attacked by microrganisms and should be discarded when any change is apparent. Some authors recommend the addition of sufficient merthiolate to obtain a concentration of 1;100,000, to control such microrganisms. Sodium azide is also used for this purpose.

The volume of the sample for protein analysis, lipoprotein analysis and glycoprotein analysis should be determined

for each species under investigation. In general, 10 microliters of either serum or plasma are sufficient for protein and lipoprotein analysis, if the total protein concentration is in the range of 3-5 g/100 ml. However, the glycoprotein content of fish serum and/or plasma is considerably lower than that of human serum. Therefore, a sample of 40-50 microliters is required for the analysis of glycoprotein in fish serum and/or plasma. In applying such large quantities of fluid to the already wet strip, one should be certain that the extra fluid does not run or smear. It is wise to apply such samples in units of 10 microliters and allow one to two minutes for the strip to assimilate this added fluid before adding more of the sample.

It cannot be overly stressed that the sample must be evenly distributed on the strip and that the sample must not extend to the edges of the strip. If these conditions are not obtained, the sample usually will smear and blur or otherwise ruin the strip for evaluation purposes. The following procedures have proved to be of value in applying samples to the strips: (1) Always keep the spinco applicator's wires in a horizontal plane. (2) Always apply the sample to dry applicator wires in such quantities that small beads of nearly equal volume are evenly distributed along the applicator wires. (3) Apply the sample to the paper with a quick thrust on the applicator depresser button. (4) The applicator should be held in contact with the paper for 20 seconds and then wiped dry for the next sample. Avoid uneven

pressure on the glass rods supporting the wet paper strips. Do not press the applicator wires so heavily that the glass rod is bent, as this may loosen the wet paper strips from their contact with the wicks.

Staining methods for paper electrophoresis. After the electrophoretic separation is completed, the strips are removed from the cells by fully extending the folding rack and then lifting the extended rack from its support. The rack is then placed in an oven preheated to 110° C. for 30 minutes to denature the proteins.

Peterson and Strong (1953) and Mertz (1959) point out that serum proteins are of the globular-type and have a coiled molecule which is profusely cross-linked within itself. Certain salts and heat have the ability to break these cross-bonds and allow the molecule to un-coil. Hence, after denaturation, globular proteins have a structure similar to fibrous proteins. The extent of conversion of protein structure (denaturation) is related to the temperature and time of exposure to this heat.

Henry, Golub, and Sobel (1957) have demonstrated that serum proteins on strips which were denatured at different temperatures have different uptakes of bromphenol blue. Brackenridge (1960) seems to have overcome this problem when using cellulose acetate as a substrate, by denaturing the protein with 30% sulfosalicylic acid. With this in mind, strict conformity to a single temperature of fixation should

be the rule. Strips treated at other temperatures should not be compared to each other.

It is interesting to note that of the serum proteins, only albumin has been reported by Smith (op. cit.) to complex with bromphenol blue before denaturation. Smith (op. cit.) describes a simple test for the presence of albumin: a crystal of bromphenol blue (the smaller the better) is added to the serum sample on the strip. The albumin will retain some of the dye and the rest of the dye will migrate freely. At the end of the separation all free bromphenol blue will usually have migrated to the end of the strip. Thus, if albumin is present, two zones of dye can be distinguished.

This method was used to determine if albumin was present in the serum of largemouth bass. This serum did not complex with bromphenol blue. If bromphenol blue is used to label a mammalian serum (i.e. bovine), the migration of albumin is readily observed. The use of such a standard allows migrations to be ended at a definite point. Such migrations are easily reproduced.

The alcoholic bromphenol blue method of staining the protein on the strips was used throughout this study. It consisted of a 10 minute pre-rinse in methanol to remove the buffer, a 30 minute bath in 0.1% bromphenol blue (acid salt) in methanol, followed by three, 5 minute rinses in 5% acetic acid. Although the stain appears to be good indefinitely, it was discarded whenever the pH rose above 4 (color change to blue) or after five series of strips have been stained in it.

After the final rinse in dilute acetic acid, the strips are removed and laid on filter paper for light blotting. The strips are then replaced on the rack and the rack is placed in the oven for 15 minutes. This leaves an alkaline residue of protein on the strips and assists in color development. To develop maximum color intensity, the strips are then exposed to an ammonia vapor bath for 15 minutes, but not longer than 30 minutes. The strips are then ready to be evaluated.

Saccharides conjugated with proteins are termed glycoproteins. Staining for glycoproteins is accomplished on separate strips with Shiff reagent after oxidizing the closed aldehyde groups in the saccharide-conjugate with periodic acid. All other substances which would be stained by fuchin-sulfite are removed in the ethanol pre-rinse.

Lipids conjugated with proteins are termed lipoproteins. Staining for lipoproteins is accomplished on separate strips by a heat-saturated ethanol solution of "Oil red O." All other lipids are removed by the warm ethanol. It should be noted that in many species freezing apparently breaks the lipid to protein bond and frozen protein samples cannot be evaluated for lipoproteins.

For a more complete description of the reagents and the staining procedures, the reader is referred to either Beckman Technical Bulletin 6095A (op. cit.) or Appendix II.

Electrophoretic Migrations on Cellulose Acetate

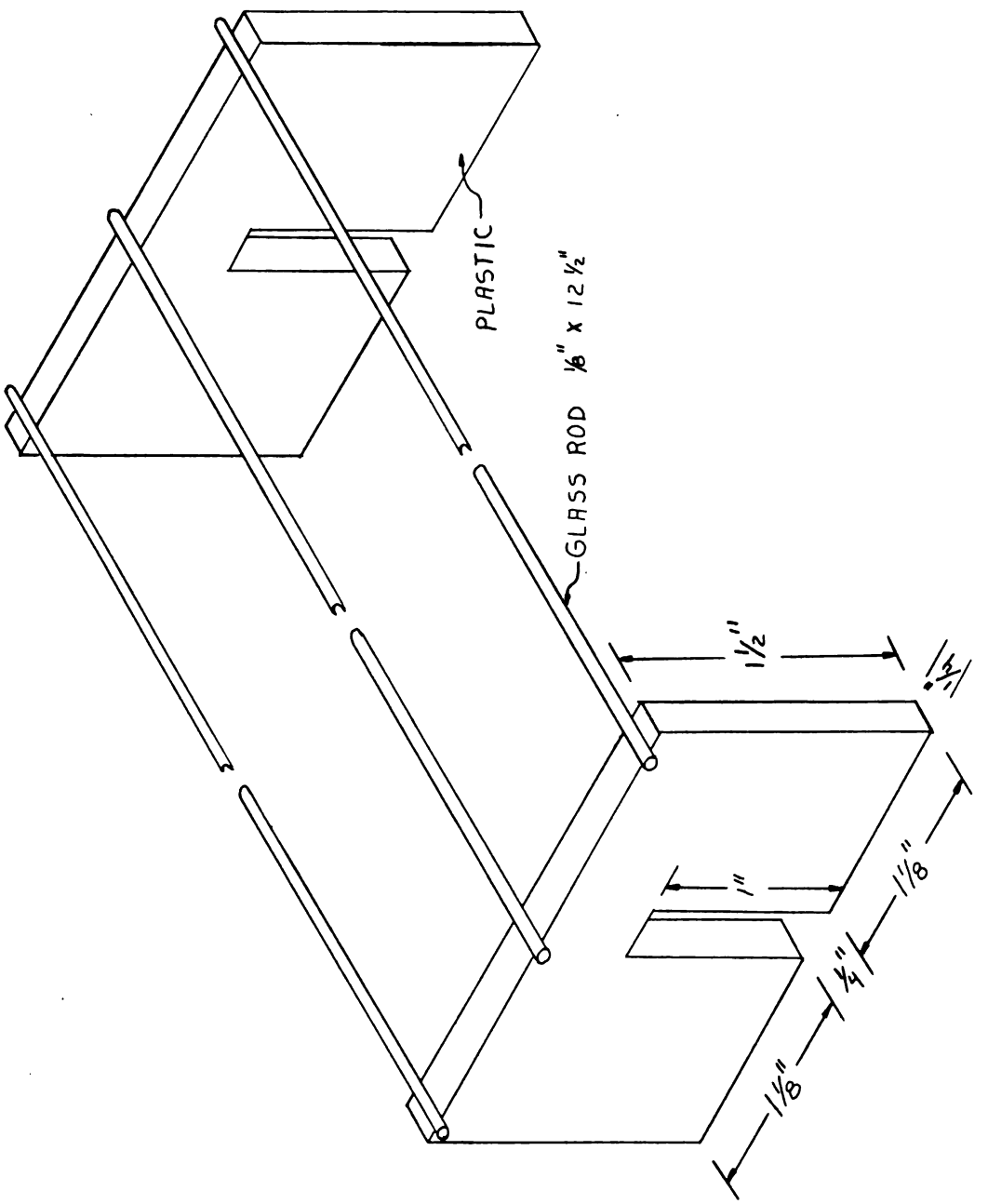
Cellulose acetate has certain advantages over paper as a substrate for electrophoretic separations. This material

does not retard the migration of protein to the extent that paper does. Tailing and streaking of the protein fraction are reduced to a minimum. The total time for migration and staining are reduced to approximately one-fourth that required for paper strips. In addition to these advantages, usually more fractions are separated and the uptake of certain dyes appears to be truly quantitative to the amount of protein on the strip. Also, these strips can be evaluated by elution easier than paper strips.

These advantages are not obtained without some disadvantages. Cellulose acetate is very expensive and costs nearly $0.50/strip. These strips are easily torn and tend to break if bent in a short radius. The absorption of the buffer solution and other solutions into the strip must be accomplished with great care. Evaporation of water off the strip by joule heat production tends to be a problem of great magnitude and it is recommended that separations on cellulose acetate be performed with an ambient temperature of less than 35° C. Dialyzing the protein sample is advised due to the small quantity of buffer solution on the strip, but has not been utilized in this investigation. All contact with low weight alcohols must be avoided since these alcohols cause the strips to curl beyond repair.

Utilization of this material was not possible or justified in the previously described electrophoresis cell until favorable modifications and optimum migration conditions could be ascertained. Figure 1 depicts a modified

Figure 1. An electrophoresis bridge to modify Spinco/Beckman (Durram type) electrophoresis cells for the use of cellulose acetate substrates.


PLASTIC
GLASS ROD 1/8" x 12 1/2"
1 1/2"
1/4"
1"
1 1/8"
1/4"
1 1/8"

electrophoresis bridge which allows the use of cellulose acetate strips on the Spinco/Beckman "Durram" type cell. Eight strips are accommodated as before.

Very good separation is obtained using the following conditions:

1. Cellulose acetate strips, 2.5 cm x 12.0 cm. (May be purchased from Gellman Instruments Company, Chelsea, Michigan.)
2. Barbital buffer previously described.
3. Constant current as previously described.
4. Migration time: two hours.

The lower current obtained by these conditions results in lowered heat production and the results are comparable to those obtained on paper.

The buffer must be absorbed into the strips by carefully floating them on the surface of the buffer solution. As the buffer is absorbed, the strips will turn light gray and any areas which have not absorbed it will remain white. The strip should not be plunged into the buffer until all such white areas have disappeared. Otherwise, air will be trapped and the strips will be ruined. At no time is it permissable to handle the strips with fingers. Microscope slide cover-glass forceps are useful for handling these strips.

Smaller sample volumes are used for protein analysis and in general 3-5 microliters provides more than enough serum for protein analysis. This must be applied approximately

one-fourth of the bridge distance away from the anode, as the protein will migrate toward the cathode. The glass supporting-rods are conveniently located in this position. Again, the sample must not extend to the edges of the strip.

Staining methods for cellulose acetate strips. After the migration is completed, the wet strips are laid on filter paper and placed for 15 minutes in an oven preheated to 110° C. This temperature will cause the strips to curl slightly and this curling should be ignored at this time.

Various dyes may be used to locate the protein on the strip. A solution of Ponceau "S" described by Smith (op. cit.) is the stain of choice in this investigation. It consists of 0.2% Ponceau "S" in 3% trichloracetic acid. The uptake of this dye is stated by Smith (op. cit.) to be quantitative to the amount of protein on the strip.

Brackenridge (op. cit.) using Light Green in an involved staining procedure was able to estimate the total protein more accurately than by the biuret method.

Alcoholic bromphenol blue is totally unsatisfactory and in this respect, all alcoholic solutions should be avoided. Any contact with alcohol must be followed by a thorough rinse in distilled water to prevent irreversible curling.

Staining is accomplished by laying the strips on the surface of the stain and allowing the stain to soak into the strip from below. This allows the air to be displaced and all areas of the strips should be devoid of air before the

strip is plunged beneath the surface of the buffer. This is the same procedure as used for impregnating the strips with buffer solution.

After staining, the excess dye is removed from the strips by repeated rinses in 5% acetic acid. While the strips are still wet, they are laid between sheets of filter paper and pressed overnight.

Cellulose acetate strips may be stained for glycoproteins and lipoproteins.

Evaluating Electrophoretically Separated Serum Proteins

Some investigators believe that the quantification of serum and/or plasma protein fractions is unnecessary. These investigators believe that only drastic changes in the relative quantities of serum and/or protein fractions are of interest. In example, Fujiya (op. cit.) stained the protein fractions with bromphenol blue, removed the excess dye, developed the maximum color intensity with ammonia vapor, and then impregnated the strips with paraffin. After compensating for the optical density of the paper strip and the paraffin, the optical density of the dye was recorded at 2 mm intervals along the strip. From these data, the electrophoretic patterns were determined. This method obviously precludes the evaluation of subtle changes in plasma proteins.

Another method of evaluation is the elution of the stain from the individual fractions. The eluted dye in a standard volume is then evaluated in a colorimeter or

spectrophotometer and compared to a standard curve to obtain the grams of dye present. Thus the per cent of total protein in each fraction can be quantified for statistical analysis.

Two major difficulties are encountered with the elution-evaluation method. First, many volumetric centrifuge tubes are necessary to accommodate the fractions from several strips. Accompanying this difficulty is the increased number of man-hours required to evaluate each fraction of the several strips. A second difficulty experienced in the elution of paper strips is that paper fibers contribute to the optical density of the solution. These fibers are very difficult to remove even with prolonged centrifuging. Cellulose acetate yields no fibers and, therefore, does not present this difficulty.

Strips stained with Ponceau S may be eluted in 0.1 N. NaOH followed by an equal amount of acetic acid. Strips stained with bromphenol blue are eluted in 0.5% sodium carbonate in water.

The solution of the previous problems is found in the use of a scanner which both draws the electrophoretic pattern and accurately records the area under each curve. This evaluation method was used throughout this study by utilizing the Beckman Analytrol scanner.

To scan the bromphenol blue stained paper strips, the following procedure is used: (1) At a point three-quarters of the distance between the anode end of the strip and the first protein band, make a thin pencil mark parallel to the

bands. This will serve as a reference point. Creasing the paper is equally effective. (2) At the time the strips are placed in the ammonia bath, the power and lamp switches of the scanner are turned to "on." (3) While the maximum color intensity is developing in the ammonia bath, select the slit width, remove any dust from the interference filters, place the recording pens in position on the cable riders and fill the pens with ink. (4) After the strips have been exposed to the ammonia for 15 minutes, a strip is placed in the scanning channel and the pen switch is turned "on." The strip should be placed so that the light beam is between the anode end of the strip and the pencil mark. (5) A recording chart is placed in position and the traveling pen is adjusted to zero. This pen is then calibrated with the use of a 0.9 neutral density filter. (6) Turn the motor switch to "on" and scan the strip. Turn the pen switch and motor switch to "off" before the paper strip passes out of the light path. Each strip was individually calibrated and recorded using the above procedure.

The analytrol scanner requires specific adaptation and adjustments if glycoprotein and lipoprotein stained strips are to be scanned. The reader is referred to the operators' manual for these specific instructions. An error in these instructions is that two, not one 0.9 neutral-density filters are required for scanning glycoprotein stained strips. Without using two such filters, the potential differences between the photoelectric cells cannot be balanced.

To scan cellulose acetate strips stained with Ponceau S stain, the 0.5 mm slit width is used. However, the slit height must be reduced to approximately 0.5 cm. The previous interference filters (500 mu) may be used, but a 0.9 neutral density filter must be placed in front of the rear photoelectric cell. Clearing of the strips with immersion oil, or other reagents appears to be unnecessary.

Cellulose acetate strips must be mounted on some media such as filter paper strips, or on plastic strips which have one side adhesive. Otherwise, the drive wheel will tear the cellulose acetate. Also, it is best to place the paper strip between the acetate and the photoelectric cell to prevent the pressure foot from tearing the acetate strip.

Results obtained on cellulose acetate stained with Ponceau S differ slightly from the results obtained from paper strips stained with bromphenol blue. This is to be expected and is the product of reduced chromatographic capture and sharper resolution.

In this study, cellulose acetate was used to separate the plasma samples obtained from invertebrates. It also was used to provide a check on the quality of the separation obtained on filter paper.

Evaluation of the Electrophoretic Method

It was of primary importance to the study as a whole to ascertain the limitations of the electrophoretic method used. In particular, it was necessary to know if the

results obtained from a single 10 cmm portion of serum accurately characterized the total serum in that sample.

Serum samples obtained from yellow bullheads were pooled to obtain approximately 2 ml of hemoglobin-free serum. This was mixed and centrifuged to remove any protein which might have been precipitated by antigen reactions. Sixteen paper strips were moistened with commercial vernol buffer (pH 8.6, ionic strength 0.075) in the previously described manner. A 10 microliter portion of the pooled serum was applied to each strip. A constant current of 0.104 MA/cm width of strip was applied for 14 hours. At the end of the separation, the protein on the strips was de-natured by placing the strips for 30 minutes in an oven heated to 110°C. These strips were then stained using the alcoholic bromphenol blue method and evaluated in the analytrol scanner as previously described.

The fractions of protein on these strips separated poorly and the commercial buffer was considered responsible for this difficulty. Nevertheless, it was decided to analyze these data. It was believed that these data would be useful since it would indicate the reproducibility of data obtained under adverse conditions. Due to the poor separation of the fractions, it was necessary to group the data into the four most prominent fractions. By doing this, certain fractions were evaluated as if they were only one fraction. Thus, for the purposes of this evaluation, this serum will be considered to consist of only four protein fractions.

A statistical analysis of the distribution of protein in the four fractions of pooled yellow bullhead serum is summarized in Table 1. It can be seen that the indicated quantity within each fraction varies slightly from portion to portion of the serum. In spite of this variability, the standard error of the mean of each fraction was less than 0.5%. This variability is obvious when the coefficients of variation are compared. It can readily be seen that variability is inversely related to the quantity of protein present within a given fraction. Thus the data obtained from fractions having low protein concentrations are likely to be less accurate than the data from fractions having higher protein concentrations.

Several factors contribute to the above relationship. Probably the most important factor and the most difficult to control is the human error involved in the delimination of a fraction's boundaries. This problem becomes particularly acute when the fractions are not resolved into distinct, well separated zones.

To test the influence of the analytrol scanner on the data, one strip of rainbow trout (Salmo gairdnerii) serum was scanned 14 consecutive times. The results of the statistical analysis are summarized in Table 2. Variability again decreased with increasing protein concentration. Although the width of the range for each fraction was 2% or less, in general plus or minus 0.5% constituted the range from the mean. The largest standard error of the mean of any fraction

TABLE 1. Summary of data obtained from electrophoretic analyses of sixteen portions of pooled yellow bullhead serum.

	Fraction "I"	Fraction "II"	Fraction "III"	Fraction "IV"
Mean	26.8	33.9	35.7	3.6
Variance	3.044	3.114	2.927	0.417
Standard Deviation	1.754	1.765	1.711	0.646
Standard Error of the Mean	0.436	0.441	0.428	0.162
Coefficient of Variation	6.54	5.21	4.79	17.94
Range	23.7-30.0	30.8-37.0	32.5-38.8	2.2-4.6
Width of Range	6.3%	6.2%	6.3%	2.3%

TABLE 2. Summary of data obtained from fourteen repeated scannings of the same strip containing rainbow trout serum protein.

