Some Effects of Inorganic Salts on the Blood Density and Tissue Fluids of the Bluegill, Lepomis Macrochirus.

Roland Abegg

Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Part of the Life Sciences Commons

Recommended Citation

https://digitalcommons.lsu.edu/gradschool_disstheses/7936

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
MANUSCRIPT THESES

Unpublished theses submitted for the master's and doctor's degrees and deposited in the Louisiana State University Library are available for inspection. Use of any thesis is limited by the rights of the author. Bibliographical references may be noted, but passages may not be copied unless the author has given permission. Credit must be given in subsequent written or published work.

A library which borrows this thesis for use by its clientele is expected to make sure that the borrower is aware of the above restrictions.

LOUISIANA STATE UNIVERSITY LIBRARY
SOME EFFECTS OF INORGANIC SALTS ON THE BLOOD DENSITY AND
TISSUE FLUIDS OF THE BLUEGILL, LEPOMIS MACROCHIRUS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Zoology, Physiology and
Entomology

by
Roland Abegg
B.A., University of Michigan, 1936,
M.S., Louisiana State University and
Agricultural and Mechanical College, 1939
August, 1948
The writer wishes to express his appreciation to the Louisiana Petroleum Refiners' Waste Control Council which financed this research project. The refineries that participated in this project are: Chalmette Petroleum Corporation, Cities Service Refining Corporation, Continental Oil Company, Esso Standard Oil Company of New Jersey, Pan-American Petroleum Corporation, and the Shell Oil Company, Incorporated. Appreciation is also due the Louisiana Department of Wildlife and Fisheries, Division of Research and Statistics for supplying this project with the laboratory space and much of the equipment which made this research possible.

The writer wishes to thank Mr. Louis Cusachs, formerly director of this division, who was largely responsible for the initiation of this cooperative enterprise between the refineries of the state and the Louisiana State University. His contributions to the work through his technical knowledge and cooperation have been substantial. Mr. Frank J. Coogan, his successor, has continued to support the project through his interest and assistance. The writer also wishes to express his appreciation to Mr. A. J. Wilson, Superintendent of the United States Fish Culture Station, Hatchitoches, Louisiana, for his generous cooperation in supplying the fish so vital to the research program; to Mr.
James Brown, Director of the Division of Fish and Game, Louisiana Department of Wildlife and Fisheries, who kindly arranged to transport the fish from Natchitoches to Baton Rouge; to Miss Caroline Sentilles whose secretarial assistance was of inestimable value and for whom no task was too much trouble; to M. T. Losavio for her assistance in analyzing the reference waters used in this study; and to the many other people for their encouragement, advice and assistance in helping to bring this work to completion. I wish to refer especially to my family whose sacrifices were manifold and to my friends and colleagues, Messrs. Robert E. Opferkuch, Jr. and Mr. James E. Williams, Jr. whose faith and counsel have been a source of encouragement to the writer throughout this work.

The writer is indebted to his committee for their encouragement and many helpful suggestions. He is especially desirous of expressing his appreciation to the Chairman of the committee, Dr. George H. Mickey, whose proficiency in and enthusiasm for research have been an inspiration to the author during the years of association with him.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I</strong> INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>II</strong> LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td><strong>III</strong> METHODS AND TECHNIQUES</td>
<td>14</td>
</tr>
<tr>
<td>- The test animal</td>
<td>15</td>
</tr>
<tr>
<td>- Acclimatizing the test animal</td>
<td>16</td>
</tr>
<tr>
<td>- Preparation of reference water.</td>
<td>17</td>
</tr>
<tr>
<td>- Test conditions</td>
<td>22</td>
</tr>
<tr>
<td>- Quantitative and qualitative testing</td>
<td>26</td>
</tr>
<tr>
<td>- Blood density</td>
<td>27</td>
</tr>
<tr>
<td>- Tissue fluids</td>
<td>32</td>
</tr>
<tr>
<td>- Phenol red experiments</td>
<td>33</td>
</tr>
<tr>
<td><strong>IV</strong> RESULTS</td>
<td>35</td>
</tr>
<tr>
<td>- The normal bluegill under laboratory conditions</td>
<td>35</td>
</tr>
<tr>
<td>- Test groups</td>
<td>38</td>
</tr>
<tr>
<td>- Sodium sulfate</td>
<td>38</td>
</tr>
<tr>
<td>- Sodium chloride</td>
<td>43</td>
</tr>
<tr>
<td>- Calcium chloride</td>
<td>44</td>
</tr>
<tr>
<td>- Sodium orthophosphate, dibasic</td>
<td>44</td>
</tr>
<tr>
<td>- Potassium chloride</td>
<td>45</td>
</tr>
<tr>
<td>- Sodium chromate</td>
<td>45</td>
</tr>
<tr>
<td>- Sodium dichromate</td>
<td>46</td>
</tr>
<tr>
<td>- Sodium carbonate</td>
<td>46</td>
</tr>
<tr>
<td>- Phenol red experiments</td>
<td>47</td>
</tr>
<tr>
<td>- Iv</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
</tr>
<tr>
<td>V DISCUSSION</td>
<td>49</td>
</tr>
<tr>
<td>Techniques</td>
<td>49</td>
</tr>
<tr>
<td>The normal fish</td>
<td>57</td>
</tr>
<tr>
<td>Test results</td>
<td>59</td>
</tr>
<tr>
<td>VI CONCLUSIONS</td>
<td>71</td>
</tr>
<tr>
<td>VII SELECTED BIBLIOGRAPHY</td>
<td>75</td>
</tr>
<tr>
<td>VIII VITA</td>
<td>80</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>I</strong> Frequency Distribution of the Percents Tissue</td>
<td>36</td>
</tr>
<tr>
<td>Fluid of Normal Fish Muscle <em>(Lepomis macrochirus)</em></td>
<td></td>
</tr>
<tr>
<td><strong>II</strong> Frequency Distribution of the Densities of</td>
<td>37</td>
</tr>
<tr>
<td>Normal Fish Blood <em>(Lepomis macrochirus)</em></td>
<td></td>
</tr>
<tr>
<td><strong>III</strong> Blood Density Means and Percent Tissue Fluid</td>
<td>40</td>
</tr>
<tr>
<td>Means of the Several Control Groups of Fish Tested</td>
<td></td>
</tr>
<tr>
<td>Between February 29 and April 7, 1948 *(Lepomis</td>
<td></td>
</tr>
<tr>
<td>macrochirus)*</td>
<td></td>
</tr>
<tr>
<td><strong>IV</strong> Solution Characteristics and Statistical Data</td>
<td>42</td>
</tr>
<tr>
<td>on Tissue Fluid and Blood Density Values for Test and</td>
<td></td>
</tr>
<tr>
<td>Control Groups of Fish for Each Chemical Tested *(Lepomis</td>
<td></td>
</tr>
<tr>
<td>macrochirus)*</td>
<td></td>
</tr>
<tr>
<td><strong>V</strong> Minimum, Mean and Maximum Blood Density and</td>
<td>53</td>
</tr>
<tr>
<td>Percent Tissue Fluid Values for the Control and Test</td>
<td></td>
</tr>
<tr>
<td>Groups of Fish for Each Chemical Solution to Which Fish</td>
<td></td>
</tr>
<tr>
<td>Were Subjected <em>(Lepomis macrochirus)</em></td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lateral View of Fish Showing Incisions for the Exposure of the Heart and the Muscle Tissue. (Lepomis macrochirus)</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>Ventral View of Fish Showing the Heart Exposed in Preparation for Obtaining Blood for a Density Determination. (Lepomis macrochirus)</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>Laboratory Work Sheet Devised for Recording Blood Density Data and Calculations Obtained by the Falling-drop Technique.</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Laboratory Work Sheet Devised for Recording Percent Tissue Fluid Data and Calculations.</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Frequency Distribution Curve of the Percents Tissue Fluid of a Sample of 81 Normal Fish. (Lepomis macrochirus)</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>Frequency Distribution Curve of the Blood Densities of a Sample of 78 Normal Fish. (Lepomis macrochirus)</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>Blood Density Means and Percent Tissue Fluid Means of the Several Control Groups of Fish as Plotted Against Their Respective Determination Dates (Lepomis macrochirus)</td>
<td>41</td>
</tr>
<tr>
<td>8</td>
<td>Blood Density Ranges and Means of the Control and Test Groups of Fish for Each Chemical Solution to Which Fish Were Subjected. (Lepomis macrochirus)</td>
<td>54</td>
</tr>
<tr>
<td>9</td>
<td>Percent Tissue Fluid Ranges and Means of the Control and Test Groups of Fish for Each Chemical Solution to Which Fish Were Subjected. (Lepomis macrochirus)</td>
<td>54</td>
</tr>
</tbody>
</table>
ABSTRACT