	Fraction "I"	Fraction "II"	Fraction "III"	Fraction "IV"	Fraction "V"
Mean ($\bar{X}$)	5.6%	14.2%	16.1%	22.5%	41.1%
Variance (s^2)	0.159	0.215	0.118	0.318	0.584
Standard Deviation(s)	0.399	0.464	0.344	0.564	0.764
Standard Error	0.106	0.124	0.092	0.150	0.764
Coefficient of Variation	7.1	3.27	2.14	2.50	1.86
Range	5.0-6.0	13.7-15.5	15.4-16.4	21.8-23.2	4.00-42.1
Width of Range	1.0%	1.8%	1.1%	1.4%	2.1%
95% Confidence Limits of $\bar{X}$	5.42% 5.88%-	13.93% 14.45%-	15.86% 16.26%-	22.22% 22.86%-	40.62% 41.51%-

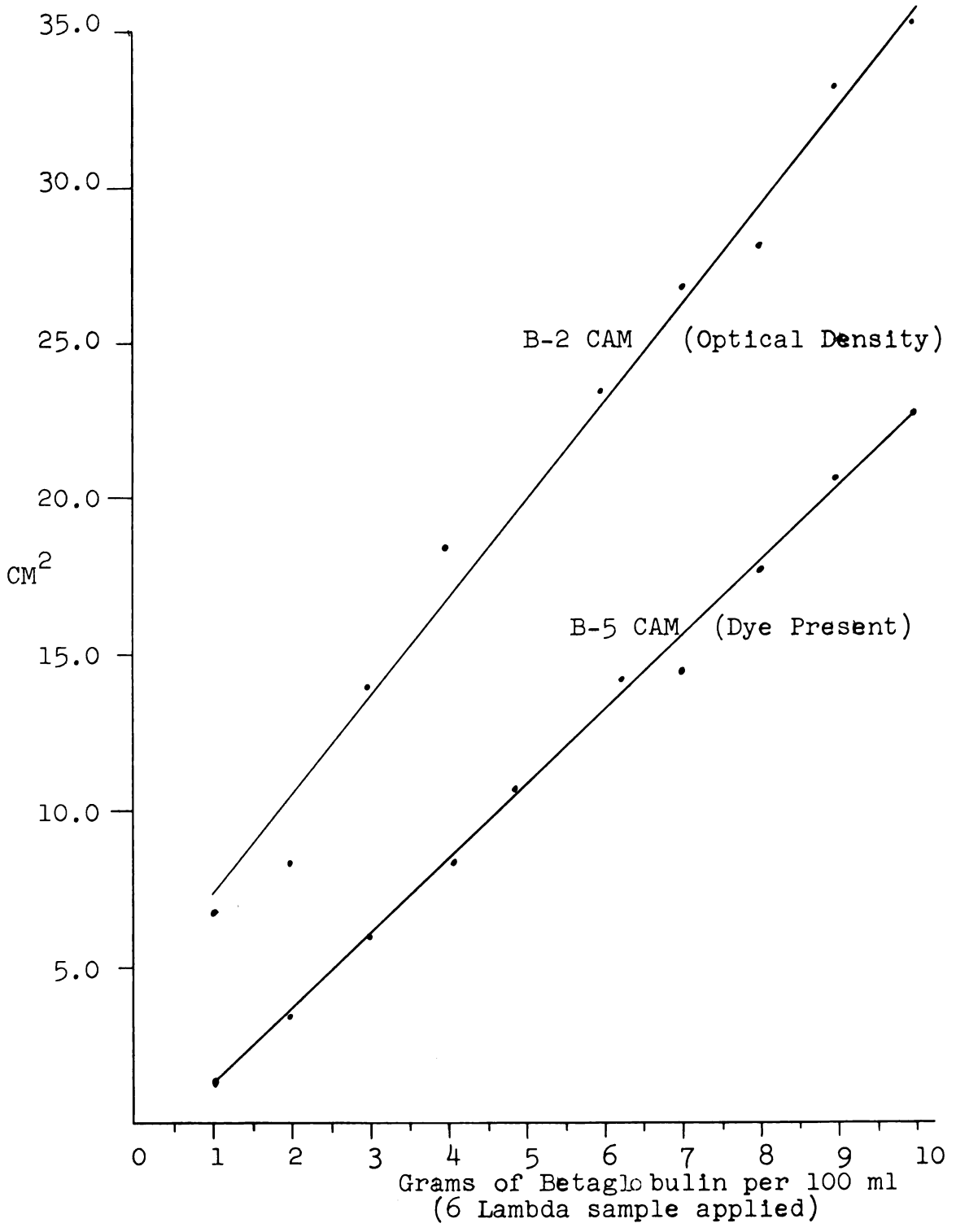
observed was 0.204%. Again, human errors in delimiting the protein fraction's area were probably the main source of variability. In this aspect of the electrophoretic analysis, experience is most important if variability is to be decreased.

The reliability of the Analytrol unit to record the area under a given curve using different slit widths was also tested. Matched observations on 16 different strips using 2.0 and 1.0 mm slit width were performed. The mean of the differences obtained were not significant at the 95% level. However, the appearance of the curves obtained using different slit widths is different. Curves drawn using the 2.0 mm slit width are considerably smoother than curves drawn using the 1.0 slit width. However, the latter may be of greater use since it is more sensitive to variations.

Since the Analytrol scanner draws the curves for each fraction and gives the area under each curve by integration, it was decided to attempt to use the area of the total curves to estimate the total protein concentration in terms of grams per 100 ml of serum. Samples of bovine serum albumin, alpha, beta, and gamma globulin were applied at various known concentrations to the strips, migrated a short distance and then fixed, stained, and evaluated.

The relationship of area under the curve to protein concentration for a given electrophoretic run was found to be linear. This is depicted in Figure 2 for bovine beta globulin. Two balancing cams were used to evaluate the curves and the B-5 cam yields data less variable than the B-2 cam. The B-5

Figure 2. Uptake of bromphenol blue by bovine betaglobulin as evaluated by scanning in a Spinco Analytrol.



cam yields curve area linear to the amount of colored material on the strip, and is corrected for Beer's law. The B-2 cam yields curve areas linear to optical density and it is also corrected for Beer's law.

On different electrophoretic runs, the values obtained for curve areas were found to be proportional to the amount of protein on the strip. However, the "Y" intercept value was found to change considerably. The slope of the regression line appeared to be so similar for each electrophoretic run that it was not tested statistically. Therefore, it was concluded that the area under the curve could not be used to estimate protein concentration when staining with bromphenol blue. However, this relationship does allow the determination of the per cent of the total protein constituted by a single curve (fraction). If one wishes to convert these values to grams per 100 ml of serum, the total protein concentration must be determined by a method such as the biuret method.

The differences observed for samples evaluated on different electrophoretic runs may be due to variation of the sample volume, differences in the temperature of fixation, differences in protein concentration, or other factors. Sample volume is strongly suspected to be the main contributor although it is known that fixation temperature is extremely important. The latter was difficult to control in the oven used for this study.

Methods for the Collection of Blood

Methods for the collection of blood samples from fish were explored and cardiac puncture was found to be the best method. Direct cardiac puncture consisted of exposing the heart by an incision and then inserting a hypodermic needle connected to a 1 ml tuberculin syringe directly into the bulbus arteriosus. Indirect cardiac puncture consisted of probing for the heart after one has ascertained its position. The former method destroys the specimen but the latter method generally incurs only low mortality. A #22 guage hypodermic needle is preferred over a #26 gauge, since the former do not plug easily. Coating the needle with mineral oil helps to reduce clots from forming in the needles. The blood should not be expelled through a hypodermic needle as this tends to induce hemolysis.

Attempts to draw blood from the dorsal aorta on Centrarchids, Ictalurids, and Percids were unsuccessful, probably due to the small size of the specimens, but worked well on Salmonids and Carp. Collection of blood by severing the caudal peduncle offered no advantage since it bled poorly, clotted rapidly, destroyed the specimen, and had the risk of contaminating the blood with mucous and body fluids.

Collection of blood from crayfish (Orconectes propinquus) was accomplished by inclining the hypodermic needle toward the pericardial cavity after entrance at the dorsal soft-membrane between the cephalothorax and the first abdominal segment.

Collection of blood from larval insect forms such as those of the dobson fly (Corydalus cornutus), burrowing mayfly (Hexagenia limbata), caddis fly (Hydropsyche sp.), and midge larvae was accomplished by drawing a melting-point determination capillary tube into a fine point, rinsing it with anticoagulant, and inserting it under the skin of the abdomen. After the initial filling by capillary action and hydrostatic pressure, it may be necessary to apply gentle pressure to the general body surface of small larvae to obtain an adequate blood sample volume.

The decision of whether to investigate plasma or serum must be decided by the investigator in view of the aspects which are to be investigated. In cases where the cellular components of the blood are of no interest to the investigation, serum will suffice. However, the use of anticoagulants to provide the investigator with plasma allows the determination of both cellular and non-cellular components. Since fish and invertebrate blood clots very rapidly at summer temperatures, it is often useful to prolong or prevent the coagulation of such blood samples. In the case of invertebrate blood it is always necessary to use an anticoagulant to obtain plasma since their blood apparently will not undergo clot retraction to express the serum.

The selection of an anticoagulant is again a matter of choice. Larsen and Snieszko (1961) have compared various anticoagulants and consider the practice of coating syringes and capillaries with 10% heparin to be the best method.

However, it must be recognized that heparin is a polysaccharide and its use may preclude the evaluation of glycoprotein in the plasma. In this study both 10% heparin and 10% citrate were used and citrate was selected for most of the work. Citrate does not need refrigeration and remains potent indefinitely. However, heparin is the choice for coating microhematocrit capillaries.

Although only 0.3 ml of whole blood are needed for analysis of microhematocrit, total non-cellular protein concentration, hemoglobin concentration, red blood cell counts, white blood cell counts, and electrophoretic analysis of the plasma protein, as much blood as possible was always obtained.

After the blood sample has been collected, the needle should be detached from the syringe. Samples for microhematocrit, hemoglobin concentration, and both erythrocyte and leucocyte counts are taken from the syringe. Smears for visual inspection of the blood also may be taken at this time. The remaining blood is gently expelled into a narrow centrifuge tube for subsequent separation of the cellular and non-cellular protein by centrifuging. Both the microhematocrit tube and the centrifuge tube should be centrifuged as soon as possible since whole blood tends to interact with the glass and results in some hemolysis and/or clotting. However, it is not necessary to read the hematocrit tube or separate the supernate immediately. Coating the glass centrifuge tube with neutral paraffin or use of plastic centrifuge tubes will eliminate this problem.

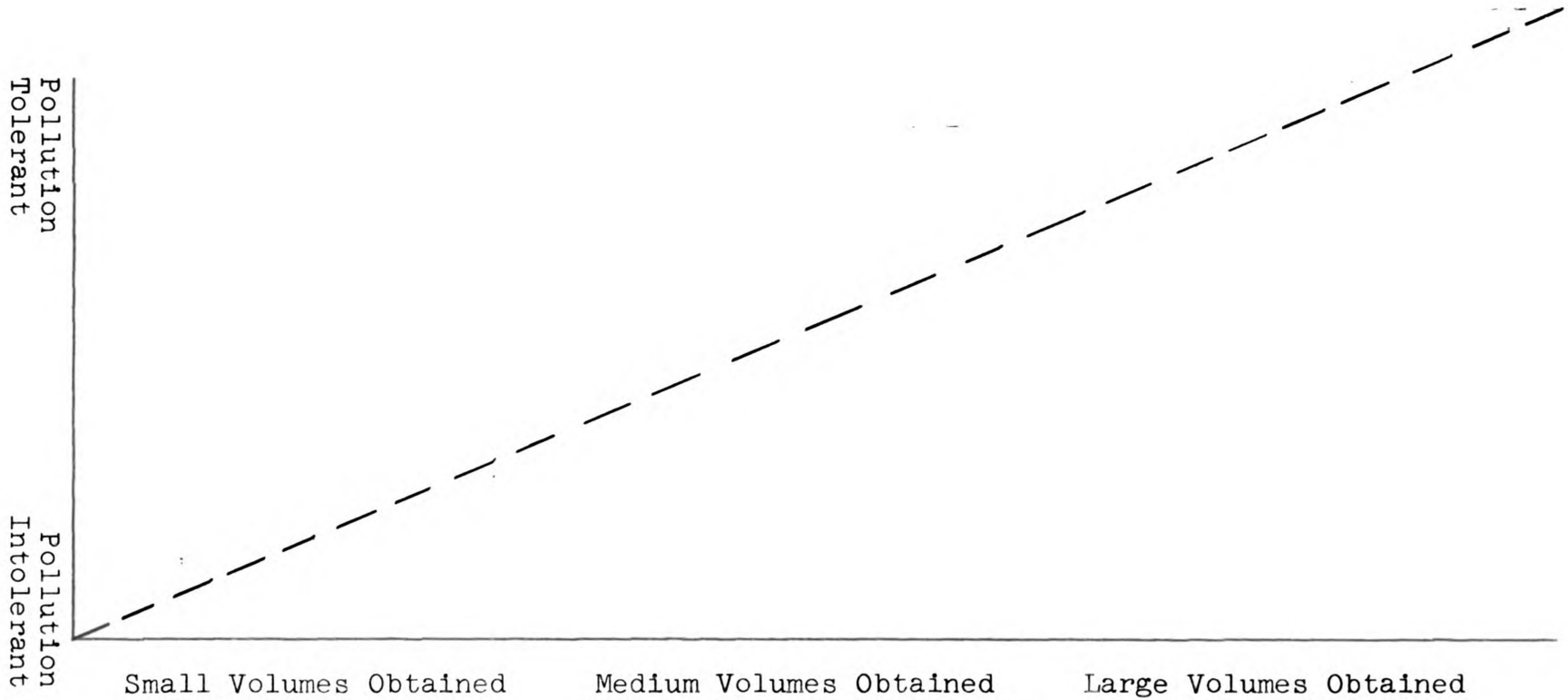
Adjustment of the total non-cellular protein concentration of invertebrate blood appears to be necessary for some species. Reduction of protein concentration to the range of 3-5 g/100 ml can be accomplished after the total non-cellular protein concentration has been determined. This adjustment assures linearity between protein concentration and the dye uptake.

Although no attempt was made to evaluate the differences which may exist between fish anesthetized with 1:60,000 tricane methyl sulfonate (MS 222) and those not so anesthetized, there is no evidence that significant differences exist. All fish used in these experiments were so anesthetized.

On the basis of taking blood samples from many species, two relationships seem apparent: (1) larger fish yield larger quantities of blood by cardiac puncture; (2) if species differences alone are considered and size differences are relatively constant, one can form a rank-order of apparent, relative blood volume based on the ease and quantity of blood obtainable. This is depicted in Figure 3.

This rank-order is based on subjective, empirical observations and may not be related to total blood volume. Peripheral dialation, changes in blood pressure, and other systemic factors could strongly alter the quantity of blood obtainable. Such influences are presumed to have been at a minimum since the specimens were anesthetized at the time of blood sampling.

Figure 3. Relationship of empirical pollution tolerance to apparent blood volume.



Small Volumes Obtained With Difficulty	Medium Volumes Obtained With or Without Difficulty	Large Volumes Obtained Without Difficulty
Largemouth Bass	Northern Pike	Bowfin
Smallmouth Bass	Green Sunfish	Chubsucker
Black Crappie	Common Sunfish	Goldfish
Bluegill	Yellow Perch	Channel Catfish
Grass Pickrel		Brown Bullhead
		Black Bullhead
		Yellow Bullhead

It readily can be seen that the "pollution tolerant" species yield the largest blood sample volumes. For example, bullheads of size 15-20 cm consistently yield samples of at least 1.0 ml of blood. By contrast, largemouth and smallmouth bass of size 20-30 cm yield only 0.5 ml of blood, or less. Shell (1962) reported a similar observation for smallmouth bass.

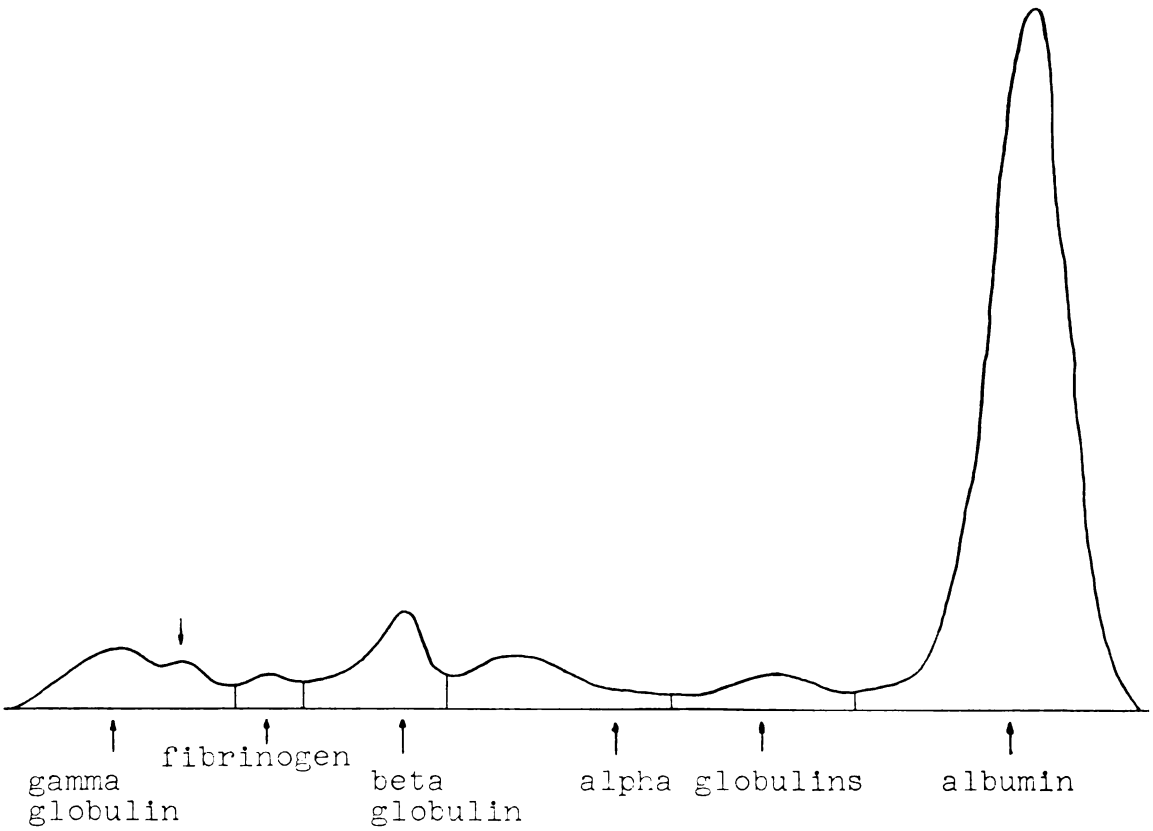
If this relationship is indicative of blood volume, then bullheads would have a much larger antibody pool than the largemouth bass. Prosser and Brown (op. cit.) point out that smaller blood volumes are more efficient in transporting oxygen, et cetera, but that a larger blood volume would be useful if it also served as a source of antibodies and phagocytic cells.

ELECTROPHORETIC PATTERNS OF FISH AND INVERTEBRATES

A survey of the electrophoretic patterns of twenty-two species of freshwater fish and two species of invertebrates was conducted. Specimens used for these determinations are presumed to be representative of the species. In most cases, the specimens were taken from their natural environment and acclimated to the laboratory for several days. Some specimens were sacrificed at the collection site to obtain blood. The electrophoretic patterns were determined using the previously listed conditions for paper electrophoresis. These patterns were then traced and are shown in Figures 4 through 29.

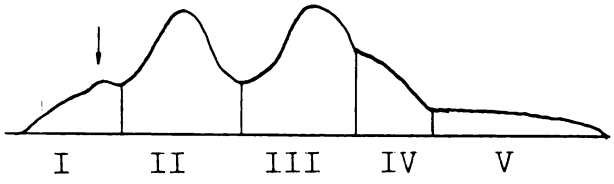
While comparing the following electrophoretic patterns, no attempt should be made to compare the various curves on the basis of curve height alone. The area of a given curve is indicative of the relative quantity of protein therein. Therefore, it is permissible to compare the areas of curves (fractions) on a single electrophoretic pattern, but it is not permissible to compare curve areas on different electrophoretic patterns. After curve areas have been converted to relative values (per cent of total protein) or absolute values (grams per 100 ml), they are readily comparable. Also, there is no evidence that proteins of similar electro-chemical characteristics have similar physiological or biochemical characteristics.