There is a large body of literature available on the effects of inorganic salts on marine animals but that dealing with fresh-water species is very small. Demands for this information are mounting steadily as state and federal authorities become more insistent upon maintaining the purity of natural bodies of water. In order to adequately and fairly control the purity of streams it is necessary to know what substances are toxic to their contained fauna, what concentrations are effective and their mode of action. This dissertation considers the effects of some inorganic salts on the internal water balance of the bluegill, *Lepomis macrochirus*. That the results of this study might be of comparative value to other investigators the conditions of the tests are standardized in every way to insure their reproducibility.

The concentrations of the chemicals used in this study are those which by testing yield 24-hour median tolerance limit values for the test animal species, 4-10 centimeters in length. The test fish used are considerably larger however, weighing between 15 and 35 grams, though the extremes in weight allowed in any one test are plus or minus 5 grams. The dilution water used for the test and control solutions is prepared from distilled water and analytical.
grade chemicals. Its composition duplicates the mean surface water of the United States. Complete directions for the preparation of this water are presented. For five days prior to their use the test animals are acclimatized to the test conditions.

Two liters of solution per fish is considered an adequate volume to prevent any marked changes in the solution characteristics during the test period. Fish are kept in the well aerated solutions for 24 hours unless it becomes evident that the number of fish surviving this time interval will be too few for the determinative testing. In these cases the testing is begun sooner and the duration of exposure is recorded. The physical data recorded for each solution includes: temperature, pH, specific resistance in ohms, concentration of the salt in parts per million, and the calculated increase in the osmotic pressure of the solution due to the added salt.

The determinative tests are designed to indicate the changes in the water balance of the fish. To obtain these data blood density and percent tissue fluid changes are measured. The blood densities are determined with a falling drop densiometer and the percents tissue fluids by the loss of weight of muscle tissue after drying for 24 hours at 110 degrees centigrade. The methods for obtaining these samples is described in detail.

In a separate series of tests phenol red is added
to the salt solutions and to the reference water of a control group in the proportions of one part in 10,000. These tests indicate that fresh-water fish swallow more water in salt solutions than in fresh water and that the fish in fresh water swallow more water than the literature would lead one to believe. There is no quantitative data given on this point. The phenol red tests further indicate that the mucus covering of the body is destroyed by the salt solutions. The mucus is dissolved by all test solutions except sodium chromate, sodium dichromate and sodium carbonate. These salts cause the mucus to precipitate.

The blood density and tissue fluid data are analysed statistically. Conclusions are based on statistical significance although significant trends are noted and suggested interpretations presented. All data from the control fish are analysed and discussed separately.

The mean blood density for 78 control fish is 1.0414 and 81 control fish yield a mean percent tissue fluid value of 81.08. The skewness of the frequency distribution curves for both sets of data indicate an internal shift of water from the blood to the tissues supporting the data presented by the various control group means relative to time. As the testing period only covers 39 days these data are only tentatively interpreted as seasonal shifts in the body fluids. Eight inorganic salt solutions are used in the testing program: sodium sulfate, sodium chloride, calcium chloride,
sodium ortho-phosphate, dibasic, potassium chloride, sodium chromate, sodium dichromate, and sodium carbonate. The chloride solutions and those of sodium dichromate and sodium carbonate cause statistically significant decreases in the percent tissue fluids. Sodium dichromate and sodium carbonate solutions cause increases in the percent tissue fluids. Statistically significant decreases in the density of the blood are observed in fish subjected to solutions of sodium chloride and sodium dichromate.