Figure 4. Electrophoretic pattern of human plasma proteins. (For comparison to fish plasma and/or serum proteins.)



↓ Denotes the application point of the sample on the paper strip. Electrical field is positive to the right and negative to the left of this point.

Figure 5. Electrophoretic pattern of the Bowfin (Amia calva).



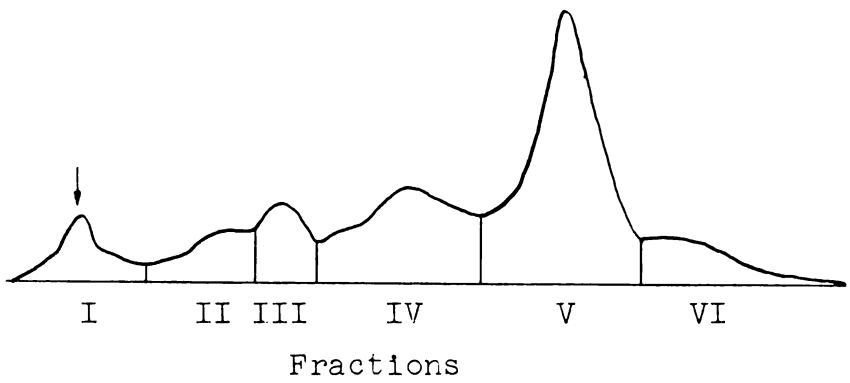
Per Cent of Total Serum Protein (three specimens)

I	II	III	IV	V
11.6%	32.5%	30.2%	16.2%	9.3%[1]
11.1%	31.4%	31.4%	18.5%	7.4%[1]
14.4%	29.1%	35.5%	20.0%	6.0%[2]

[1]These fish were acclimated to the laboratory.

[2]This fish was collected and sampled during winter-kill conditions.

Figure 6. Electrophoretic pattern of the plasma proteins of the Rainbow Trout (Salmo gairdnerii gairdnerii).

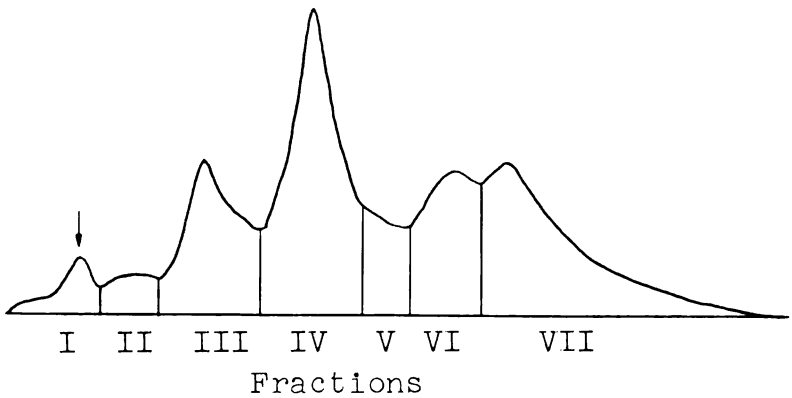


Per Cent of Total Plasma Protein (five specimens)[1]

I	II	III	IV	V	VI
6.5%	15.7%	10.5%	14.4%	40.7%	11.8%
4.3%	11.5%	9.3%	20.1%	46.0%	8.6%
5.0%	13.0%	11.0%	18.0%	40.0%	13.0%
6.1%	14.2%	12.2%	19.3%	38.7%	9.1%
5.1%	11.0%	13.2%	18.3%	41.1%	11.0%

[1]These specimens were acclimated to the laboratory.

Figure 7. Electrophoretic pattern of the plasma proteins of the Kamloops Trout (Salmo gairdnerii kamloops).

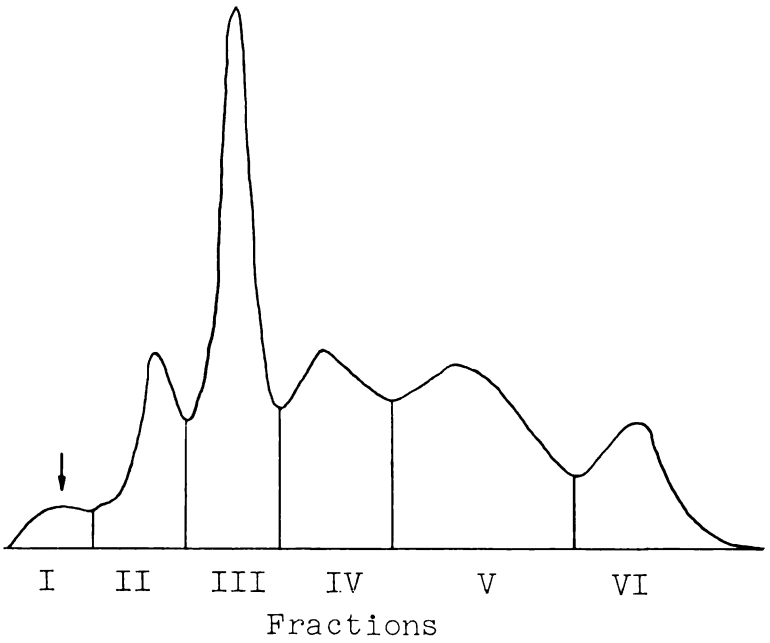


Per Cent of Total Plasma Protein (one specimen)[1]

I	II	III	IV	V	VI	VII
2.8%	3.8%	16.1%	34.2%	18.0%	20.9%	3.8%

[1]This specimen was acclimated to laboratory conditions.

Figure 8. Electrophoretic pattern of the serum proteins of the Brown Trout (Salmo trutta).

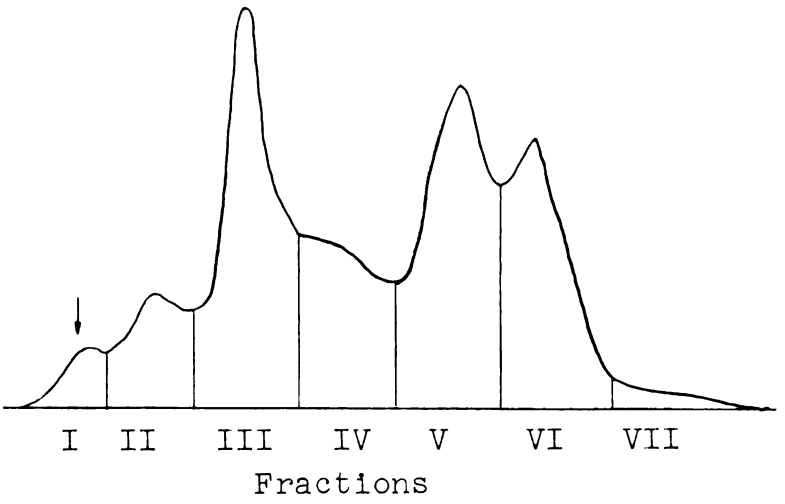


Per Cent of Total Serum Protein (four specimens)[1]

I	II	III	IV	V	VI
4.1%	9.4%	29.5%	17.7%	27.2%	11.8%
4.3%	13.6%	34.7%	19.8%	18.6%	8.6%
2.9%	13.7%	29.9%	18.6%	26.9%	7.7%
3.1%	11.1%	24.4%	19.6%	29.7%	11.7%

[1]These specimens were collected and sampled at the side of their hatchery raceway.

Figure 9. Electrophoretic pattern of the serum proteins of the Brook Trout (Salvelinus fontinalis).

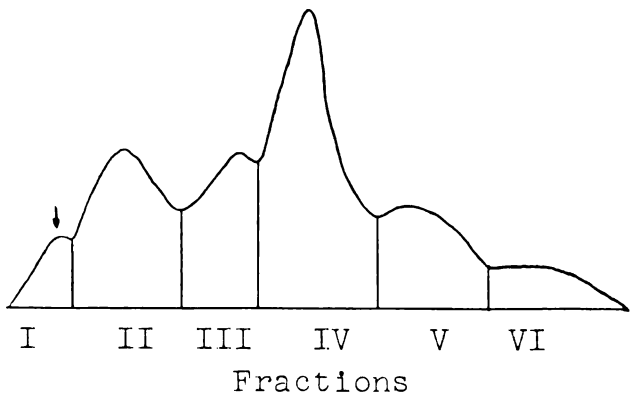


Per Cent of Total Serum Proteins (four specimens)[1]

I	II	III	IV	V	VI	VII
6.5%	13.0%	25.2%	15.2%	20.2%	16.6%	2.8%
5.2%	9.7%	28.4%	16.5%	20.3%	15.7%	3.7%
3.7%	8.3%	28.7%	13.6%	21.2%	21.2%	3.0%
3.5%	7.7%	28.9%	11.9%	25.1%	19.1%	2.9%

[1]These specimens were collected and sampled at the side of their hatchery raceway.

Figure 10. Electrophoretic pattern of the serum proteins of the Western Grass Pickrel (Esox americanus vermiculatus).

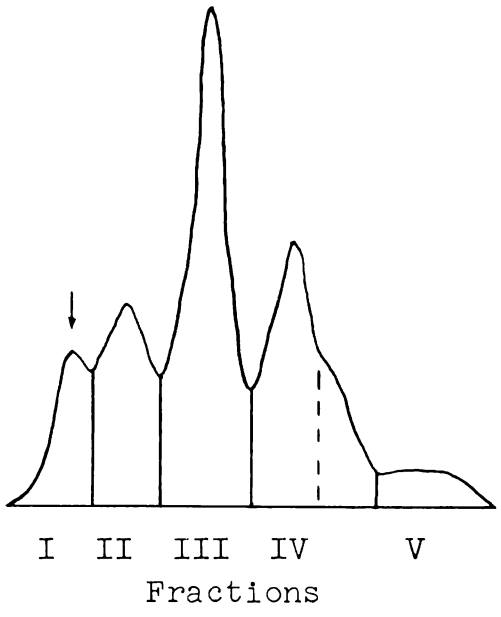


Per Cent of Total Serum Proteins (five specimens)

I	II	III	IV	V	VI
4.4%	23.0%	15.0%	35.3%	15.0%	7.0%[1]
2.0%	20.0%	17.0%	39.0%	15.0%	7.0%[1]
1.9%	23.1%	26.4%	29.8%	13.9%	4.6%[1]
4.1%	20.8%	12.5%	43.3%	13.3%	5.8%
2.7%	15.4%	15.4%	45.4%	7.2%	4.5

[1]These fish were acclimated to the laboratory.

Figure 11. Electrophoretic pattern of the Northern Pike (Esox lucius).

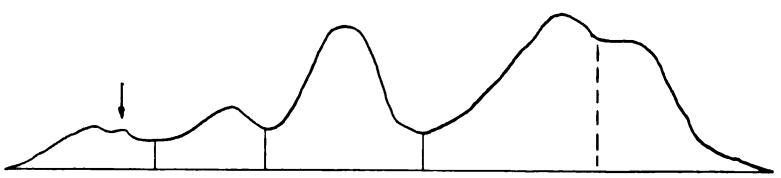


Per Cent of Total Serum Protein (six specimens)[1]

I	II	III	IV	V	
6.4%	17.6%	38.2%	31.7%	5.8%	(winter)
7.6%	25.1%	38.4%	24.4%	4.1%	(winter)
8.7%	16.2%	12.5%	8.7%	53.7%	(summer)
7.5%	18.9%	42.4%	26.5%	4.5%	(spring)
6.1%	18.4%	39.2%	30.0%	6.1%	(spring)
8.7%	17.5%	38.5%	29.8%	5.2%	(spring)

[1]All specimens were sampled at the collection site.

Figure 12. Electrophoretic pattern of Goldfish serum (Carassius auratus).

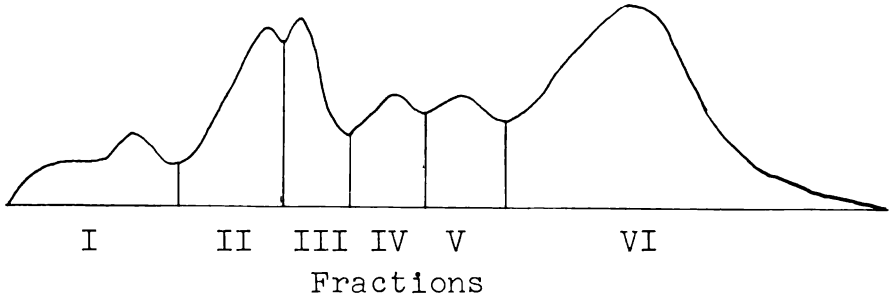


Per Cent of Total Serum Protein (nine specimens)[1]

I	II	III	IV
18.6%	16.6%	10.2%	54.4%
8.5%	8.5%	25.6%	57.3%
9.4%	9.4%	24.3%	56.0%
7.0%	5.0%	25.0%	62.0%
6.7%	8.3%	16.8%	68.0%
7.0%	9.5%	25.0%	58.0%
12.0%	7.3%	18.3%	62.0%
12.0%	10.3%	14.4%	65.0%
9.8%	8.8%	18.8%	63.0%

[1]These specimens were acclimated to the laboratory.

Figure 13. Electrophoretic pattern of the plasma proteins of the Carp (Cyprinus carpio).

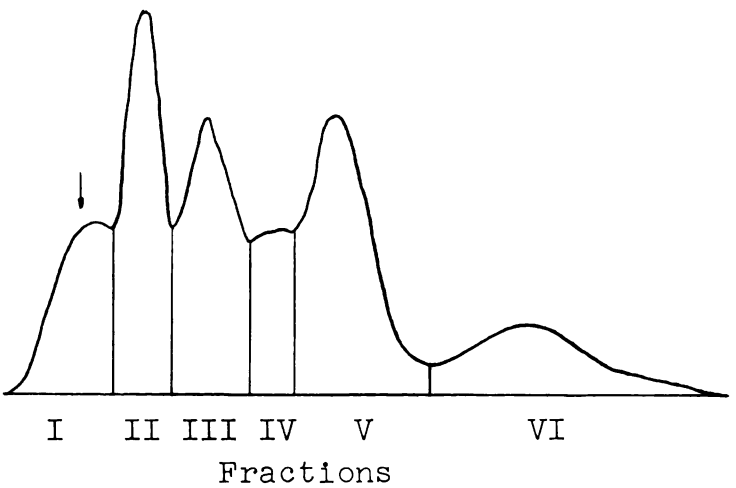


Per Cent of the Total Plasma Protein (two specimens)[1]

I	II	III	IV	V	VI
16.2%	12.6%	10.2%	13.2%	10.1%	37.6%
24.2%	14.6%	12.1%	9.7%	9.7%	29.1%

[1]These specimens were acclimated to the laboratory.

Figure 14. Electrophoretic pattern of the serum proteins of the Lake Chubsucker (Erimyzon sucetta).

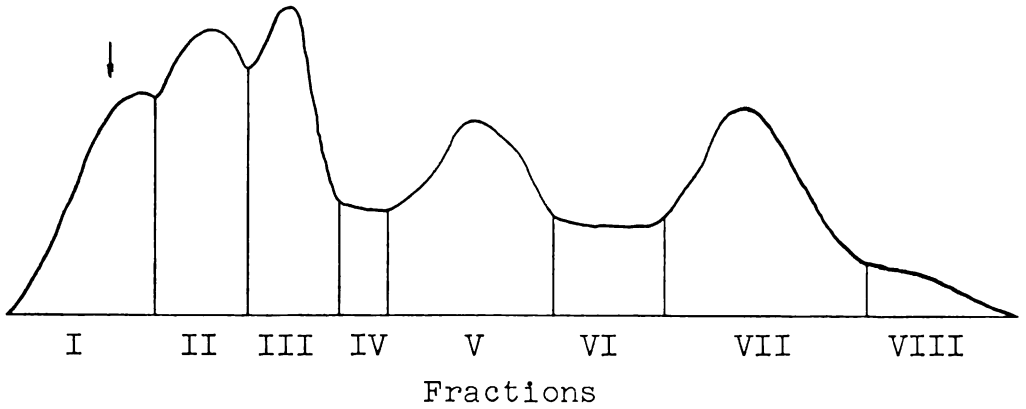


Per Cent of Total Serum Protein (one specimen)[1]

I	II	III	IV	V	VI
12.2%	23.0%	16.0%	10.8%	25.0%	12.8%

[1]This specimen was collected and sampled during winter-kill conditions.

Figure 15. Electrophoretic pattern of the plasma proteins of the Northern Hog Sucker (Hypentelium nigricans).

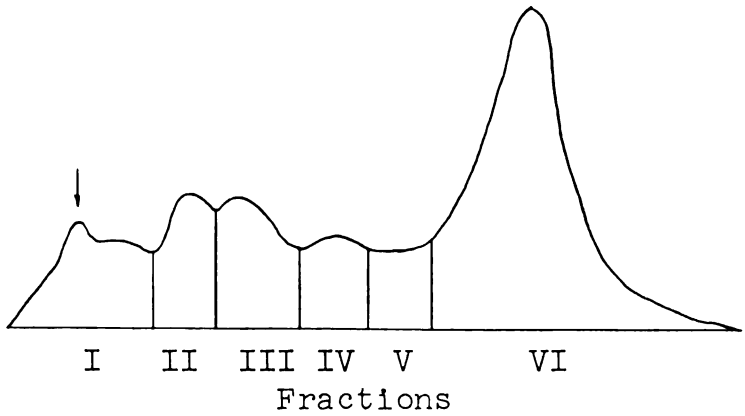


Per Cent of Total Plasma Proteins (two specimens)[1]

I	II	III	IV	V	VI	VII	VIII
13.2%	12.4%	18.6%	8.2%	18.6%	7.4%	18.2%	3.3%
12.9%	17.9%	14.6%	5.4%	17.1%	6.6%	20.9%	4.1%

[1]These specimens were collected by electro-shocking and blood was collected at the sample site, June, 1962.

Figure 16. Electrophoretic pattern of the serum proteins of the Black Bullhead (Ictalurus melas).

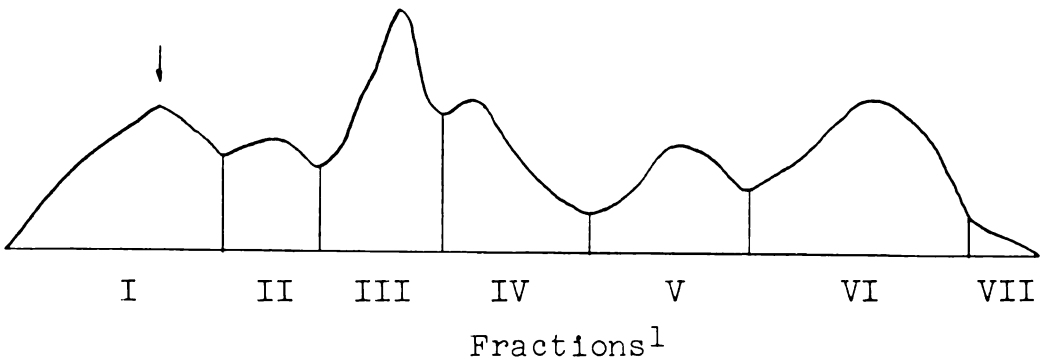


Per Cent of Total Serum Protein (two specimens)[1]

I	II	III	IV	V	VI
13.9%	10.8%	10.8%	8.5%	9.1%	56.8%
6.7%	5.7%	7.6%	7.6%	4.8%	67.3%

[1]These specimens were acclimated to the laboratory.

Figure 17. Electrophoretic serum pattern of the Yellow Bullhead (Ictalurus natalus).