The effects of the solutions on the water balance is explained on the basis of osmotic effects and relative mobility of the ions, except in the cases of the dichromate and carbonate sodium. These two salts are toxic at very low concentrations and their effects are thought to involve precipitation reactions with the proteins of the gill membranes altering the permeability of the latter.

The shifts in ranges of the data of the test groups in relation to the control groups indicate incipient changes in the water balance of the fish which do not reach statistical significance in the time the fish are exposed to the solutions. These shifts are however considered significant and in no case do they conflict with the general interpretations suggested.

The literature dealing with the general problem of osmotic regulation in marine and fresh-water fishes is extensively reviewed and the conclusions and interpretations
presented are integrated with the more pertinent literature on the subject.
INTRODUCTION

The material to be presented in the following pages is an attempt to add certain basic knowledge to the existing literature on fish physiology and particularly on that phase which deals with the toxic effects of salt solutions on fresh-water fishes. The efforts of other workers in this field have been directed mainly toward an understanding of the physiological effects on marine fishes of changes in osmotic pressure. This is understandable for the sea has a fish fauna which is exploited commercially and yields a wealth of material to the physiologist in the form of large readily available specimens. Furthermore the outstanding biological stations of the world are located near the sea and the contributions to the literature from these stations have logically dealt with the physiology of marine species.

In the past decade greater emphasis has been laid on the ecological study of fresh-water species. This has been due in part to the rapid expansion of the industrial facilities of this country. Consequently a greater burden has been placed on the capacities of the fresh-water streams to carry the effluents discharged into them. The life in these streams has been seriously affected by the increased volume of industrial wastes, and many streams, which once supported a rich and varied fauna and flora are now barren.
This problem of stream pollution is receiving a great deal of attention from state and federal authorities with the resulting enactment of a great deal of stringently restrictive legislation. Much of this legislation is basically unsound for it is founded more on good intentions than on scientific facts. This study proposes to supply some few facts which may help in placing the conservation of the fishes of the streams and the effluents of industry in their proper relationship.
Bert (1871) was the first to observe the effects of transferring fish to an environment of a density higher or lower than the one to which they were accustomed. Freshwater fish lost weight when placed in sea water; most of this loss he assigned to the muscle tissue, though he did not state the reason for this assertion. Fresh-water fish killed by exposure to sea water floated in sea water, while marine fish killed by exposure to fresh water sank in the fresh water. These facts led Bert to conclude that the specific gravity of the fresh-water fish was less than that of the salt-water fish. He made numerous attempts to acclimatize fresh-water fish to sea water by gradually increasing the concentration of sea-water salts in fresh water. The end result in all cases was death, although the fish died more slowly after acclimatization than they did when plunged directly from fresh water into sea water.

Bert’s grasp of the whole problem was exceptionally good and his reasoning, relative to the role the gills played in the osmotic regulation of the fish, has stood the test of time. Numerous investigations since then have borne out his hypothesis. Particularly active in this line of research have been Summer ('06, '12), Dakin ('08a), Scott and White ('10), Scott and Denis ('13), H. W. Smith ('29b, '30, '32) and Keys ('31a, '31b, '33, '37) and Keys and Willmer ('32).
such cells in fresh-water fishes, but has as yet been
limited to a single species. However, these secretory cells are also located at the base of the Gill lamellae. The main characteristic of these cells is their ability to resemble the mucous cells which are also located at the water-trough in a little dorsal (key, 37) as they are in marine species. The presence of these cells in fresh-water fishes, led him to conclude that the same mode of regeneration of the cartilaginous crest is in both sea water and fresh water. He also noted that the cartilaginous crest, although supported by the cartilaginous crest, must be present in the head region of these fishes, but their exact location is not yet known. For these reasons he concludes that the cartilaginous crest is at least in the case of the marine teleosts the testum are excoted, at least in the region of the head, and that, at least in the case of the marine teleosts, concentration showed that the cartilaginous crest is at least in the head, and

H. W. Smith (1904) did some very careful research on the problem which finally established the role of the kid-
eosinophilia to a more active state of mucous secretion.
Thus the chain of evidence, built up since the time of Bert,
has been completed and the broad outlines of water regulation
in the fishes have been delineated.

Aquatic organisms meet the conditions of their var-
ious environments in several ways and with different degrees
of tolerance. The marine invertebrates assume the osmotic
pressures of their environments (Fredericq, 1885; Quinton,
'00a, '00b; Dakin, '08b; Dakin and Edmonds, '31; Bethe, '30).
Schleiper ('30), Duval ('28) and Weil and Pantin ('31) re-
port that some species of marine invertebrates have the
ability to maintain their body fluids at an osmotic press-
ure slightly superior to their environments. Fresh-water
and euryhaline species have body fluids superior to their
environments but greatly inferior to the osmotic pressures
exhibited by the marine species. Marine forms are able to
withstand considerable changes in the osmotic pressures of
their environments, the osmotic pressures of their body
fluids changing in the same direction as the environment until
equal values are reached (Fredericq, '03; Dakin, '08b).
Garry ('05) suggests that the marine invertebrate membranes
are permeable to both water and salts and merely act as
dialyzers. Adolph ('25) reports that marine invertebrates
can survive abrupt changes in the osmotic pressures of their
environment as great as that produced by diluting sea water
up to 98 per cent with distilled water. Fresh-water forms
cannot stand such changes, even when the change is effected very gradually. Osmotic pressure may be an effective limitation of distribution in its own right, but another consideration of equal importance is the ionic balance of the water. Coutance (1884) reports the complete toxicity to mollusks of changes in the proportions of the salts in environments of equal osmotic pressures. Loeb (1900) has reported the same condition in respect to Fundulus, a teleost.

Fishes exhibit a similar diversity in respect to their ability to adjust to changes in their environment. The mode of this adjustment is, however, of a different nature from that found among the invertebrates. Smith (1932) divided fishes into two classes, according to their mode of adjustment: the elasmobranchs and the teleosts. Since the appearance of the findings of Keys and Willmer (1932) on the chloride secreting cells of the gills, the special modifications exhibited by the fresh-water and euryhaline teleosts makes it more practical to divide the fishes into four types. These types, classified according to the tonicity of the body fluids in relation to their environment, are:

1. the elasmobranchs (both marine and fresh-water species) are isotonic with or hypertonic to their environment; 2. the marine teleosts are inferior to their environment; 3. the fresh-water teleosts are hypertonic to their environment but
inferior to the marine teleosts; and 4, the euryhaline species which may be either hypotonic in a marine environment or hypertonic in a fresh-water environment. The diversified environmental conditions that must be met by the various types of fishes in this last category require that each type be so equipped that its requirements are met. Not only is the mode of regulation different in the several groups but the degrees of tolerance are also various which is the principal reason for dividing the teleosts into three groups instead of in one as suggested by Smith (132).

The elasmobranchs exhibit the most peculiar form of osmotic pressure regulation of any of the types. They withstand relatively great variations in the osmotic pressures of their environments but are able to withstand only about a 20 per cent variation in the salt content of the body fluids. The percentage concentration of the salts of the body fluids is always below the salt content of the sea (Bottazzi, 1897). Nevertheless they maintain a superior tonicity to their environment by maintaining a constant state of uremia (Rodier, 1899). Staedler and Freich (1858) were the first to observe the high urea content of the elasmobranch blood. Fresh-water elasmobranchs are also superior to their environment and in both environments the elasmobranchs secure water in the same manner, by its absorption through the oral membranes. Like all the lower
vertebrates the urine is isotonic with, or hypotonic to the
blood (Bottazzi, '06 and Burian, '08). This is true of all
vertebrates below the mammals. Only in this highest class
of vertebrates is found the loop of Henle which enables the
kidney to reabsorb water, (Marshall, '30), and thus excrete
a urine which is hypertonic to the blood. The kidneys and
the oral membranes are relatively impermeable to urea and
thus the elasmobranch maintains a uremic condition. Smith
('29a) asserts that in the elasmobranchs urea is not pri-
marily a mode of nitrogen excretion, but must be considered
a metabolite in its own right. The urea is lost by simple
diffusion at a steady rate, regardless of the salinity of
the environment and can be separated from the excretion of
chlorides, which is an active process to be utilized at the
demands of the environment. In summary then, the elas-
mobranchs like the invertebrates are isotonic with, or
hypertonic to, their environments but maintain this condi-
tion by their own activity through a state of uremia, whereas
the marine invertebrates, in general, merely react passively
to their environment and the salt content of their body
fluids is isotonic with it.

The marine teleost is an organism which is hypotonic
to its environment and is required to maintain this condition
by conserving water and excreting salts. As the kidneys can
only excrete urine which is hypotonic to the blood, the
excretion of salts must be done extrarenally. The amount of
salts to be thus excreted must be considerable for the only source of water available to the marine teleost is the sea water. The fish must therefore separate the salts from the sea water to produce a hypotonic urine. To accomplish this the marine teleost swallows copious quantities of water but produces only small quantities of urine. According to Smith ("30a) calcium carbonate is largely excreted by the intestinal tract in the form of pellets while the magnesium and sulphate ions are excreted by the kidneys along with small quantities of sodium and potassium chloride, some carbon dioxide and the phosphates. The bulk of the chlorides are excreted extrarenally by the gills as suggested by Bert as early as 1871, and by Smith ("31), who arrived at this conclusion by elimination of other structures through testing and logic. Keys and Willmer ("32) were the first to describe the actual cells responsible for this extrarenal secretion of hypertonic salt solutions. The cells which they termed "chloride secreting cells" are found at the base of, and between the gill lamellae of all fish examined by them. They are apparently much more numerous in marine teleosts than in the fresh-water species.

In the continual fight to conserve water the glomeruli of the marine teleost kidney have been greatly reduced in numbers or have been eliminated (Marshall and Grafflin, '28). The glomerulus has been shown to be primarily associated with the excretion of large quantities of urine from
which the fish would be unable to resorb the water due to
the lack of Henle's loop.

In comparison to the marine teleosts, the fresh-
water teleosts have exactly the opposite conditions to meet.
They are hypotonic to their environment which requires that
they conserve salts and excrete large quantities of water.
For these conditions they are much better adapted to their
environment than the marine teleosts are to theirs and it is
due to these and other considerations, that Smith ('32)
postulates that the marine teleosts were derived from fresh-
water ancestors. In contrast to the marine teleosts, the
fresh-water teleosts swallow very small quantities of water,
obtaining most of their water by means of the oral membranes
and gills (Smith '30). Furthermore the kidneys of all fresh-
water teleosts are glomerular which results in a urinary ex-
cretion which is fifteen to thirty times the volume found in
the marine forms (Smith '32). To conserve salts, the gills
are almost devoid of chloride secreting cells and their
very presence is doubtful (see page 2 and Keys, '37).

The last type of water-regulation found in the tel-
eoasts, the euryhaline type, is in reality a combination of
that found in the marine stenohaline forms, rather than a
distinct mode of regulation of its own. The teleost of this
type which has received the greatest attention from the phy-
siologist is the catadromous eel, though the anadromous
salmon probably exhibits the same method of regulation. In the former case no cyclic phenomena are involved in the ability to adjust from one environment to another, but whether this obtains in the salmon is not known. The only evidence available for this species is the presence of land-locked salmon and the presence of the necessary cellular structures for the survival of such changes. In both fish, but especially in the eel, the chloride secreting cells are very abundant but as these only function when the internal environment demands the elimination of salts, their presence when the fish is in fresh-water is no disadvantage. Keys ('33) states that the eel acts like a normal fresh-water teleost in fresh-water, and like a normal marine teleost in sea water. The eel and the salmon are the extremes of the euryhaline group but there are many intermediate forms that are able to withstand relatively great changes in the osmotic pressures of the environment but may never adjust to either extreme, or, as will be pointed out, they exhibit what may be physiological races within the same species. Fundulus, one of the better known euryhaline species, varies the osmotic pressure of its tissue fluids to some extent to meet its environment. Stenohaline marine forms have osmotic pressures like strictly marine forms (Δ 0.76° C.) while the fresh-water species are like the stenohaline forms (Δ 0.57° C.) Despite the wide changes in osmotic pressure some species can
tolerate, they generally cannot stand as abrupt changes as the eel. Summer ('06b), Scott ('13). Scott ('13), E. Smith ('12) and especially Summer ('06b, '12) have shown that Fundulus exhibits all degrees of flexibility to adjust to changes in the environment, not only between species but between individuals of the same species. Purely marine forms never acclimatize to low salinities while others will do so if the acclimatization is extended over a period of one or two weeks. It is interesting to note that some species will never adjust to fresh-water but will survive in water containing as little as one per cent sea water (Summer '12). Summer ('06) describes the surprising condition of Fundulus diaphanus which is known to normally inhabit brackish waters and fresh waters but which he was unable to transfer from mildly brackish water to fresh-water. This may be a case of physiological speciation in its early stages.