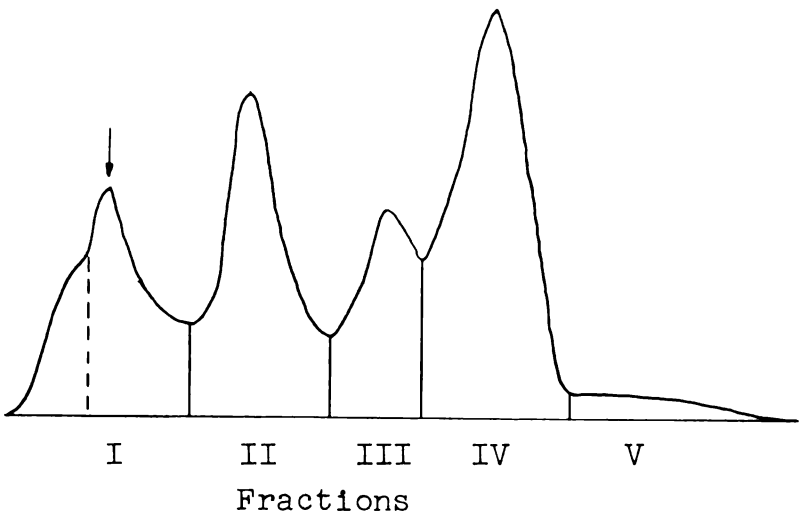


Means of Data Obtained from Seven Specimens[1]

I	II	III + IV	V	VI	VII
18.29%	9.10%	28.91%	14.92%	25.67%	2.74%

[1]These data obtained from the control fish (Appendix I).

Figure 18. Electrophoretic pattern of the serum proteins of the Brown Bullhead (Ictalurus nebulosus).

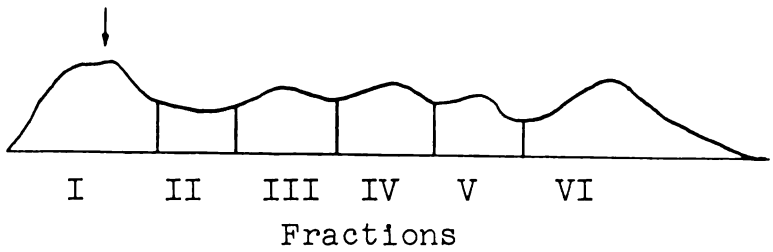


Per Cent of Total Serum Proteins (one specimen)[1]

I	II	III	IV	V
21.5%	24.4%	14.7%	38.6%	4.5%

[1]This specimen was collected and sampled during winter-kill conditions.

Figure 19. Electrophoretic pattern of Channel Catfish serum (Ictaluris punctatus).

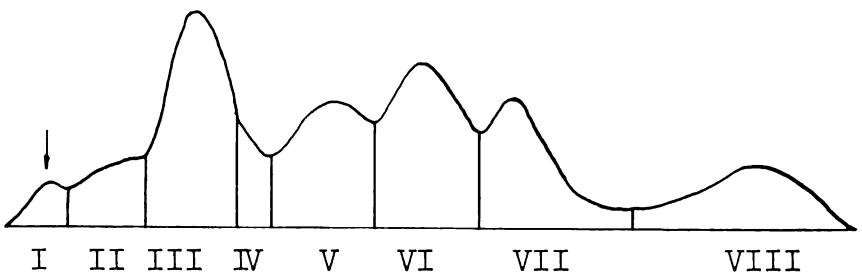


Per Cent of Total Serum Protein (one specimen)[1]

I	II	III	IV	V	VI
14.5%	11.2%	9.6%	12.9%	21.2%	30.1%

[1]This specimen was acclimated to the laboratory.

Figure 20. Electrophoretic pattern of the plasma proteins of the Rock Bass (Ambloplites rupestris).



Per Cent of Total Plasma Protein (six specimen)[1]

I	II	III	IV	V	VI	VII	VIII
2.4%	5.8%	14.9%	5.4%	18.4%	23.3%	16.4%	12.9%
1.2%	11.0%	14.7%	6.1%	11.0%	29.4%	15.9%	10.4%
1.5%	6.6%	14.5%	5.1%	16.5%	26.9%	17.0%	11.3%
2.2%	6.0%	16.2%	6.1%	26.2%	24.5%	12.2%	6.1%
4.2%	9.0%	16.9%	7.2%	15.1%	21.8%	17.5%	7.8%
2.7%	9.0%	20.8%	5.8%	14.9%	22.6%	17.1%	6.7%

[1]These specimens were collected by electro-shocking and their blood was drawn at the collection site with the use of heparin. Collection was made June 27, 1962.

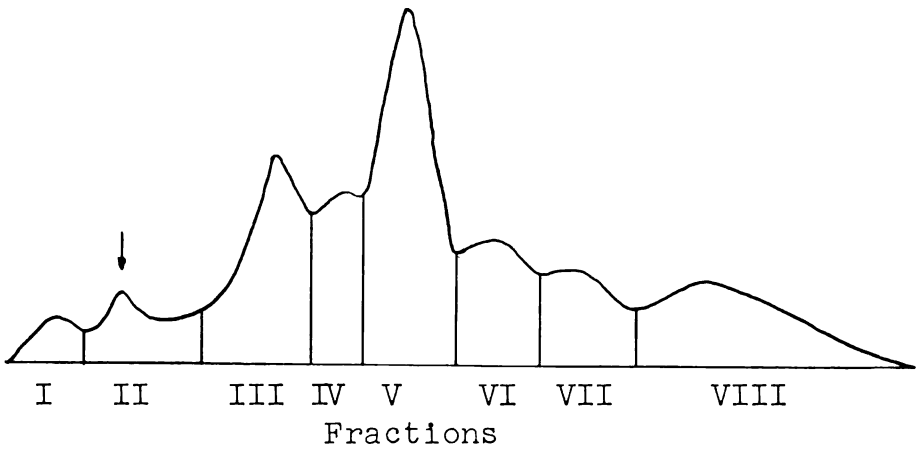
Figure 21. Electrophoretic pattern of the serum proteins of the Green Sunfish (Lepomis cyanellus).

Per Cent of Total Serum Protein (two specimens)[1]

I	II	III	IV	V	VI
24.0%	14.8%	20.3%	18.5%	11.1%	11.1%
11.8%	14.0%	7.4%	29.6%	22.2%	14.8%

[1]These specimens were collected during winter-kill conditions, and may not be typical of the species due to this stress.

Figure 22. Electrophoretic serum pattern of the Pumpkinseed (Lepomis gibbosus).

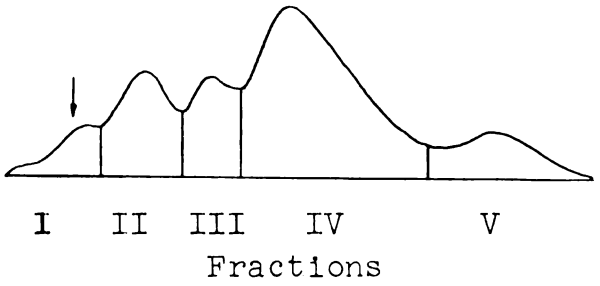


Per Cent of Total Serum Protein (one specimen)[1]

I	II	III	IV	V	VI	VII	VIII
2.6%	5.3%	17.4%	10.7%	26.8%	12.2%	8.7%	16.1%

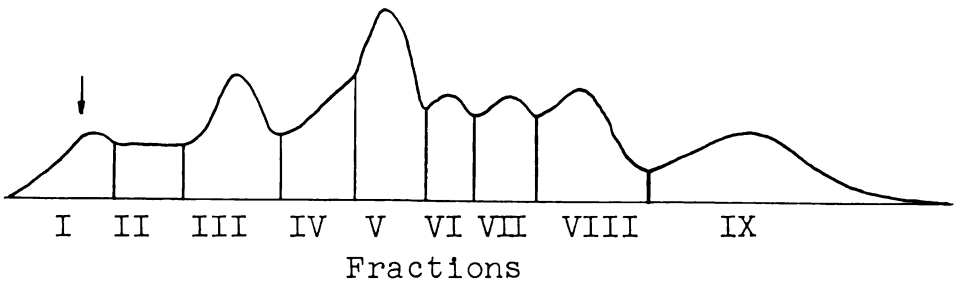
[1]This specimen was acclimated to the winter conditions in a lake and sampled there.

Figure 23. Electrophoretic pattern of the serum proteins of the Bluegill (Lepomis macrochirus).



For the per cent of total serum proteins see Appendix I, Table 4, control specimens.

Figure 24. Electrophoretic pattern of the serum proteins of the Smallmouth Bass (Micropterus dolomieui).

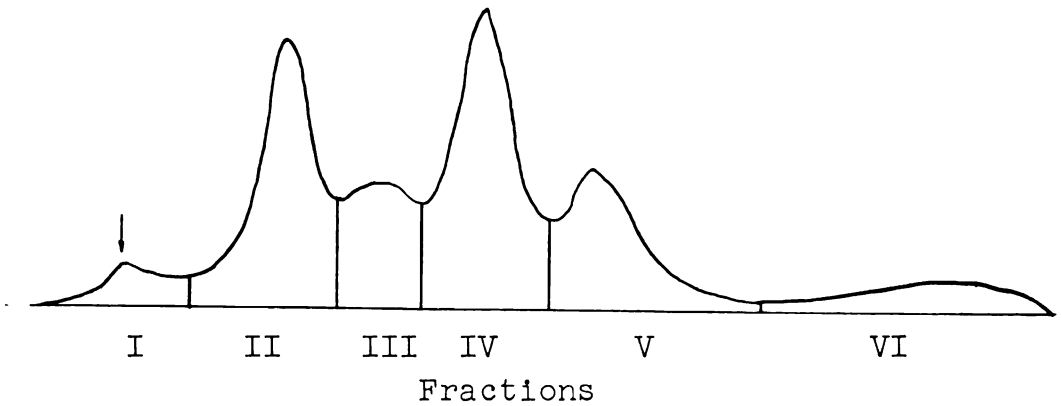


Per Cent of Total Serum Protein (three specimens)[1]

I	II	III	IV	V	VI	VII	VIII	IX
1.9%	10.7%	13.7%	9.8%	16.6%	7.8%	10.7%	11.7%	16.6%
1.8%	12.8%	13.7%	8.2%	16.5%	9.1%	9.1%	11.9%	16.5%
4.5%	10.0%	14.5%	8.2%	15.5%	4.5%	9.0%	12.7%	16.3%

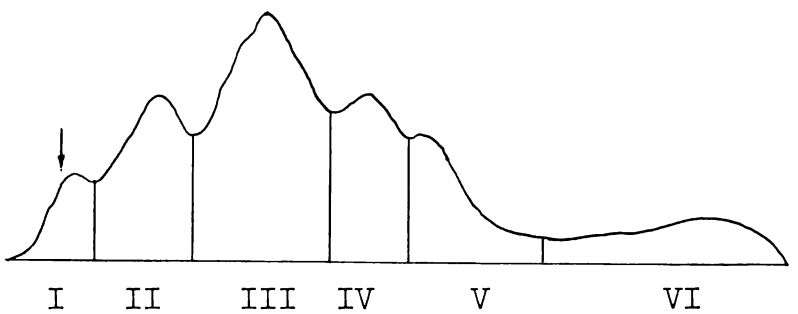
[1]These specimens were acclimated to the laboratory.

Figure 25. Electrophoretic pattern of the serum proteins of the Largemouth Bass (Micropterus salmoides).



Per cent of total serum proteins see control specimens Appendix I.

Figure 26. Electrophoretic pattern of the serum proteins of the Black Crappie (Pomoxis nigromaculatus).

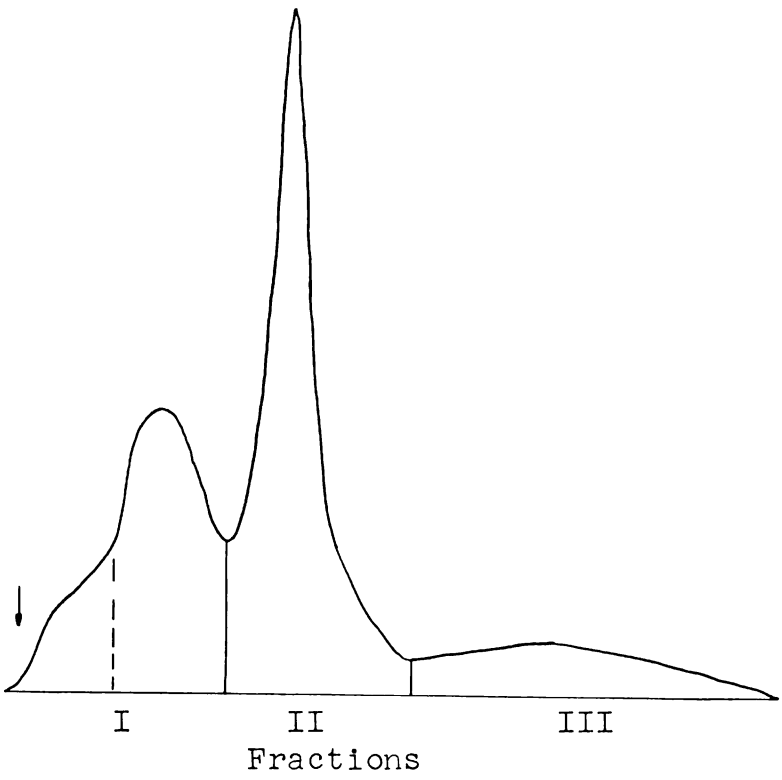


Per Cent of Total Serum Protein (two specimens)[1]

I	II	III	IV	V	VI
5.4%	19.0%	25.0%	14.5%	13.6%	10.9%
6.4%	18.4%	35.2%	16.0%	12.8%	11.2%

[1]These specimens were acclimated to the laboratory.

Figure 27. Electrophoretic pattern of the plasma proteins of the Yellow Perch (Perca flavescens).

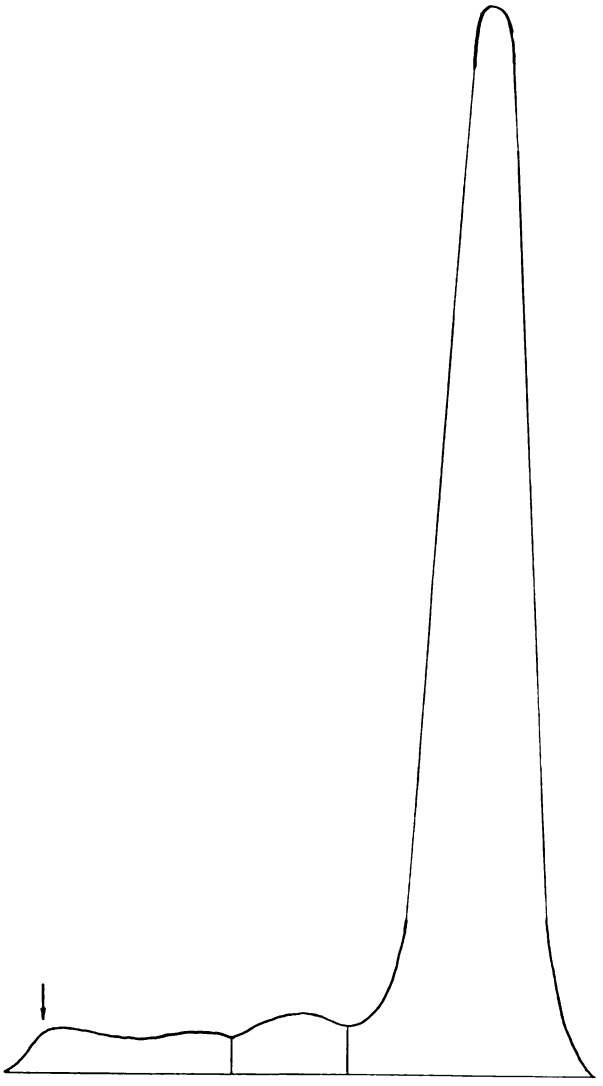


Per Cent of Total Plasma Protein (nine specimens)[1]

I	II	III
42.6%	48.2%	9.0%
35.5%	49.4%	15.0%
34.3%	54.6%	11.0%
40.7%	45.1%	14.2%
40.5%	48.4%	11.1%
41.9%	43.0%	15.1%
41.3%	40.7%	18.0%
40.9%	46.8%	12.4%
31.2%	54.7%	14.1%

[1]These specimens were acclimated to the laboratory.

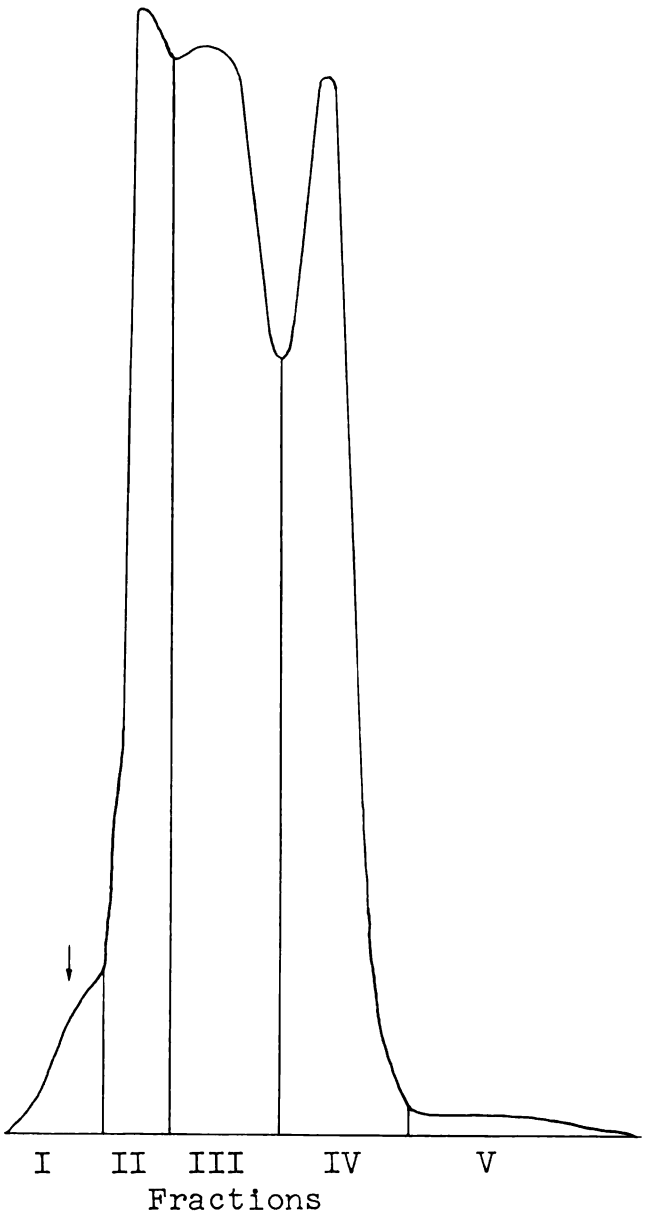
Figure 28. Electrophoretic pattern of the plasma proteins of the freshwater crayfish (Orconectes propinquus).



Per Cent of Total Plasma (one specimen)

I	II	III
9.3%	11.8%	78.8%

Figure 29. Electrophoretic pattern of plasma proteins of the dobson fly larvae (Corydalus cornutus).



Per Cent of Total Plasma (one specimen)[1]

I	II	III	IV	V
2.0%	36.0%	47.2%	14.3%	0.6%

[1]Acclimated to the laboratory.

The migration of each fraction is not a true function of time and because the electrophoretic separation conditions vary slightly from separation to separation, the position of each fraction for a single species will vary slightly from one electrophoretic migration to the next. Also, there is some variation from specimen to specimen. In spite of these variations, the number and position of the fractions allows species identification. Similar results were obtained by Lillevik and Schloemer (1961) who used the free proteins of skeletal muscle to show species differences.

An attempt was made to determine the morphological differences which distinguished rainbow trout (Salmo gairdnerii gairdnerii)from kamloops trout (Salmo gairdnerii kamloops), based on a taxonimic key by Schultz (1938). This failed to distinguish between specimens of kamloops trout purchased from a licensed fish breeder, from rainbow trout obtained from the Michigan Conservation Department hatchery at Wolf Lake.

The serum proteins of these specimens were analyzed electrophoretically by the methods previously listed. Tracings of the electrophoretic patterns of rainbow trout and kamloops trout are shown in Figures 6 and 7. It can readily be seen that their electrophoretic patterns differentiate these subspecies. In addition to having a different number of fractions, these fractions contained considerably different proportions of protein, glycoprotein, and lipoprotein.

The electrophoretic patterns of one species of immature aquatic insect and one species of crayfish were determined, and these appear to be species specific also. Van Sande and Karcher (1961) found species differences in the hemolymph protein of insects. Ball and Clark (1953) reported species differences in the amino acids of Culex mosquitoes.