The euryhaline species, if they are all similar to the eel, are capable of adjustment to changes in the salinity of the environment by virtue of the possession of the chloride secreting cells of the marine species combined with the fresh-water characteristics of their primitive condition.

Regardless of the mode of regulation employed by the various types of fishes it seems probable that the blood is the mediating system in each case. The final effect of
osmotic regulation lies in the water content of the fish's tissues. This water content has been suggested (Smith, '32) as the fundamental steady state and that this water content is maintained in relation to the protein of the tissue.

The effect of changes in the environment on the blood has been extensively studied in marine and euryhaline species from the standpoint of osmotic pressure relationships. The data on fresh-water species is much more limited. The question of blood density has been largely ignored by most investigators. Scott ('10) followed the simultaneous changes in the number of red blood cells and blood densities when marine fish were placed in waters of different densities. He found that the densities of the water and the blood and the number of red blood cells varied together. The only other reference to blood densities of fish is that of Westfall ('43). He subjected goldfish (Carrassius) to low oxygen tensions so that the fish developed a rapid anoxia. This produced a low blood density and a simultaneous decrease in the red blood cell count. When the fish were placed in a one percent solution of sodium chloride and subjected to rapid anoxia the density changes were not obtained.
METHODS AND TECHNIQUES

The methods employed in setting up the testing conditions were, in general, those proposed by Hart, Doudoroff and Greenbank ('45). The quantitative determinations performed upon the fish at the completion of the testing periods were suggested in the literature. Many of the early workers in the field of fish physiology employed the techniques of measuring the changes in the degree of hydration of fishes after they were subjected to changes in their environment. Most of this work was done with marine or brackish-water species or with fish which periodically move from a marine to a fresh-water environment like the anadromous salmon or vice versa, like the catadromous eel.

The use of the falling drop densiometer for measuring changes in the specific gravity of small quantities of fish blood was original with the writer, though later it was discovered that this method had been employed by Westfall ('43). Scott ('10) used the more cumbersome Hammerschlag benzole-chloroform method.

The description of the methods and techniques employed during the course of this study is extended because it is hoped that the material may benefit those who may continue the work on the present problem. The techniques are not well established and only a very limited number of
laboratories employ them.

**The Test Animal**

At the outset of the present study it was decided that the conditions of the tests should meet the minimum specifications of the procedure for reference testing as outlined by Hart, Doudoroff and Greenbank ('45). It was found, however, that the limitation placed upon the size of fish to be used, (4-10 centimeters) could not be adhered to because it was impossible to obtain sufficient blood for density determinations from fish of this small size.

The test fish, *Lepomis macrochirus*, used in this study belongs to the family Centrarchidae, which includes the sunfish and bass. These fish are designated as warm-water reference test animals by Hart, Doudoroff and Greenbank ('45). This designation indicates that they are moderately sensitive test animals, and that they are suitable for tests in which the test temperatures do not fall below twenty degrees centigrade.

Fish were obtained from the United States Fish Culture Station in Natchitoches, Louisiana, an agency of the United States Department of the Interior. The fish were transported from the station to the laboratory in Baton Rouge, in trucks specially designed for the transportation
of fish. Although some mortality occurred among the fish of the first few shipments, this condition was corrected before the actual testing for the present study was begun. It was found that the pH change to which the fish were subjected when transferred from Cane River water, pH 7.3, to Baton Rouge tap water, pH 8.6 was so great as to cause considerable mortality. Upon arrival in Baton Rouge, the fish were placed in outdoor concrete holding tanks. The water in these tanks was well aerated and its temperature was adjusted to 3°C of the transportation water temperature. The pH was adjusted with dilute hydrochloric acid to within 0.4 units of the transportation water. Tap water was slowly sprayed into the tanks so that after a few days the tank water had assumed the pH of the tap water. This allowed the fish ample time to adjust to the higher pH.

The fish were fed daily a commercial, pellet-form, goldfish food. The pellets are quite hard and sink to the bottom of the tank. If they are not eaten immediately they imbibe water, swell and disintegrate, eventually causing the water to become cloudy. This is an important factor in holding fish in acclimatizing tanks wherein it is impractical to change the water frequently. Consequently, the fish were fed only what they would consume immediately.

**Acclimatizing the Test Animal**

Several days before a test was to be made, a given
group of fish was brought into the laboratory to be accli-
matised. Usually the temperature of the tap water in the
holding tanks was quite different from that of the tap water
in the laboratory tanks. To avoid sudden temperature
changes (which are detrimental to the fish) the temperature
of the water in the laboratory tanks was adjusted to that
of the water in the holding tanks. The water in the labora-
tory tanks was then allowed to assume the room temperature.
A temperature of $22^\circ \pm 1^\circ$ C was maintained in the air con-
ditioned laboratory. The temperature of the acclimatizing-
tank water was $21^\circ \pm 1^\circ$ C. This temperature was sufficiently
close to the test temperature of $22.5^\circ$ C to avoid secondary
effects on the fish due to temperature shock.

After the fish had been acclimatized to temperature,
a group of 26 to 30 were transferred to reference water of
the same temperature for a minimum period of four days.
These fish were graded roughly for size, to assure a rela-
tively homogeneous group. As the reference water was the
same temperature as the tap water in which the fish had
been, they received only the shock of the changed chemical
composition of the water.

**Preparation of Reference Water**

The term "reference water" designates a water con-
taining all the major ions in concentrations and proportions
typical of a mean surface water of the United States. In
addition, the solution contains minor mineral elements in concentrations typical of natural waters. Reference water was prepared in the laboratory using distilled water and analytical grade chemicals.

To satisfy the large demand for reference water it was made up in 100 gallon lots. Distilled water was obtained from a five-gallon-per-hour steam-heated still which produced a distillate of very high quality, 350,000 to 400,000 ohm specific resistance at 25° C. This water was collected in a 100 gallon capacity tin-lined tank. The first 16 gallons of distilled water were drawn off into ten gallon capacity earthenware crocks, to be used in making up an intermediate concentrate of the chemicals required for the 100 gallons. This intermediate step was necessary as the reference water formula calls for 90 p.p.m. bicarbonate ion, most of which must be in the form of calcium bicarbonate. This chemical exists only in solution, and was made by converting calcium oxide, with carbon dioxide gas, to a calcium carbonate suspension. The water to which the suspension was to be added was saturated with the gas until the pH was constant, usually 5.0. The suspension then was added slowly and gas was bubbled through the solution until the conversion was complete and the solution had become crystal clear. The necessity of lowering the pH to 5.0 was the reason for making the intermediate concentration in the earthenware crocks. At this low pH the tin lining of the tank tends to change to the
stannic form and powder off, exposing the copper of which the tank is made. Copper salts are notoriously toxic to fish and their appearance in the test solutions would have invalidated any experiments in which such water was utilized.

The maximum and minimum limits of chemical composition to which this water must conform are set forth by Hart, Doudoroff and Greenbank (145) as follows:

Total alkalinity (as CaCO$_3$) 60 - 120 p.p.m.
Total hardness (as CaCO$_3$) 75 - 150 p.p.m.
Sulfates (as SO$_4$) 20 - 50 p.p.m.
Dissolved solids (maximum) 500 p.p.m.

Reference water meeting these specifications was prepared using the following solutions:

1. MgSO$_4$.7H$_2$O .................................. 71.0 grams
   K$_2$SO$_4$ ........................................ 6.5 grams
   MnSO$_4$.4H$_2$O .................................. 0.2 grams
   Made up to one liter

2. CaCl$_2$.2H$_2$O .................................. 18.6 grams
   Made up to one liter

3. NaHCO$_3$ ........................................ 25.0 grams
   NH$_4$NO$_3$ ..................................... 3.0 grams
   K$_2$HPO$_4$ ..................................... 1.1 grams
   Made up to one liter

---

1 The author was supplied with this procedure through the courtesy of the Waste Control Laboratory of the Atlantic Refining Company, Philadelphia, Pennsylvania.
4. CaO .................................. 32.2 grams
   Made up to one liter and pure
   carbon dioxide blown through this
   mixture to make a CaCO₃ suspension.
5. Na₂SiO₃·9H₂O  ................. 62.6 grams
   Made up to one liter.
6. FeCl₃·6H₂O  ................. 1.2 grams
   Made up to one liter.

The reference water was prepared as follows from the above solutions:

1. One milliliter of each of the solutions 1, 2, and
   3 were added for each liter of the final volume
   of water. Gentle agitation was used during the
   addition of the solutions.
2. Pure carbon dioxide was blown through the solu-
   tion at a medium rate, using a carborundum diffuser,
   until the solution reached a pH of about 5.0.
3. One milliliter of solution 4 was added for each
   liter of final water. Carbon dioxide was blown
   through the solution until it became crystal clear.
4. One milliliter of solution 5 was added for each
   liter of the final water. Air was blown through
   the water until the pH was about 7.4.
5. The intermediate concentrate prepared above was
   then added to the distilled water in the 100 gallon
   storage tank.
6. Just before use, one milliliter of solution 6 was added to each liter of water. The ferric chloride was not added prior to this time in order to prevent the formation of complex ferric ions which might form in the water during storage.

Reference water, as used in this laboratory, was not entirely satisfactory. Fish placed in this water showed definite signs of distress expressed as an orientation defect, the fish darting nervously about the tank, with their bodies inclined at a 45-degree angle, head down. After a few hours the fish became adjusted to the new water. After two or three days the fish again showed signs of distress, congregating in a corner of the tank, behaving sluggishly and with their bodies at a 45-degree angle but with their heads at the surface. This problem requires further attention if reference water is ever to be accepted as a standard laboratory reagent. Even with its faults reference water has enough advantages to justify its use if appropriate accommodations in testing procedures are made to circumvent these faults. Thus a period of four days for acclimatization to the water has been used to minimize the effects a longer period would produce. Its strongest single advantage lies in its reproducibility. This water can be reproduced in any laboratory by modifying either tap water or distilled water. A survey of the literature dealing with toxicity testing shows that each investigator has set up his or her own standards as
to the type of water used as the diluent. This fact, com-
plicated by the use of a variety of test-fish species of 
 dctering sensitivity, and a multitude of testing procedures 
and individual methods of reporting results, makes it 
 virtually impossible to compare the results reported by 
different authors. The universal utilization of a dilution 
 water, comparable in its standardization to Ringer's solu-
tion, would be of great value to all investigators interested 
in fish physiology.