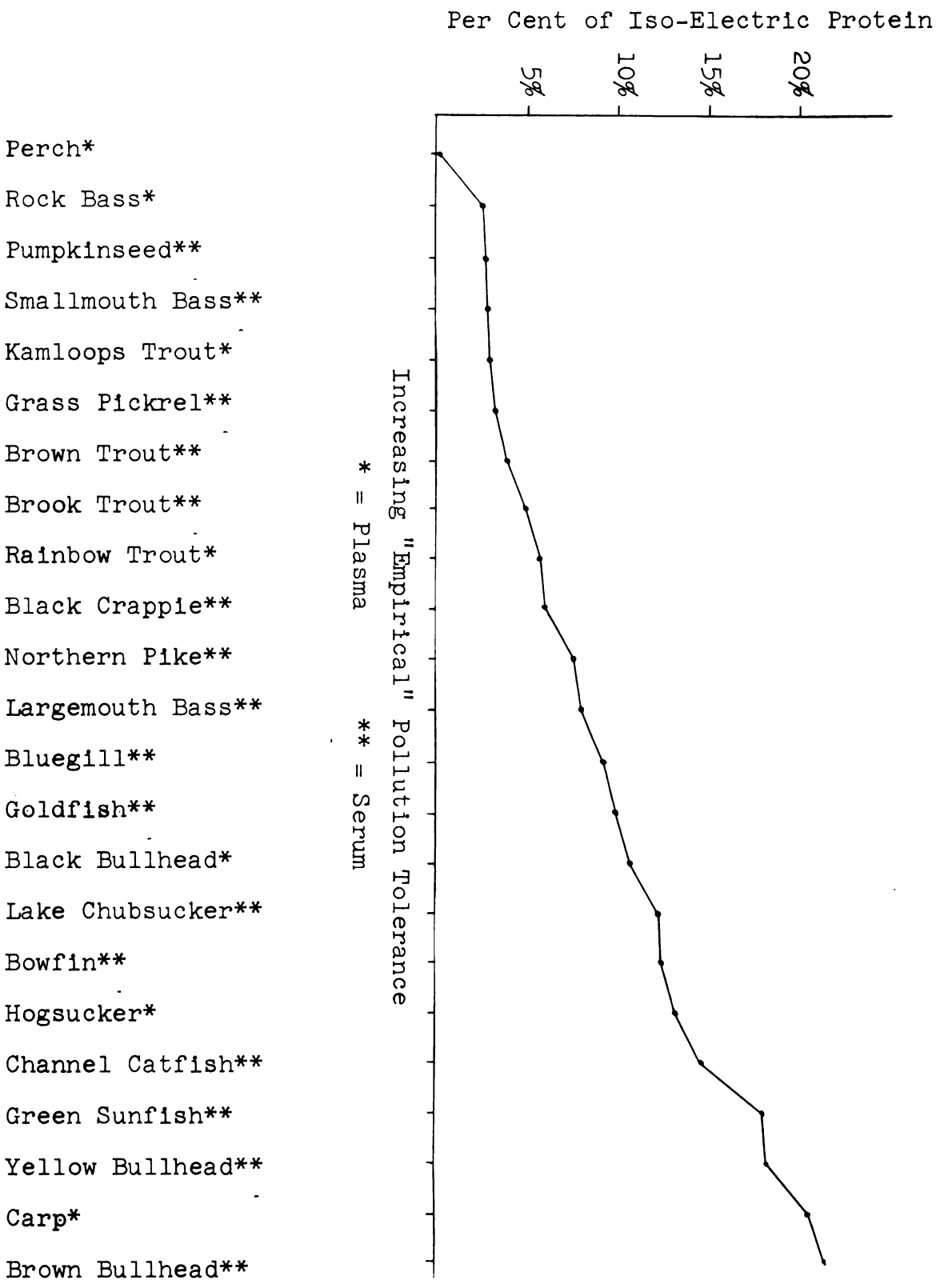
This procedure may be useful to aquatic entomologists in constructing taxonomic keys to the immature insect larvae, by associating the electrophoretic patterns of immatures with adults.

Protein fraction I when separated using the described conditions is relatively immobile due to its iso-electric character. In this respect it conforms to the description of human gamma-globulin known for its antibody power. The means of fraction I for the various species (derived from Figures 5 through 27) were arranged in rank-order. This rank-order is presented in Figure 30 and proceeds from lowest to highest quantities of fraction I.

When the species are compared by this rank-order, it can be seen that empirically-determined pollution-intolerant fish have low quantities of fraction I. By contrast, empirically-determined pollution-tolerant fish have large quantities of this fraction. Ictalurids contain large quantities of this fraction and are well known for their pollution tolerance. Centrarchids and salmonids usually have less than half of the quantity of fraction I normally present in bullhead serum.

Figure 30. Rank-order of fish species according to the mean per cent of iso-electric protein in their plasma and/or serum at pH 8.6 (vernol buffer).[1]

[1]Specimens not living in the same water.


Per Cent of Iso-Electric Protein
5%
10%
15%
20%
Perch*
Rock Bass*
Pumpkinseed**
Smallmouth Bass**
Kamloops Trout*
Grass Pickrel**
Brown Trout**
Brook Trout**
Rainbow Trout*
Black Crappie**
Northern Pike**
Largemouth Bass**
Bluegill**
Goldfish**
Black Bullhead*
Lake Chubsucker**
Bowfin**
Hogsucker*
Channel Catfish**
Green Sunfish**
Yellow Bullhead**
Carp*
Brown Bullhead**
Increasing "Empirical" Pollution Tolerance
* = Plasma
** = Serum

Yellow perch have no apparent iso-electric protein at pH 8.6 and may present an exception to this generality.

This relationship of the quantity of iso-electric protein to empirical pollution tolerance is based on small sample sizes. However, this relationship appears to occur with regularity too great to have occurred by chance alone. If this protein fraction is related to the "hardiness" of the species it may be due to the environment or to genetic differences. Stress tends to increase the quantities of the iso-electric protein in fish and the above listed relationship may be due to the stresses of the environment. Thus, bullheads could be comparable to other fish living in the same water conditions. A comparison of the common warm-water fish indicates that it is not the gross condition of the water which is responsible for high levels of this iso-electric protein. Fish of several species were held in clean tap-water for two months, with feeding. At the end of this period, they had not changed the relative quantity of this fraction.

This relationship of pollution tolerance to the quantity of iso-electric protein (in vernol buffer at pH 8.6) may be of value in classifying species of fish in regard to their relative hardiness. Several reasons support this hypothesis as follows: (1) this material is found in highest relative quantities in "pollution" tolerant species; (2) since "pollution" tolerant species appear to have larger blood volumes, and larger relative quantities of this fraction, the product would produce a larger absolute quantity of this fraction per

gram of body weight; (3) the abundance of this material appears to be related to the tolerance potential of the species; and (4) this material tends to increase during "acute stress" periods.

Although further information concerning the relationship of iso-electric protein plasma and/or serum to hardiness will be required, it appears possible to use this criterion as an aid in ranking hardiness in fish species. One possible use of this relationship would be in the selection of breeding stock. If one stock was found to exhibit higher quantities of this material than other stocks, providing this increase was not due to stress, then one could reason that this stock had a higher genetic-antibody-potential than the other stocks in question. By in-breeding fish which exhibit high quantities of iso-electric plasma and/or serum, one might produce hardier offspring. These offspring, due to their "genetic antibody-potential" might be more able to withstand the difficulties of hatchery or stream life.

Mertz (op. cit.) indicates that the optimum pH for enzyme activity is near its iso-electric pH. At the iso-electric pH the protein molecule exists in the di-pole (zwitter ion) form and retains its maximum number of reactive sites. This suggests that the blood of pollution tolerant fish contains a greater quantity of proteins capable of maximum activity at or near physiologic pH.

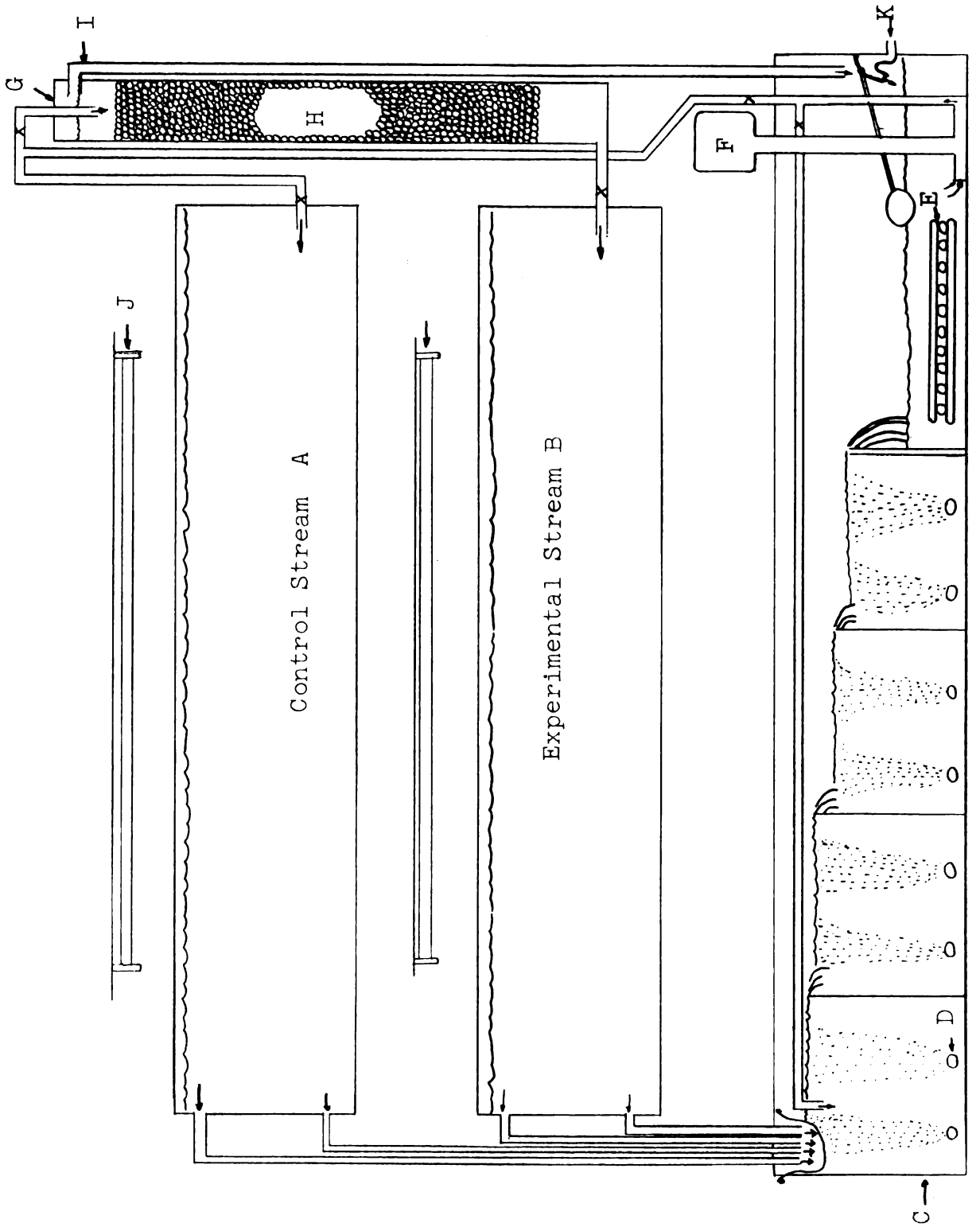
ARTIFICIAL STREAM SYSTEM

An artificial stream system was constructed which would allow the investigation of the influence of a diurnal oxygen pulse on the serum protein fractions of fish. This system was so constructed that only the oxygen content of the experimental section would be varied, and all other aspects of water quality would be approximately equal between the control and experimental section. This condition was achieved by utilizing a re-circulating water system which was both drained into and supplied from a single reservoir.

Figure 31 presents a schematic representation of this stream system. The control and experimental sections (A and B) were constructed of one-fourth inch thick acrylic plastic. Their measurements were: 30 cm deep, 60 cm wide, and 150 cm long. Inlets were subsurface and outlets were located at both surface and subsurface levels. In operation each stream contained approximately 225 liters of water.

Water drained by gravity flow from the artificial streams through a nylon organdy filter and into the regeneration reservoir (C). This reservoir was constructed of three-quarter inch marine plywood which was coated with plastic resin. Its dimensions are: 16 inches deep, 30 inches wide, and 96 inches long. Beginning with the entrance end, the reservoir contained four aeration compartments, which were

Figure 31. Re-circulating artificial stream system for controlling dissolved oxygen content, temperature, and photoperiod.


I
G
H
K
F
E
J
Control Stream A
Experimental Stream B
D
C

constructed to form water-falls. In each of these compartments, compressed air was forced through six air-stones (D). The result is much mixing and subsequent near saturation of the water with oxygen.

The fifth and final compartment contained a flat, block-tin refrigeration coil (E) and a bronze-stainless steel sump-pump (F). The continuous flow of water passes over the refrigeration coil and the sensitivity of the thermostat was such that the temperature of the water in both units remained within $\pm$ 1° C. of 20° C.

The flow of water from the pump (F) is divided between the control (A), the experimental (B) sections, and the regeneration reservoir (C). Before the water passes into the experimental section, it must pass through two oxygen-stripping columns (G), one of which is not shown. These columns were constructed of one-quarter inch thick, 4-inch inside-diameter acrylic plastic tubes, which measured four feet high. Each column was filled with unglazed ceramic raschig rings (H) to a height of approximately 42 inches. A constant head of pressure is maintained by overflow outlets (I).

During degassing, nitrogen gas is introduced into the bottom of these columns and allowed to bubble to the top. The rings help to control the size of the nitrogen bubbles and smaller rings produce smaller bubbles with proportionately greater surface area.

The removal of the oxygen from the water by nitrogen gas bubbles is effected by oxygen's greater solubility in nitrogen gas than its solubility in water. Brown (1957) points out that this process will not produce "nitrogen gas bubble disease" in fish.

Each section was illuminated by two 40 watt fluorescent tubes (J) which were suspended approximately four inches above the lids of the control and experimental sections. An automatic timer switch controlled these lights, turning them on at 6:00 P.M. and off at 6:00 A.M. Black polyethylene sheets were used to shield exterior light from the stream system. It was found that two 40 watt tubes were too bright and caused the fish to be rather excitable. To correct this, a single layer of brown wrapping paper was laid on top of the section lids.

The flow of water into each section was approximately 5 liters per minute. This flow was rapidly dissipated as the water entered the stream and the fish appeared to be in a resting condition at all times.

Temperature, pH, alkalinity, total carbonate alkalinity, and dissolved oxygen were determined for the control and experimental sections during non-degassing periods, and the mean of the differences between the streams was not found significant at the 20% level. The same results were obtained during degassing with the exception of the dissolved oxygen content.

A float valve inlet (K) connected to the reservoir was installed in the fifth compartment to resupply water lost by evaporation and that water used in chemical tests.

The Influence of a Diurnal Oxygen-Pulse on Bluegills

The bluegills used in this experiment were collected by seining pools of the Lake Lansing drain, Ingham County, Michigan during May, 1961. These fish were held in the laboratory in aquaria for three weeks, during which time they were fed commercial fish-food pellets. Twenty-four specimens were placed in the artificial stream by non-random assignment in the following manner: pairs of nearly equal size fish were formed, one of each pair being placed in either the control or the experimental section. Acclimation continued for another two weeks before degassing began. Feeding continued throughout the experiment.

Degassing consisted of reducing the oxygen content of the experimental section from an average of 8.3 ppm to an average of 3.5 ppm, eight hours per day, for nine days. Water samples were collected non-randomly during the non-degassing and degassing periods to provide a check on water quality. The means of the chemical data determined during degassing periods are listed in Table 3.

On the tenth day specimens were sacrificed and blood samples were collected by indirect cardiac-puncture. Although citrate was used to coat syringes and capillary tubes, it failed to prevent the rapid coagulation of the

blood taken from the experimental specimens. The collection of serum then became the primary objective.

TABLE 3. Summary of the water chemistry during the degassing periods for bluegills.

	Temp. °C.	Dissolved Oxygen ppm	pH	Total Alkalinity ppm
		Control		
Mean	20.25	8.3	8.56	203
Range	19.0-21.0	8.0-8.6	8.4-8.8	192 - 216
		Experimental		
Mean	20.25	3.5	8.52	201
Range	19.0-21.0	2.5-4.1	8.4-8.7	188 - 210

The samples were allowed to clot at room temperature for four hours. During this time, much of the serum was expressed from the clots before centrifuging. Serum samples were then collected and stored in individual three-inch glass test-tubes. A 10 microliter portion of each serum sample was electrophoretically separated using commercial vernol buffer and a fourteen hour separation period. The unused serum was stored at -5° C. until other portions could be separated and analyzed for glycoproteins. This storage precluded lipoprotein analysis.

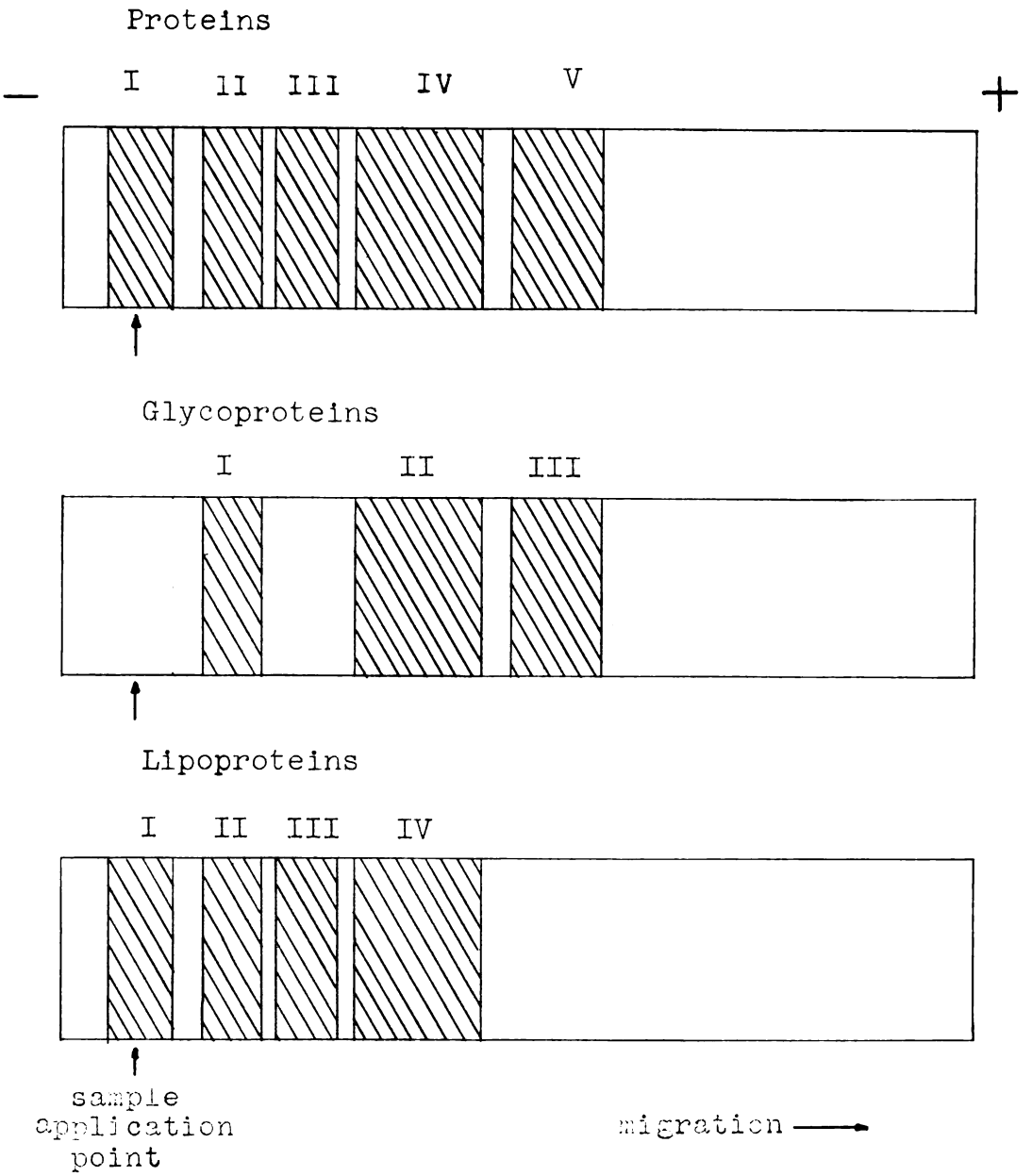
Five clearly distinguishable serum protein fractions were consistently separated. A sixth fraction was obtained

occasionally and consisted of separating fraction II into two separate fractions. This difficulty may have been due to genetic differences, but it is believed that the commercially prepared buffer powder was responsible for the poor separation. This buffer is prepared for the separation of human serum and such buffer is here considered to be totally unacceptable for the separation of fish serum.