**Test Conditions**

The test containers were five gallon, round, glass 
battery jars. Each jar was in a strap-brass handled basket 
to facilitate handling. Four jars were used in each test: 
two for the test solution and two for the reference water 
for the control group. Each jar contained twelve liters of 
solution or of reference water. The jars were placed in a 
constant temperature bath and the solutions aerated until 
they had reached the test temperature, 22.5°. To minimize 
evaporation and consequent alteration of the strength of 
the solutions, each jar was covered with a glass plate. 
Aeration of the solutions was accomplished through the use 
of compressed air forced through carborundum air dispersers 
which broke the air up into a fine stream of bubbles. In 
addition to the compressor which supplied the whole building 
with compressed air, a small compressor was installed in the
laboratory to serve as a stand-by unit. All air entering
the manifold supplying the aquaria tables passed through a
porous stone air filter to remove all oil, water and grit.
An air-pressure regulator was also installed to insure a
relatively even flow of air through the solutions. The air
dispersers were cleaned after each test by boiling them in a
strong solution of trisodium phosphate. To remove this
cleaning solution the dispersers were placed in a jar into
which flowed a steady stream of water. Air was forced
through the dispersers during this rinsing period. The
dispersers were adjudged clean and rinsed when they no
longer affected the pH of the water in which they were placed.
To further reduce the probability of contamination from
equipment used in previous tests, the hose connecting the
disperser with the air manifold was a combination of three
elements. A one-inch piece of tygon tubing was placed on
the nipple of the disperser. Into this was inserted a ten-
inch section of seran tubing which, in turn, was connected
to the air manifold by a piece of rubber tubing. This rather
complicated combination served its purpose effectively.
The rubber tubing never was reached by the solutions and,
therefore, did not require cleaning. The other two elements
could be scrubbed and placed in a potassium dichromate clean-
ing solution, and the tygon permitted the easy removal of
the disperser from the hose connection so that the disperser
might be boiled.

The constant temperature bath was equipped with a flexible-type immersion heater which was controlled by a mercury thermoregulator through a supersensitive, single tube, normally closed, mercury relay. The bath kept a constant temperature of 22.5°C ± 0.2°C. A laboratory-type centrifugal circulating pump insured adequate circulation of the water to maintain an even temperature throughout the bath. All of the test containers were accommodated in the bath simultaneously.

The test temperature 22.5°C was decided upon after careful consideration of the laboratory conditions, both summer and winter. The factors taken into consideration were the minimum winter temperature and the maximum summer temperature, with due weight being given to the capacities of the air conditioning unit (without heat control) and the constant temperature bath heater, as well as the temperature limitations placed upon the testing program by the adopted procedure. The temperature decided upon was readily maintained throughout the year without exerting an undue strain on the available equipment.

The strengths of the solutions used in the testing program were those concentrations found to yield the 24 hour median tolerance limits\(^1\) for the chemicals used. These values

\(^1\) The 24 hour median tolerance limit (24 hour TL\text{\textsubscript{24}}) is the concentration of a chemical or solution which one-half of the test animals can survive for a period of twenty-four hours. It may be expressed as either per cent or parts per million.
were determined in this laboratory by Mr. James E. Williams, Jr., ('48) and in part by the author, ('47a) working on that particular phase of the general program of toxicity testing. It must be noted that in some cases the 24 hour median tolerance limit concentrations were not as lethal to the fish of the size used in this study as they were to the smaller fish used in the median tolerance limit tests. This was a great advantage in the present study. It supplied a tested toxic concentration value for each chemical used, usually without killing the fish before they could be used in the investigation. Theoretically, then, all tests were made using the same base.

In addition to the concentration of the chemical used in the test, three characteristics of the test solutions, and of the water in the control containers, were recorded: the temperature, the pH and the specific conductance. These factors, and the known composition of the reference water, supplied the essential information necessary for comparative purposes.

When the temperature and oxygen concentration of the solutions had stabilized at the test conditions, six fish were placed in each of the four test containers.

Fish to be used for testing purposes were not fed during the twenty-four hour period preceding the beginning of the test. This gave ample time for the alimentary tract
to be cleared, thus reducing the degree to which the test solutions were modified by the introduction of the fish. To further reduce modifications of the test solutions, the fish were rinsed for a short time in clean reference water before being placed in the test containers.

Fish were selected at random from the clean reference water rinsing bath and added alternately to the test and to the control containers. Fish that were obviously diseased or injured were rejected as test animals.

It has been stated that the chemical concentrations used were not generally lethal to the large-size test fish. Exceptions to this were encountered and the employment of even two extra animals per test was no guarantee that ten test animals would survive the twenty-four hour exposure to the test solutions. In those cases in which it became evident that the test animals would not survive the period of exposure, the determinative testing was begun immediately.

Quantitative and Qualitative Testing

The testing program can be conveniently divided into two phases: quantitative testing and qualitative testing. The former includes the blood density and the tissue hydration determinations; the latter, the effects of the solutions on the body mucus and the ingestion of water by the use solutions colored with phenol red. The techniques employed in making these determinations will be discussed in the order mentioned above.
Blood Density. Fish were removed one at a time from the test containers, were pithed, weighed and the heart was exposed with a curved cutting probe. After the pithed fish were weighed the opercula were cut away (see Figures 1 and 2). An incision (AB) was made on each side of the isthmus penetrating to the parietal pericardium, and extending to the junction (A) of the isthmus with the ceratohyal. The narrow connection between the isthmus and the ceratohyal was cut and the isthmus was raised so that the connective tissue between it and the parietal pericardium could be separated. The isthmus was then cut away (BC) and the pericardial membranes teased to expose the bulbus. The pericardial fluid was removed with absorbent paper. A three-quarter inch needle tube (broken from a 23 gauge hypodermic syringe-needle) was held in a bulldog clamp and was used to pierce the bulbus arteriosus. The action of the heart pumped the blood out through the needle tube which was held so that the blood could be collected on a paraffin block. When sufficient blood had been collected, the needle tube was removed and the fish were covered with a piece of cardboard to prevent excessive evaporation of surface water while the blood density determination was being made.

The blood density determinations were made using the methods of Barbour and Hamilton ('26 and '27). The pipettes were calibrated to deliver two successive drops, each with a
Figure 1. Lateral View of Fish Showing Incisions for the Exposure of the Heart and the Muscle Tissue. (*Lepomis macrochirus*).