The positional relationships of the protein fractions, glycoprotein fractions and lipoprotein fractions of this species is depicted in Figure 32. The width of the fractions is not necessarily indicative of the amount of material contained therein. Rather, this illustrates the location of proteins which are conjugated to polysaccharides (glycoprotein) and/or lipids (lipoprotein). Three glycoprotein fractions and four lipoprotein fractions were separated. It should be noted that protein fraction I contained lipoprotein and did not appear to contain glycoprotein. Protein fraction II contained both glycoprotein and lipoprotein, as did protein fraction III and IV. Protein fraction V contained glycoprotein but did not appear to contain lipoprotein.

The above results are important since they clearly demonstrate fundamental differences between bluegill serum and mammalian serum. Weil (op. cit.) points out that in human serum the majority of the lipoproteins are carried in the beta fraction and that the glycoproteins are contained mainly in the alpha fraction.

Figure 32. Positional relationship of proteins to glycoproteins and lipoproteins in the serum of the bluegill (Lepomis macrochirus).



Since male specimens predominated both the experimental and control groups, the serum collected from females was not statistically analyzed. Therefore, differences due to sexual dimorphism were not ascertained. Samples collected from bluegills during the winter of 1961-1962 indicate that such a condition exists.

The statistical analyses of the influence of this diurnal oxygen-pluse onthe distribution of serum proteins in the control and experimental bluegill specimens are shown in Table 4.

Protein fraction I most closely resembles gamma globulin in its electro-chemical characteristics. In general, the specimens of the experimental lot appeared to increase the relative quantity of this fraction. This increase was not statistically significant, but it is believed that a larger sample would have demonstrated statistical significance.

The mean of protein fraction II was also higher in the experimental specimens, but like fraction I this was not statistically significant. The variance of fraction II in the experimental fish was significantly different at the 95% level from the variance of the controls.

The mean of fraction III in the control lot was significantly greater at the 95% level than the mean of fraction III in the experimental lot. The variance of this fraction was also significantly greater at the 95% level in the control lot than in the experimental lot.

TABLE 4. Summary of the influence of a diurnal oxygen-pluse on the distribution of proteins in the serum of male bluegills.[1]

	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V
			Control		
Mean	9.14%	32.64%	24.60%*	25.68%	7.86%
Range	7.3-11.8%	30.4-35.5%	19.7-30.1%	20.5-29.0%	7.2-8.9%
Standard Error of the Mean	1.147%	1.376%	2.587%	2.278%	0.453%
Coefficient of Variation	18.8	6.3	15.7	12.9	8.6
Variance	2.963%	4.26%	15.055%	11.050%	0.462%
			Experimental		
Mean	9.94%	33.32%	21.06%	27.10%	8.56%
Range	8.5-11.9%	24.6-38.2%	18.9-22.9%	22.0-38.9%	7.1-9.3%
Standard Error of the Mean	0.872%	3.546%	1.155%	4.584%	0.580%
Coefficient of Variation	13.2	16.1	8.2	25.4	10.1
Variance	1.713%	28.277%*	3.00%*	47.285%	0.757%

[1]Data in Appendix I. *Significantly greater at the 95.0% level.

The mean of fraction IV showed a general increase in the experimental lot and a general decrease in fraction V in the experimental lot was noted. Variance between the control and experimental lot for these two fractions was not statistically significant.

Correlation analyses were performed to determine the relationship of these protein fractions to body length and weight. These correlation coefficients are listed in Table 5, and show the influence of low dissolved-oxygen.

Fractions I and II have a significant negative correlation at the 95% level to body weight in the control lot. Fractions I and II were not significantly correlated to body length. Other fractions of bluegill serum protein were not significantly correlated to either body weight or body length. In general, the highest correlation coefficients were found in the relationship of the fractions to body weight.

Previous mention has been made of the electro-chemical similarity between "gamma globulin" and bluegill serum protein fraction "I." In the control lot this fraction is negatively correlated with both body length and body weight. While a "larger" specimen would be expected to have lower quantities of this fraction, the larger ones of the experimental lot tended to have more of this fraction.

The stress from low-oxygen apparently reverses this relationship and the "size" of the specimen appears to have

some influence on the amount of fraction "I" produced. This may be related to the lower metabolic rate of the larger fish.

TABLE 5. Coefficients of correlation for the distribution of serum proteins to total body length and body weight of bluegills.

	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V
			Weight		
Control	-.90%*	-.87*	+0.59	+0.40	-0.44
Experimental	+0.14	-0.38	-0.35	+0.03	-0.63
			Total Length		
Control	-0.79	-0.79	+0.49	+0.08	-0.44
Experimental	+0.19	+0.56	-0.10	+0.08	-0.26

*Significant at the 95% level.

It should be noted that the correlation coefficients of the control lot were generally higher than those of the experimental lot. The latter were lower than those of the control lot in nine of the ten analyses. It also should be noted that a general correlation sign difference exists between the control and experimental lots. However, in cases where this correlation sign-difference was absent, the correlation coefficient of the experimental lot was usually much lower than that of the control lot. Therefore, not only a reduced correlation, but also an opposite relationship contrasted the control from the experimental lot. As such, this

suggests both quantitative and qualitative changes in serum protein fractions of bluegills subjected to a low dissolved-oxygen pulse.

Three fractions of glycoprotein were separated in the serum of bluegills. In general, glycoprotein fraction I (Figure 32) was contained in protein fraction II, glycoprotein fraction II was contained in protein fraction IV, and glycoprotein fraction III was contained in protein fraction V.

The distribution of glycoprotein in the serum fractions of the control and experimental lot was tested statistically, and the results are summarized in Table 6. The mean of fraction I was 10.8% in the control lot and 10.3% in the experimental lot. The mean of fraction II was 26.2% in the control lot and 35.0% in the experimental lot. The difference between the means of the control and experimental group of fraction II was statistically different at the 95% level. The mean of fraction III was 70.8% in the control lot and 54.8% in the experimental lot. The difference between these means was also statistically significant at the 95% level.

As previously noted, the protein in the glycoprotein fractions were not significantly different between the control and experimental lots. Thus the glycorprotein content of these fractions had changed in the experimental lot, without significantly altering the levels of the protein. To demonstrate this further, Pearsonian correlation analyses were performed on the protein to glycoprotein levels in each

TABLE 6. Summary of the influence of a diurnal oxygen-pulse on the distribution of glycoproteins in the serum of male bluegills.

Item	Control			Experimental		
	Fraction I	Fraction II	Fraction III	Fraction I	Fraction II	Fraction III
Mean	10.8%	26.2%	70.3%*	10.3%	35.0%**	54.8%
Range	6.0-14.0%	18.0-35.0%	53.0-91%	8-16%	21-50%	41-63%
Standard Error of the Mean	2.181%	4.545%	9.244%	2.521%	7.117%	5.447%
Coefficient of Variation	30.2	26.0	19.4	35.6	30.5	15.4
Variance	10.7%	46.7%	192.3%	14.3%	114.0%	69.3%

*Significantly greater at the 95% level.

**Significantly greater at the 90% level.

fraction. These data are summarized in Table 7. From these data it can be seen that in the experimental lot, the levels of glycoprotein were strongly related to the levels of protein. In the control lot, the levels of glycoprotein were not strongly related to the protein levels. These data further demonstrate the qualitative and quantitative changes induced by low dissolved-oxygen on bluegill serum proteins.

TABLE 7. Influence of a diurnal low oxygen-pulse on the relationship of glycoprotein to protein in male bluegill serum.

Item	Correlation Coefficients	
	Control	Experimental
Glycoprotein "I" to Protein "I"	+0.18	+0.74
Glyco protein "II" to Protein "II"	+0.18	+0.90*
Glycoprotein "III" to Protein "III + IV"[1]	-0.43	+0.77

*Significantly correlated at the 95% level.

[1]The glycoprotein carried by these two protein fractions did not distinguish between the protein fractions.

A comparison of the distribution of serum proteins of the control and experimental specimens to that of specimens living in their native habitat was made. Table 8 lists the values obtained for specimens collected from three lakes.

All specimens were taken by hook and line and blood samples were collected without benefits of anesthetics as soon as the fish were caught.

TABLE 8. Comparison of the distribution of serum and plasma protein fractions of bluegills collected at different locations and months.

Location and Date	Per Cent Composition I	II	III	IV	V	N*
Lake Lansing, Ingham County, Michigan June 16, 1961 (serum)	14.6	25.7	22.1	26.2	13.6	6
Lake Chemung, Livingston County, Michigan January 16, 1962 (plasma)	6.2	10.8	21.6	41.0	18.4	6
Lake "Taylor," Livingston County, Michigan January 20, 1962 (plasma)	7.6	12.8	15.6	45.3	18.5	6
Control Fish used in Experiment (serum)	9.1	32.6	24.6	25.7	7.9	5

*Number of fish in the sample.

In general, samples taken at the same time of the year are comparable to each other. These apparent seasonal differences actually may be due to differences in habitat, diet, genetic strain, or other factors.

One factor ignored at the time of this study was the influence of the method of capture. This has now been demonstrated to have a significant influence on the blood

properties of rainbow trout and probably all fish. In spite of the influence of the method of capture, it is believed that the differences observed in bluegills were largely seasonal differences. Katz (1949) reported seasonal differences in various blood-cell levels of silver salmon. Shields, Platner, and Neubeiser (1961) reported differences in the distribution of serum proteins of rats during cold acclimation. Recently, Meisner and Hickman (1962) reported that rainbow trout acclimated to different water temperatures had significantly different distributions of serum protein in their electrophoretic patterns.

Another difference noted in bluegills collected in the winter was that their blood coagulation time was much greater than in the summer. This increase in coagulation time maybe associated with the differences already noted in the electrophoretic pattern of their serum and/or plasma.

Influence of a Diurnal Oxygen-Pulse on Yellow Bullheads

Specimens for this experiment were collected from the Red Cedar River, Ingham County, near Okemos, Michigan, with the aid of a 230 volt D.C. shocker during July, 1961. These fish were acclimated to the laboratory for two weeks and were fed beef liver and pellets twice daily. The specimens were then randomly assigned to the control and experimental section. Acclimation to the artificial stream continued for two weeks before degassing began. All length and weight data were obtained immediately after blood was drawn from the specimens. These data are listed in Appendix I.

Degassing consisted of the reduction of the oxygen content of the control section from approximately 7.5 ppm to approximately 2.7 ppm, eight hours a day, for nine days. Means of the water quality data are listed in Table 9. On the tenth day specimens were sacrificed and blood samples were collected by cardiac puncture. Serum was collected as before but was stored at 2.0 °C, to allow the analysis of lipoprotein fractions.

TABLE 9. Mean chemical data of the control and experimental sections during degassing for yellow bullheads.

	Temperature	Oxygen Dissolved	pH	Total Carbonate Alkalinity
		Experimental		
Mean	24 °C.	7.6 ppm	8.20	121.0 ppm
Range	23.0-25.0	7.0-7.9	7.7-8.3	100-131
		Control		
Mean	24 °C.	2.7 ppm	8.17	125.0 ppm
Range	23-25.0	2.3-3.4	7.7-8.3	118-134

Seven protein fractions were separated in the serum, but for unknown reasons fractions "II" and "III" separated poorly. This was somewhat the case for fractions "IV" and "V." No explanation for this difficulty is known since within the same electrophoretic run samples which did separate

well and those which did not were found. In this case, it is also suspected that the use of commercially prepared buffer solution caused the difficulty. Due to the poor resolution, the areas of fraction "III" and "IV" were added together for each specimen to allow a better comparison of the data. This grouping may have hidden significant changes within these fractions, but no alternative was available.

The results of the analyses of the distribution of the serum proteins from male yellow bullheads is listed in Table 10. Neither the means nor the variances of the fractions were statistically different between the controls and the experimentals. This conforms to their empirically determined pollution tolerance. Correlation coefficients of the yellow bullhead serum-protein fractions to body length and weight are listed in Table 11. Fraction I is significantly correlated at the 95% level with both body weight and length in the control lot. In the experimental lot, fraction I was significantly correlated at the 95% level only to body weight.

Fraction II was significantly correlated at the 95% level to body weight in the control lot, but not in the experimental lot. Other significant correlations of body weight and length to protein fractions were lacking.

In general, the highest correlation coefficients were obtained in relation to body weight, rather than body length. The correlation coefficients of the experimental lot were usually lower than those of the control lot. Where this was not the case, a sign difference existed between the control

TABLE 10. Summary of the influence of a dirunal oxygen-pulse on the distribution of proteins in the serum of male yellow bullheads.

	Fractions					
	I	II	III + IV	V	VI	VII
	Control					
Mean	18.29%	9.10%	28.91%	14.92%	25.67%	2.74%
Range	12-25%	6-12%	27-33%	11-21%	20-35%	2-4%
Standard Error of the Mean	0.721%	0.392%	0.582%	0.487%	0.698	0.092%
Coefficient of Variation	25.5	32.8	13.1	21.1	17.6	21.9
Variance	21.86%	6.47%	14.25%	9.96%	20.47	0.36%
	Experimental					
Mean	19.74%	10.31%	33.03%	14.03%	21.70%	2.37%
Range	15-25%	5-16%	25-44%	10-20%	13-30%	1-4%
Standard Error of the Mean	0.539%	0.519%	1.038%	0.628%	0.981%	0.169%
Coefficient of Variation	61.8	32.6	20.3	26.8	27.1	46.2
Variance	12.21%	11.35%	45.30% *	14.20%	34.63%	1.20%

*Significantly greater at 90% level.

TABLE 11. Coefficients of correlation for the distribution of serum proteins to total body length and body weight of male yellow bullheads.

	Fractions					
	I	II	III + IV	V	VI	VII
			Weight			
Control (N=7)	+0.99**	+0.88*	-0.63	-0.33	-0.42	-0.47
Experimental(N=7)	+0.95**	-0.53	-0.11	+0.33	-0.30	+0.57
			Length			
Control (N=7)	+0.87*	+0.18	-0.51	-0.13	-0.60	-0.28
Experimental(N=7)	+0.33	-.30	+0.16	+0.18	-0.39	+0.59

*Significant at the 97.5% level.

**Significant at the 99.9% level.

and experimental lots. This has been discussed previously concerning bluegills.

The positional relationship of proteins, glycoproteins and lipoproteins in the serum of yellow bullheads is depicted in Figure 33. The width of the fractions is not necessarily indicative of the amount of material contained therein. Rather, this illustrates the location of proteins which are conjugated to polysaccharides (glycoprotein) and/or lipids (lipoprotein). Four glycoprotein and four lipoprotein fractions were separated in the yellow bullhead serum. Protein fractions I, IV, V, and VI carried glycoprotein. Protein fractions II, IV, V, and VII carried lipoprotein.

The results of the analyses of glycoprotein of yellow bullheads is summarized in Table 12. The mean of fraction I was 20% in the control lot and 27% in the experimental lot. This difference was statistically significant at the 95% level.

The mean of glycoprotein fraction II was 38% in the control lot and 31% in the experimental lot. This difference was statistically significant at the 95% level.

Glycoprotein fractions II and IV remained rather constant and no significant differences existed between the control and experimental lots. None of the variances of the glycoprotein levels were statistically significant.

It was previously shown that no significant differences existed in the protein fraction levels of yellow bullhead serum. Therefore, without changing the protein levels

Figure 33. Positional relationship of proteins to glycoproteins and lipoproteins in the serum of the yellow bullhead (Ictalurus natalus).

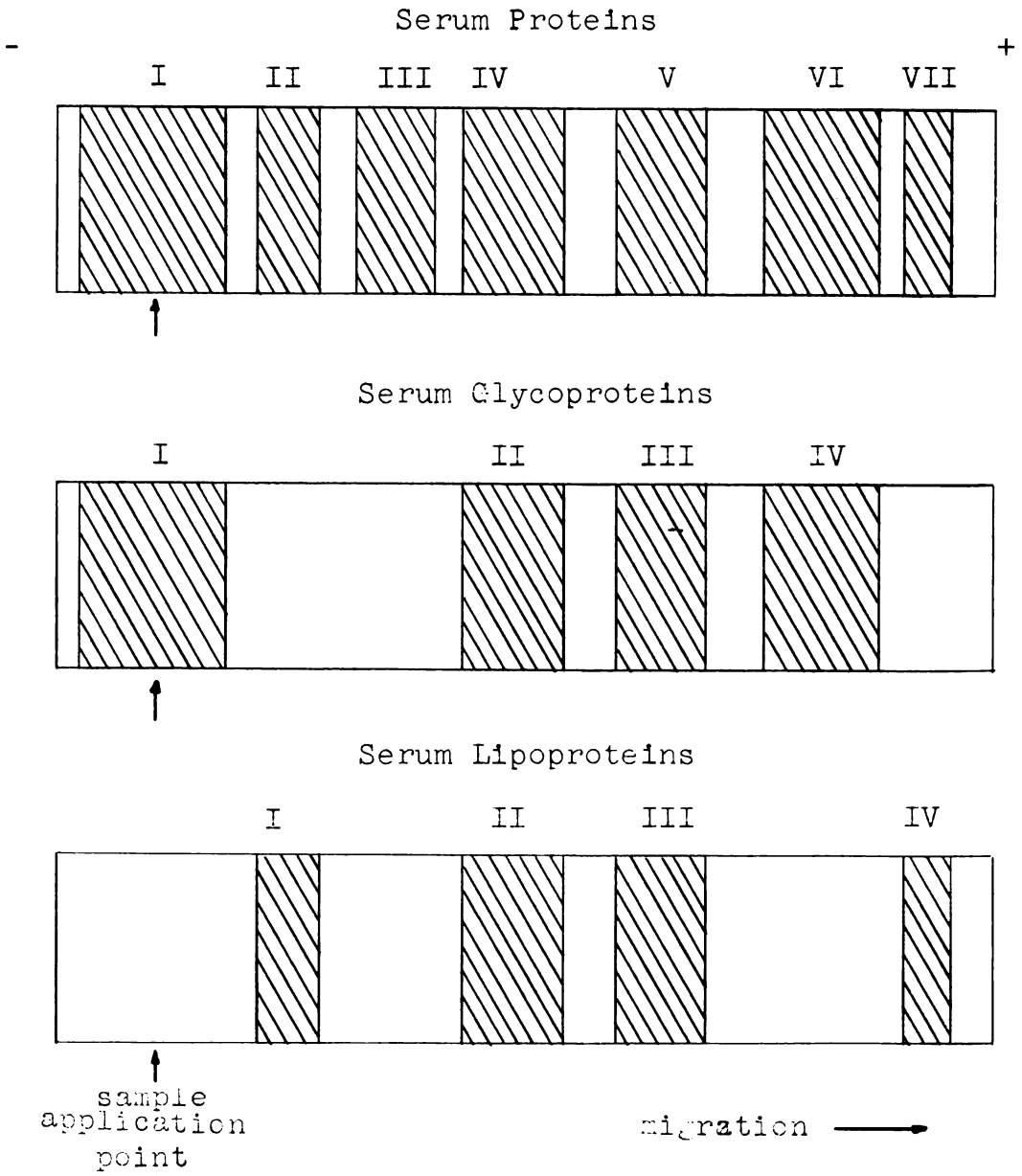


TABLE 12. Summary of the influence of a diurnal oxygen-pulse on the distribution of glycoproteins in the serum of male yellow bullheads.

	Fraction I	Fraction II	Fraction III	Fraction IV
		Control (N=7)		
Mean	20.14%	22.74%	38.27%	18.84%
Range	11-26%	19-25%	32-44%	15-25%
Standard Error of the Mean	1.842%	0.752%	1.637%	1.285%
Coefficient of Variation	24.2	8.7	11.3	18.0
Variance	23.75%	3.96%	18.79%	11.56%
		Experimental (N=7)		
Mean	27.81%*	21.74%*	31.86%	19.16%
Range	24-33%	18-26%	22-37%	15-22%
Standard Error of the Mean	1.339%	0.957%	1.999%	1.001%
Coefficient of Variation	12.7	11.6	16.6	13.8
Variance	12.56%	6.41%	27.98%	7.02%

*Significantly different at 95% level.

significantly, yellow bullheads altered the levels of polysaccharides conjugated to these fractions. To further illustrate this, Pearsonian correlation analyses were performed on each glycoprotein fraction and its protein carrier. The resultant correlation coefficients are listed in Table 13.

Glycoprotein fractions I and IV were significantly correlated at the 95% level to their protein carrier in the control lot. In contrast, the experimental lot had much lower and/or opposite signed correlation coefficients. These data show a change in the correlation coefficients between the control and experimental lots of bullheads as was noted for bluegills.

TABLE 13. Influence of a diurnal low oxygen-pulse on the relationship of glycoproteins to protein in male yellow bullheads.

Item	Correlation Coefficients	
	Controls	Experimentals
Glyco protein "I" to Protein "I"	+0.75*	-0.42
Glycoprotein "III" to Protein "V"	+0.59	+0.15
Glycoprotein "IV" to Protein "VI"	+0.96**	+0.43

*Significant at the 95% level.
**Significant at the 99.95% level.

Such changes may have resulted in increased quantities of the proteins which conjugate with polysaccharides or perhaps a change in the ability of these proteins to conjugate.

The analyses of the distribution of lipoprotein in yellow bullhead serum are listed in Table 14. The values obtained for these fractions are highly variable and may be due to storage of the samples. This variability is described by their individual coefficients of variation.

The mean levels of lipoprotein for the respective fractions between the control and experimental lot remained rather constant and no significant differences between these means were obtained. Only the variance of lipoprotein fraction II in the experimental lot was found to be significantly different from that of the control lot.

Influence of a Diurnal Oxygen-Pulse on Largemouth Bass

The largemouth bass used in this experiment were collected by hook and line from Wintergreen Lake, Kalamazoo County, Michigan, by using artificial lures. At the time of capture these fish appeared to be in poor physical condition. To improve their condition, these fish were placed in a local minnow raisers pond and were allowed to feed for two months. At the end of this period all specimens appeared to be in good condition. The bass were then collected by seining the pond and were removed to the laboratory.

Feeding was attempted in the laboratory as with the previous experiments, using live minnows as food. This

TABLE 14. Summary of the influence of a diurnal oxygen-pulse on the distribution of lipoproteins in the serum of male yellow bullheads.

	Fractions			
	I	II	III	IV
	Control (N=7)			
Mean	10.70%	29.63%	43.23%	16.10%
Range	5-18%	21-35%	31-58%	8-23%
Standard Error of the Mean	1.010%	1.896%	3.111%	2.031%
Coefficient of Variation	61.1	16.9	19.0	18.7
Variance	42.83%	25.17%	67.75%	173.18%
	Experimental (N=7)			
Mean	12.65%	25.01%	41.21%	21.07%
Range	4-33%	8-43%	31-51%	10-40%
Standard Error of the Mean	3.691%	4.200%	2.589%	3.624%
Coefficient of Variation	77.1	9.2	14.6	10.4
Variance	95.35%	117.45%*	46.91%	91.92%

*Significant at the 95% level.

rapidly fouled the water. It appeared that such feeding was unnecessary since the fish in the experimental section tend to vomit their stomach contents during periods of low dissolved oxygen. Fujiya (op. cit.) indicated that up to two months of fasting would not alter the serum proteins of fish and on this basis food was withheld from the bass for the remainder of the experiment.

The bass were acclimated to the conditions of the laboratory for two weeks and then were randomly assigned to the control and experimental sections of the artificial stream. Acclimation to the artificial stream continued for two weeks before degassing began.

Degassing consisted of reducing the oxygen content of the experimental section from approximately 8.1 ppm to approximately 3.0 ppm. This was done for eight hours/day for the first eight days. At this time the nitrogen supply was exhausted and degassing was temporarily suspended until the tenth day. This break in the degassing operation may have lessened the stress load. A summary of the water chemistry data obtained during degassing periods is listed in Table 15.

On the eleventh day the specimens were sacrificed and blood samples were collected by direct cardiac puncture. This method was necessary due to the small quantity of blood obtainable from a single specimen. No more than 0.5 ml of blood was obtained from the largest specimen (23.0 cm). Shell (1961) reported a similar difficulty when working with smallmouth bass.

TABLE 15. Summary of the water chemistry data collected during the degassing periods for largemouth bass.[1]

	Temp. °C.	Dissolved Oxygen ppm	pH	Total Alkalinity ppm
		Control		
Mean	20.5	8.32	8.32	159
Range	19.0-21.0	8.1-8.7	8.0-8.5	110-195
		Experimental		
Mean	20.5	3.1	8.30	151
Range	19.0-21.0	2.8-3.5	7.9-8.5	114-200

[1]These data excluded water quality on the ninth day when degassing could not be performed.

Micro-hematocrit samples were taken in commercially prepared heparinized capillaries, but these did not prevent the coagulation of the blood. All serum samples were prepared as before. Due to the small quantity of serum obtained from each specimen, the samples could be analyzed only for the per cent composition of the serum protein.

As before, all length, weight, and sex data were obtained immediately after the blood had been drawn from the specimen. Due to the small number of females in the groups, only the data from the males were analyzed.

Six clearly distinguishable fractions were separated from the serum of largemouth bass. One of these fractions

was considered to be a subfraction and is labeled accordingly. Smith's (op. cit.) buffer was utilized and no difficulty was experienced in resolving these into separate fractions.

The positional relationship of proteins, glycoproteins, and lipoproteins in largemouth bass serum is shown in Figure 34. The width of the fractions is not necessarily indicative of the amount of material contained therein. Rather, this illustrates the location of proteins which are conjugated to polysaccharides (glycoprotein) and/or lipids (lipoprotein). Differences between serum fractions of bass and those of human serum (see Figure 4) are clearly evident.

The results of the analyses of the distribution of serum proteins from male largemouth bass are summarized in Table 16. Fraction I was iso-electric and remained at the application point. The mean of this fraction was 7.88% in the control lot and 6.34% in the experimental lot. Neither the difference of these means, nor the differences in the variances were found to be statistically significant.

The mean of fraction II was 17.12% in the control lot and 26.30% in the experimental lot. While the difference in these means was not significant, a larger sample size might have achieved such significance. The variance of the experimental lot was found to be significantly different at the 95% level.

The mean of fraction IIa was 10.48% in the control lot and 10.02% in the experimental lot. Neither the differences

Figure 34. Positional relationship of proteins, glycoproteins, and lipoproteins in the serum of largemouth bass.

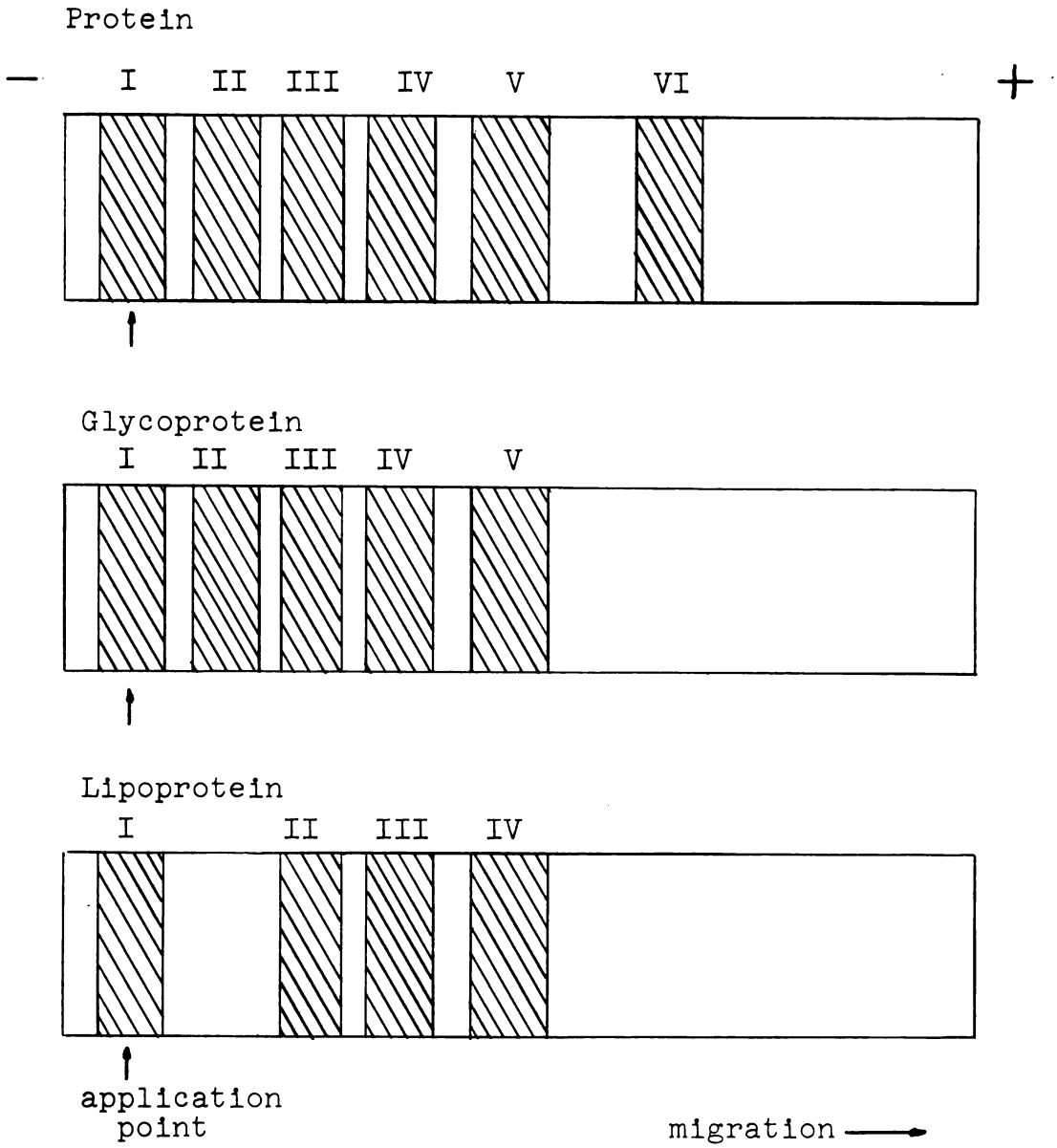


TABLE 16. Summary of the influence of a diurnal oxygen-pulse on the distribution of proteins in the serum of male largemouth bass (Micropterus salmoides).

	Fractions					
	I	II	IIa	III	IV	V
	Control (N = 4)					
Mean	7.88%	17.12%	10.48%	26.02%	23.50%	14.72%
Range	4.9-7.9%	14.4-33.0%	8.8-12.5%	20.1-33.0%	22.8-24.7%	11.8-18.3%
Standard Error of the Mean	0.524%	0.477%	0.495%	2.658%	0.414%	1.342%
Coefficient of Variation	13.2	5.5	16.4	20.4	3.5	18.2
Variance	1.097%	0.910%	2.970%	5.317%	0.829%	2.685%
	Experimental (N = 5)					
Mean	6.34%	26.30%	10.02%	25.06%	21.44%*	10.02%*
Range	6.7-8.9%	16.0-18.3%	8.4-12.5%	20.1-33.0%	22.8-24.7%	11.8-18.3%
Standard Error of the Mean	0.729%	3.395%	0.957%	0.652%	1.181%	0.393%
Coefficient of Variation	17.2	19.4	14.3	7.8	16.5	11.7
Variance	1.195%	25.940%*	2.062%	3.830%	12.562%	1.392%

*Significant at the 95% level.

in the means, nor the differences in the variances were found to be significant statistically.

The mean of fraction III was 26.02% in the control lot and 25.06% in the experimental lot. Again, no significant differences were found between the control and experimental lots.

Fraction IV had a mean of 23.50% in the control group and a mean of 21.44% in the experimental group. This difference in the means was found to be significant at the 95% level. No other differences were detected in this fraction.

Fraction V had a mean of 14.72% in the control group and a mean of 10.02% in the experimental group. This difference was found to be significant at the 95% level.

The Pearsonian correlations of body weight and body length to each protein fraction are presented in Table 17. It should be noted that fractions IIa, III, and V were significantly correlated at the 95% level with body length of control fish. Fraction V of the experimental fish was significantly correlated at the 95% level with body length. Only fraction V in both experimentals and controls was significantly correlated at the 95% level with body weight. Correlation coefficients are influenced by sample size. In general, large sample sizes tend to produce lower correlation coefficients than small samples. Increasing the number of bass tested will probably result in the reduction of the correlation coefficients as herein obtained for fractions III and V. It should be noted that, with two exceptions, the

correlation coefficient of the experimental specimens shows a reduction in the intensity of the relationship. In four fractions, this relationship became the reverse of the relationship exhibited in the controls. This was noted in both the bluegills and the yellow bullheads.

During the experiment one fish of the control group and two fish of the experimental group died. The cause or causes of these deaths is unknown.

TABLE 17. The influence of a diurnal oxygen-pulse on the coefficients of correlation of serum protein distributions to total body length and body weight in male largemouth bass.

	Fractions					
	I	II	IIa	III	IV	V
	Total Body Length					
Controls (N=4)	+0.51	+0.24	+0.88*	-0.96*	+0.76	+0.88*
Experimentals (N=5)	+0.34	-0.53	+0.31	+0.53	=0.42	+0.86*
	Body Weight					
Controls (N=4)	+0.17	+0.69	+0.51	-0.97	+0.15	+0.99*
Experimentals (N=5)	+0.34	-0.51	+0.35	-0.24	+0.33	+0.89*

*Significant at the 95% level.

SUMMARY

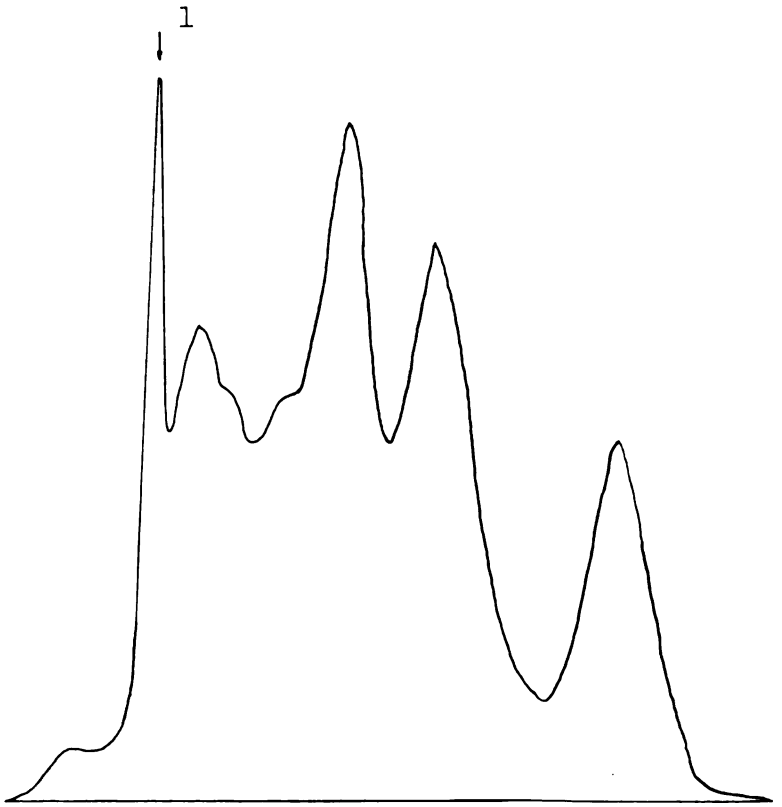
In view of the previous evidence, it is certain that at least six factors influence the electrophoretic serum and/or plasma protein patterns of a fish species. These are:

1. Conditions used for the electrophoretic separation and evaluation.
2. Quality of the protein sample.
3. Body weight and/or body length of the fish.
4. Reproductive cycle and sex.
5. Acclimation temperature.
6. Stress.

The first factor can be controlled by utilizing the same electrophoretic conditions for each analysis. This includes the various characteristics of the buffer solution, electrical current, temperature of fixation, protein stain, and evaluation method. If these factors are reproduced as closely as possible then the electrophoretic method can be considered a constant. The use of bovine serum with its albumin labeled by bromphenol blue increases the reproducibility of migration characteristics.

The quality of the protein sample should be as uniform as possible. Plasma and/or serum must be hemoglobin free. Hemoglobin rarely migrates on paper at pH 8.6 in vernol buffer and will cause a sharp peak at the application point, as illustrated in Figure 35. Usually a visual inspection is

Figure 35. Hemoglobin contamination of the serum of pumpkinseed (sunfish) which was exposed to copper toxicity.



[1]Application point, showing a sharp peak of immobile hemoglobin. Other fractions are larger than usual due to the influence of toxicity and increased sample size.

sufficient to determine if hemolysis has occurred. Sterility of the container may be important, especially when the samples must be stored for several hours. Storage conditions can produce changes in the electrophoretic pattern but agreement among investigators is not universal. Meisner and Hickman (op. cit.) reported that freezing did not influence the electrophoretic patterns of rainbow trout serum. However, this writer experienced difficulties with samples which have been frozen. Although these difficulties appeared to be universal with frozen samples, it now appears that freezing has a differential effect upon samples from different species and it is probably better not to freeze samples of serum, plasma, or hemoglobin. This aspect must be tested upon the species concerned before freezing the samples is justified.

Body weight and body length have been demonstrated in this study to be strongly correlated with certain protein fractions in the electrophoretic patterns of yellow bullheads, largemouth bass, and bluegills. However, it should be noted that the fractions which are significantly correlated with body weight are not necessarily significantly correlated with body length (i.e., Table 7 bluegill fractions I and II; Table 13, bullhead fraction II; Table 17, bass fraction IIa). This is unusual since a strong correlation between weight and length exists. One would expect that the correlation of a fraction with body weight would also produce an equally strong, and significant correlation with body length.

Apparently, the relationship between the quantities of certain fractions are dependent upon body weight (condition of the organism) and other fractions are more dependent upon the body length (growth history of the organism). Further evidence of this hypothesis is lacking.

The sex of the specimen and the reproductive cycle has been demonstrated by Vanstone and Chung-Wai Ho (1961) to influence the serum protein patterns of coho salmon (Oncorhynchus kisutch). The difference noted was the appearance of a new serum protein fraction which occurred with the onset of the reproductive cycle in females. This fraction was associated with lipids and was thought to be a mixture of the lipovitellin and lipovitellenin complexes in transit to the developing ova. This writer has made a similar observation in the blood of northern pike on their spawning-run. Females had an extra lipoprotein fraction when compared with males. This condition probably exists in other species of fish, but has not been demonstrated.

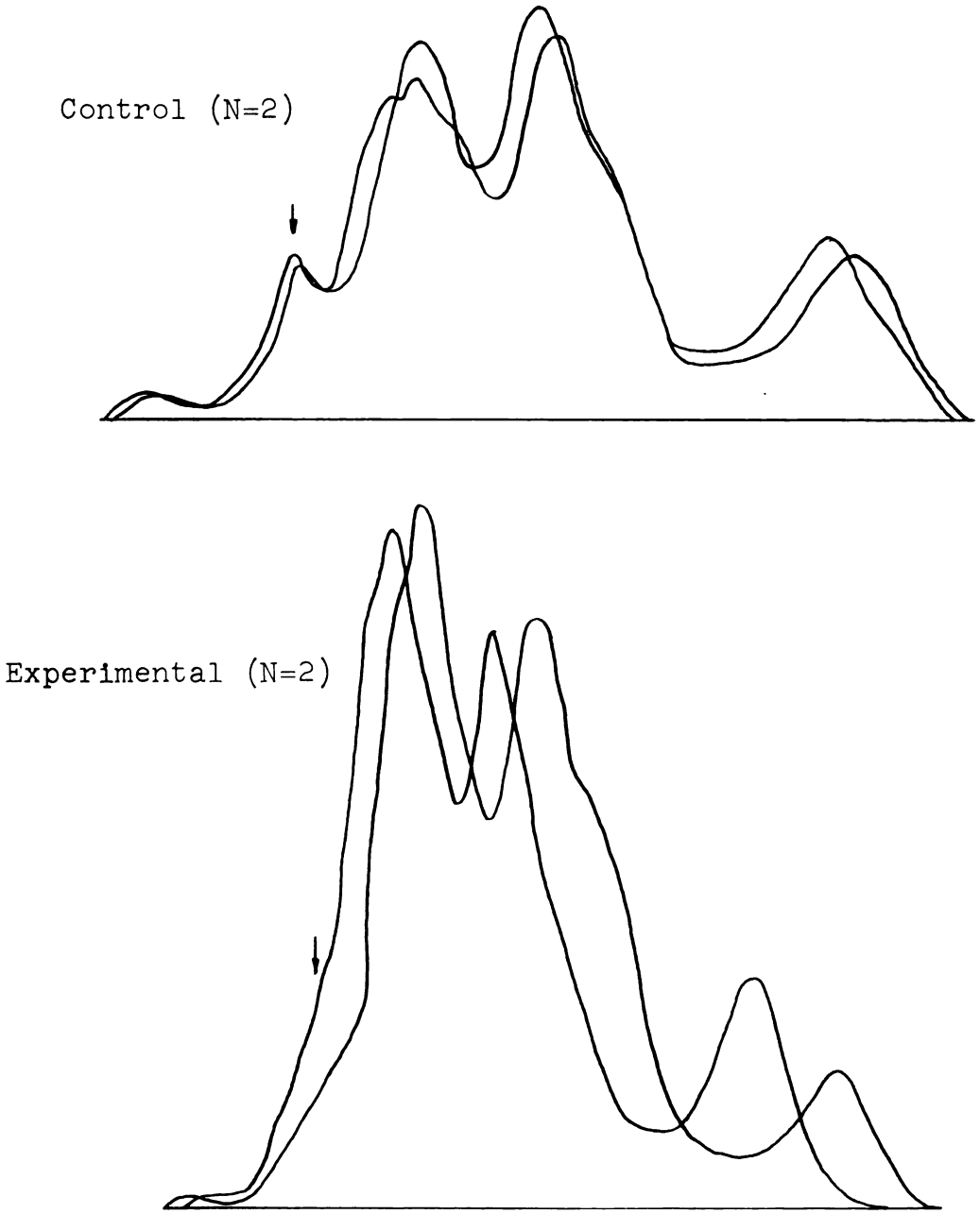
Acclimation temperature has been demonstrated to influence the serum proteins of rainbow trout by Meisner and Hickman (op. cit.). Although the differences noted may be due to temperature alone, it is possible that this may be the result of reproductive-cycle stimulation. Photoperiods used by Meisner and Hickman (op. cit.) did not alone influence the serum proteins of rainbow trout, but it is possible that the combination of photoperiod and temperature change promoted reproductive development.

It has been demonstrated in this study that each fish species has a unique electrophoretic pattern. In one case, it was possible to distinguish between subspecies based on their electrophoretic pattern. Therefore, hybrid specimens are to be avoided since their patterns tend to be intermediate to the patterns of their parents.

Stress from both acute and chronic toxicity has been demonstrated to influence the electrophoretic patterns of fish by Fujiya (op. cit.), and Neuhold and Sigler (1960). Fujiya (op. cit.) also demonstrated that stress resulting from fasting for six weeks will not significantly alter the electrophoretic pattern of some fish. However, Lysak and Wojcik (1960) demonstrated that the number of erythrocytes, hemoglobin, and the hematocrit usually decrease with diminishing protein content of the diet and may strongly influence the total serum protein of the blood.

Two basic differences can be noted in the action of stress due to toxicity on the electrophoretic serum protein pattern of fish, as compared to stresses of a milder nature. First, toxicity stress produces faster changes in the electrophoretic pattern. Secondly, the changes induced by toxicity are of a greater magnitude than those produced by low-level stress. Figure 36 shows these differences, as exhibited by the 30 hours exposure of green sunfish to 6 ppm cupric copper in a static bio-assay. Fujiya's (op. cit.) work also demonstrates these fundamental differences between toxicity stress and "non-specific" low levels of stress.

Figure 36. Influence of sub-lethal cupric copper on the serum electrophoretic pattern of green sunfish.[1]



[1]Exposed 30 hours in 6 ppm Cu (cupric).

Stress from low dissolved oxygen does not conform to the definition of toxicity and requires special consideration. Low dissolved oxygen has been demonstrated by Blazka (1958) to produce oxygen debt in brown trout and a metabolic shift in carp. Thus, for fish which incur oxygen debt, the period of stress from low dissolved oxygen is not ended when oxygen levels are returned to normal, but continues until oxygen debt is "repaid."

The role of acclimation to a diurnal oxygen pulse was not investigated. It is possible that the changes noted in the serum of bluegills and bass would have been greater or lesser after fewer days at these conditions. Also it is possible that continuation of these conditions would have resulted in a return to "normal" levels. However, it is believed that further exposure to a diurnal oxygen pulse would increase the differences between the control and experimental lots.

Acclimation probably has a more important role during gradual, progressive reduction of dissolved oxygen, i.e. winter stagnation of lakes. A diurnal oxygen pulse appears to present a daily traumatic experience to fish. This is supported by the vomiting which occurred each day as the oxygen concentration was reduced to 3 parts per million.

Other investigators have observed that fish become "excited" and tend to increase their movement when the oxygen content of water is reduced. This was also observed in this study for bass, bluegills, and bullheads. However,

it must be pointed out that after a short period of such activity, the fish generally remained as motionless as possible.

An increase in ventilation activity is indicative of the inability of fish to obtain sufficient oxygen from the water. Mount (1961) has demonstrated that increases in ventilation rates of several fish species is inversely related to oxygen concentration. The ventilation rates of bluegills and bass appeared to be strongly increased during this diurnal oxygen pulse. Bullheads were affected to a lesser extent. Also, during periods of increased ventilation activity, bluegills and bass lost their "normal" color and appeared "faded."

During the periods of low oxygen the experimental bluegills and bullheads vomited ingested food. This occurred each day of the test period. Furthermore, these fish refused food offered to them during periods of low dissolved oxygen (largemouth bass were not fed). A low oxygen pulse, therefore, interferes may with feeding behavior and cause nutritional difficulties.

A final observation is noteworthy. Mucus strands were observed in the water and/or trailing from the anus of largemouth bass in the experimental lot. Fromm and Shiffman (1957) observed mucus strands trailing from largemouth bass exposed to sublethal concentrations of hexavalent chromium. Fromm and Shiffman (op. cit.) associated these mucus strands with the necrosis of the lamina mucosae. Fujiya (op. cit.) and

King (1962) found similar necrosis in their specimens when exposed to sublethal concentrations of pulpmill wastes and DDT, respectively. While it is possible that these toxicants actually reached the stomach wall, no proof of this hypothesis was exhibited in these studies.

These mucus strands appear to be characteristic of stress and associated with necrosis of the intestinal wall. Selye (1955) describes mammalian reaction to non-specific stress as follows: (1) the stimulation is conveyed to the hypothalamus which caused the anterior pituitary to produce adrenocorticotropic hormone (ACTH); (2) ATCH reaches the adrenal cortex and thereby releases hormones which stimulate various organs, among which are the gastric glands of the stomach; (3) pepsin and HCl produced in the stomach attack its contents and/or the stomach lining. Continuation of this condition leads to gastric ulcers and both liver and kidney malfunctions.

If fish react to stress as described for mammals, then gastric production of proteolytic enzymes, et cetera, would occur. This could account for the production of tissue necrosis and mucus in the gastrointestinal tracts of fish under stress. Since the largemouth bass were fasting, their stomachs would have been especially susceptible to any overproduction of gastric juices. Bluegills and bullheads were fed but tended to vomit. This probably would cause them to be less susceptible to such necrosis and may account for the lack of such mucus strands in the experimental lots of

bluegills and bullheads. Histochemical and histological evidence to support this hypothesis is lacking.

It is certain that a diurnal oxygen pulse as investigated here, produced both qualitative and quantitative changes in the electrophoretic serum protein fractions. This is illustrated by:

1. A significant change in the distribution of protein in the electrophoretic patterns of bluegills and bass.
2. A significant change in the distribution of serum glycoproteins in the electrophoretic patterns of bluegills and yellow bullheads (bass were not investigated).
3. A change in protein composition as shown by the conjugation of protein to polysaccharides in bluegills and yellow bullheads.
4. A change in the relationship of certain fractions to total body weight and/or length in bluegills, bullheads, and bass.

A change in the electrophoretic pattern assumes the reduction of certain fractions and the increase of other fractions. A word of caution is necessary since these fractions were measured on a relative basis (per cent of total serum protein).

Stimulated production of "X" protein fractions with normal production of "Y" fractions would yield both relative and absolute increases of "X." Conversely, normal production

of "X" with decreased production of "Y" would also produce a relative increase of "X." However, the latter case would not constitute an absolute increase of the "X" fractions. Allfrey, Mirsky, and Osawa (1957) demonstrated that the active incorporation of isotopic amino acids into the nuclear protein of isolated thymus cells is strongly inhibited by anoxia. Thus, it was believed that the changes noted in this study were due to inhibition of production of certain proteins, rather than the increase of other proteins. Increased serum protein production would be expected to raise the osmolarity of blood, thereby drawing water into the fish at a faster rate. Such a fish would either become edemic or exhibit increased osmoregulatory function and increased oxygen consumption. Subsequent investigations using carp, hogsuckers, and rock bass have shown that total plasma protein concentration tends to increase during exposure to a similar low oxygen pulse.

Blaska (op. cit.) has demonstrated that lactic acid is not stored in the body of carp and brown trout. This does not mean that this toxic product is not produced in their bodies. The increase of serum proteins may account for the detoxification of such product, by conjugation and subsequent transportation to elimination sites.

The true meaning of the changes in serum protein fractions cannot be readily described since their physiological ramifications are largely unknown. While some may contend that these changes were adaptive changes and mean nothing,

it is probable that most deviations from "normal" do not benefit the organism. Further evidence that the changes observed in the serum represent physiological degradation are: these changes are associated with vomiting, increased ventilation activity, normal body color loss, reduction of movement, appearance of mucus strands, toxicity, and other deleterious conditions.

In view of these data, a diurnal oxygen pulse allowable by Tarzwell's (op. cit.) criterion (not lower than 3 ppm at any time, and not lower than 5 ppm for more than 8 hours per day) is probably physiologically detrimental to bluegills and bass. Further investigation will be needed to determine the levels of oxygen in a diurnal pulse which are not physiologically detrimental to these species.

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APPENDICES

APPENDIX I

Raw data from male bluegills, yellow bullheads, and largemouth bass exposed to a diurnal oxygen-pulse and tested electrophoretically.

Raw Data from Male Bluegills Tested Electrophoretically

Fish No.	Total Length (cm)	Weight (grams)	Serum Protein Fractions*					Glycoprotein Fractions*		
			I	II	III	IV	V	I	II	III
Controls										
1	13.0	37	9.7	33.9	22.6	25.8	8.0	6.3	3.1	90.7
2	15.5	58	7.3	31.2	26.0	28.1	7.3	11.6	35.4	53.0
3	14.0	45	8.2	32.2	30.1	20.5	8.9	13.1	22.2	64.7
4	14.5	49	8.7	30.4	24.6	29.0	7.2	8.8	17.5	73.5
5	13.3	34	11.8	35.5	19.7	25.0	7.9	14.3	25.0	69.7
Experimentals										
6	14.0	41	11.9	33.9	22.9	22.0	9.3	13.2	30.2	56.5
7	14.0	46	8.5	36.9	22.3	23.8	8.5	7.7	38.5	54.0
8	12.7	30	9.1	33.0	21.6	27.3	9.1	5.9	35.5	59.2
9	14.2	46	10.4	24.6	18.9	38.9	7.1	15.8	21.0	63.2
10	13.8	35	9.8	38.2	19.6	23.5	8.8	9.1	50.0	41.0

*All electrophoretic fraction values are expressed as per cent of total protein or glycoprotein.

Raw Data from Male Yellow Bullheads Tested Electrophoretically

Fish No.	Total Length (cm)	Standard Length (cm)	Weight (grams)	Serum Protein Fractions (Per Cent of Total Serum Protein) I	II	III+IV	V	VI	VII
				Control					
1	18.7	15.6	72	17.4	7.1	30.9	20.6	20.0	3.9
2	17.2	14.4	66	19.9	7.4	30.1	15.9	23.9	2.8
3	14.0	11.5	32	13.0	9.1	28.4	12.4	34.5	2.6
4	23.0	19.5	161	25.6	11.8	21.5	11.8	25.0	2.0
5	18.5	15.4	84	20.6	8.8	32.3	12.4	23.5	2.4
6	16.0	14.5	50	12.0	13.2	27.1	16.9	27.7	3.0
7	17.0	15.5	66	19.5	6.3	32.1	14.5	25.1	2.5
				Experimentals					
8	17.5	16.0	50	15.5	12.7	39.5	20.4	20.4	1.4
9	17.5	15.0	77	24.5	15.6	31.9	13.6	12.9	1.4
10	20.4	17.3	105	18.2	4.8	44.3	9.7	20.6	1.5
11	15.0	12.9	40	15.6	10.6	25.6	12.3	30.5	2.6
12	17.0	14.2	56	22.3	8.5	29.2	12.3	25.4	2.3
13	23.5	19.5	190	22.3	9.5	27.4	15.9	20.4	4.4
14	23.0	20.0	183	19.8	10.5	33.3	-- 33.3 --		3.0

Raw Data from Yellow Bullheads Tested Electrophoretically

Fish No.	Glycoprotein Fractions*				Lipoprotein Fractions*			
	I	II	III	IV	I	II	III	IV
				Control				
1	20.0	25.0	40.0	15.0	4.9	26.2	47.2	21.3
2	22.0	22.0	39.0	17.0	5.7	31.0	39.4	22.5
3	20.0	25.0	32.0	24.8	9.5	35.1	41.9	13.5
4	24.6	19.3	38.6	17.5	8.7	32.6	39.2	19.5
5	25.7	22.2	33.0	17.8	17.9	34.3	31.3	16.4
6	11.1	22.2	44.0	22.2	13.1	21.3	57.6	8.0
7	17.6	23.5	41.3	17.6	15.3	26.9	46.1	11.5
				Experimentals				
8	33.0	19.7	34.8	15.3	11.8	23.5	39.2	25.5
9	25.8	25.8	29.0	19.4	33.4	19.0	31.0	16.7
10	27.4	18.4	36.4	18.4	4.0	8.0	48.0	40.0
11	25.0	21.8	33.3	20.8	9.3	21.8	51.5	17.2
12	24.4	22.2	31.0	22.2	14.8	26.2	37.5	21.3
13	26.5	20.4	36.8	16.3	7.4	33.4	42.5	16.7
14	32.6	23.9	21.7	21.7	7.9	43.2	38.8	10.1

*Expressed as per cent of total lipoportein or glycoprotein.

Raw Data from Male Largemouth Bass Tested Electrophoretically

Fish No.	Total Length (cm)	Total Weight (grams)	Serum Protein Fractions *					
			I	II	III	IIa	IV	V
			Control					
1	23.0	143	5.9	24.4	12.5	28.1	17.4	11.1
2	23.0	141	7.9	19.4	8.8	24.7	25.6	11.0
3	22.1	103	6.6	33.3	9.4	23.5	18.8	8.4
4	21.5	114	4.9	25.7	9.8	25.7	24.5	9.2
5	20.2	114	6.4	29.0	9.6	23.3	20.9	10.4
			Experimentals					
6	21.5	103	8.6	17.3	10.0	26.0	23.3	14.6
7	22.2	98	8.9	16.0	12.5	25.0	23.2	14.2
8	22.5	122	7.3	18.3	11.0	20.1	24.7	18.3
9	20.5	82	6.7	16.9	8.4	33.0	22.8	11.8

*Expressed as per cent of total.

APPENDIX II

Staining methods for serum and/or plasma proteins, glycoproteins, and lipoproteins after paper electrophoresis.

These staining methods are condensed from Beckman Technical Bulletin 6095A, and are included here in the chance that this publication will not be available to the reader of this thesis.

I. Staining method for serum and/or plasma protein, using alcoholic-bromphenol blue.

1. Place strips in staining rack and immerse in methanol pre-rense to remove the alkaline buffer on the strips. Do not aggitate the strips and remove after six minutes.
2. Place strips in 0.1% methanolic bromphenol-blue for 30 minutes. Do not aggitate.
3. Place strips from dye bath directly into 5% acetic acid, for six minutes.
4. Repeat step No. 3, two times.
5. Remove strips from acetic acid bath and blot. Transfer the strips to a drying rack.
6. Place drying rack in the previously described oven for 15 minutes.
7. Transfer dried strips to a staining rack and suspend in an ammonia vapor-bath for 15 minutes.
8. Evaluate by scanning as previously described.

II. Staining method for serum and/or plasma glycoprotein.

1. After heat fixation, place strips in staining rack and immerse in 95% ethanol for 10 minutes, to remove the buffer and other materials not bound to protein.

2. Transfer the rack to the periodic acid solution for five minutes.
3. Transfer the rack to a bath of running tap water for 10 minutes.
4. Transfer the rack to the staining solution (Schiff reagent) and allow staining to continue for 30 minutes.
5. Transfer the straining rack immediately to the first sulfite rinse for 10 minutes.
6. Transfer the staining rack from the first sulfite rinse to a running water bath for 10 more minutes.
7. Transfer the staining rack from the running-water bath to the second sulfite rinse for 10 more minutes.
8. Blot the strips and transfer to a drying rack. Allow to dry in front of a fan.
9. Evaluate the strips when thoroughly dry in the Analytrol scanner, using the previously listed conditions.

III. Staining method for serum and/or plasma lipoprotein.

1. After heat fixation, place strips in staining rack and immerse in ethanolic oil red "O" for 24 hours.
2. Remove strips from dye bath and place in a running tap-water bath. Blot and allow to air-dry.
3. Evaluate by scanning in the Analytrol scanner.

APPENDIX III

Staining solution used to stain protein, glycoprotein, and lipoprotein in serum and/or plasma.

1. Acetic acid rinsing solution--

   Five per cent glacial acetic acid (USP grade or better) in distilled water. One liter of rinse is used for each rinse. Discard first rinse, after it has been used once. Second rinse may be re-used as the first rinse for the next batch of strips. Third rinse may be re-used as the second rinse on next batch of strips. Third rinse for each batch must be fresh.

2. Alcoholic bromphenol-blue--

   Dissolve 1 gram bromphenol (acid salt) in one liter methanol, purified grade. When the stain turns blue, discard it.

3. Ethanolic rinse--

   95% ethanol. May be re-used many times.

4. Ethanolic oil red "O"--

   Add 0.8 gm of solid oil red "O" to 1200 ml of 95% ethanol in a 3000 ml flask. Allow this to stand 15 minutes, with occasional swirling. Add 800 ml of boiling water and cover the flask-mouth with aluminum foil. Heat to boiling using a steam or electrical heating unit. Allow the mixture to cool in a 37° C. incubator overnight while stirring constantly with a magnetic stirrer. On the following day, heat the flask to about 41° C. and filter through a pre-warmed funnel, using a large fast filter paper. At no time should the temperature drop below 37° C. or the stain will be ruined. Replace the stain in the incubator for permanent storage.

5. Methanol pre-rinse--

   Methanol, purified grade. One liter required and may be re-used several times.

6. Periodic acid--

   Dissolve and mix the following materials: 8 g periodic acid, 300 ml water, 700 ml 95% ethanol, and 2.72 g sodium acetate. When not in use, the solution should be stored in a dark refrigerator.

7. Shiff reagent--

   Dissolve 6 g. basic fuchsin in 1200 ml of 90° C. water; cool to 50° C. and filter. Add 30 ml of 2 N. hydrochloric acid and 4 g potassium metabisulfate to the filtrate. Stopper and allow to stand in a cool, dark place overnight. Add 3 g powdered animal charcoal to this mixture and allow to stand for one minute. Filter and keep in a refrigerator when not in use.

8. Sulfite rinses--

   Dissolve 8 g potassium metabisulfite in 2000 ml water. Add and mix 20 ml of concentrated HCl. Discard rinses after their first use.