Figure 2. Ventral View of Fish Showing Heart Exposed in Preparation for Obtaining Blood for a Density Determination. (*Lepomis macrochirus*)
|-----------|---------|-------|----------|------------------------|-----------------------------------------------|------------------|------------|---------|

Figure 3. Laboratory Work Sheet Devised for Recording Blood Density Data and Calculations Obtained by the Falling-drop Technique.
<table>
<thead>
<tr>
<th>Solution Tested and Concentration:</th>
<th>Date and Time Test Started</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date &amp; Time of Determination</td>
<td></td>
</tr>
<tr>
<td>Test Number</td>
<td></td>
</tr>
<tr>
<td>Test or Control</td>
<td></td>
</tr>
<tr>
<td>Wt. Wet Tissue and Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Tissue</td>
<td></td>
</tr>
<tr>
<td>Wt. Wet Tissue and Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Dry Tissue and Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Tissue</td>
<td></td>
</tr>
<tr>
<td>Wt. Dry Tissue and Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Dish</td>
<td></td>
</tr>
<tr>
<td>Wt. Dry Tissue</td>
<td></td>
</tr>
<tr>
<td>Wt. Dry Tissue - Wt. Wet Tissue</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Laboratory Work Sheet Devised for Recording Percent Tissue Fluid Data and Calculations
volume of 0.01 cubic millimeter. To cover adequately the
range of fish blood densities, three dropping tubes were
required, containing xylene-bromobenzene mixtures of the
following approximate densities, 1.015, 1.030 and 1.040.

The density determination is based upon a comparison
of the apparent density differences between the xylene-
bromobenzene mixture on the one hand and the standard po­
tassium sulfate solution and the blood on the others. These
apparent density differences were measured by observing
the times taken for a drop of blood and a drop of standard
solution, each 0.01 cubic millimeter in volume, to fall 30
centimeters at a given temperature. These data were recorded
and later used to scale off the apparent density differences
of the standard and of the blood. The apparent density dif­
ference of the standard was subtracted algebraically from
the apparent density difference of the blood and the result­
ant added algebraically to the density of the standard po­
tassium sulfate solution. The standard was prepared to
the approximate density desired. The true density was then
determined by the use of a piconometer. To prevent changes
in the densities of the standards because of evaporation,
they were placed in siphon bottles and the surface of the
liquid covered with a layer of mineral oil. As the den­
sities of the xylene-bromobenzene mixtures needed to be
known only approximately, they were determined with a
Westphal balance. According to Barbour and Hamilton (126)
the falling-drop method is accurate to $1 \times 10^{-4}$, if the falling times of the drops are greater than eighteen seconds. Falling times were measured with a stop watch calibrated in one-tenth second intervals.

The laboratory data sheet which was devised for the density determinations is presented here as Figure 3.

**Tissue Fluid.** The measurement of water in the tissues was determined in the usual manner. After the completion of each density determination a strip of muscle tissue was removed from one side of the fish (see Figure 2). This was accomplished as follows: After the removal of the scales, two incisions were made, one from the nape to the posterior end of the dorsal fin (DE), and other from the nape to the pectoral fin just posterior to the girdle (DF). The skin was grasped at the angle formed by these incisions and stripped back, exposing the lateral musculature. The removal of the skin was necessary to overcome errors introduced through the loss of scales and the different degrees of surface drying experienced by the fish in the interval between its removal from the solution and the weighing of the tissue. A strip of this tissue was removed with a scalpel, care being taken to exclude neural spines, ribs or other tissues. The strip was weighed in a tared, covered, weighing dish on an analytical balance. The weight was recorded and the tissue placed in an electric oven, thermosatically controlled at a temperature of $110^\circ$ C. After 24
hours the tissue was placed in a dessicator to cool and was then weighed. The results were calculated and expressed as percent water of the wet tissue weight. The laboratory data sheet devised for the tissue fluid determinations is presented as Figure 4.

The dried tissue was crushed with a glass rod and poured into individual vials for chemical analyses not included in this study.

**Phenol Red Experiments.** A series of tests was set up to determine whether or not fish drink water when placed in salt solutions. No quantitative measurements were attempted. Phenol red was added to the salt solutions in the proportions of 1:10,000. After three hours the fish were pithed and autopsied. The presence of the dye in the gastrointestinal tract was considered proof that the fish had swallowed water.

If the stomach was empty, through the regurgitation of the water prior to the examination, it was cut open and the acid neutralized with alkali. In most cases the stomach was distended with water and the presence of color was determined by the withdrawal of the liquid into a syringe containing a small amount of strong alkali. The alkalinity of the intestinal tract made neutralization unnecessary. To insure that no phenol red was accidentally introduced on to the lining of the gastrointestinal tract when it was exposed, the fish was first washed in a stream
of tap water to remove all excess dye. If the gastrointestinal tract did not contain obvious quantities of the dye the whole tract was removed before being cut open.
RESULTS

The Normal Bluegill Under Laboratory Conditions. The bluegills used in this study were from pond-reared stocks, adequately acclimatized to the test conditions prior to being used. All fish were subject to the same preliminary laboratory acclimatizing period, feeding schedule and water conditions. The only known variable was the length of time the fish were held in the outside holding-tanks prior to acclimatization and testing.

Seventy-eight control animals, for which both tissue fluid and blood density values are available, were used during the testing period of 39 days.

The results from the complete data on normal fish, when plotted separately, showed a normal frequency distribution of blood densities for 78 determinations, with a mean of 1.0414 and a standard deviation of 0.0041003. The percent tissue fluids for 81 fish likewise gave a normal frequency distribution curve, with a mean of 81.08 percent and a standard deviation of 1.1366. A summary of this data is presented in Tables I and II, and graphically in Figures 5 and 6.

The tissue fluid and blood density mean values have been plotted against time in Figure 7. The mean values for the tissue fluids indicate a progressive hydration of the tissues between February 29 and April 4, 1948 whereas the blood density means of the several control
TABLE I  Frequency Distribution of the Percents Tissue Fluid of Normal Fish Muscle  
*(Lepomis macrochirus)*

<table>
<thead>
<tr>
<th>Tissue Fluid Percent Class</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>78.25-78.74</td>
<td>2</td>
<td>2.47</td>
</tr>
<tr>
<td>78.75-79.24</td>
<td>2</td>
<td>2.47</td>
</tr>
<tr>
<td>79.25-79.74</td>
<td>4</td>
<td>4.94</td>
</tr>
<tr>
<td>79.75-80.24</td>
<td>4</td>
<td>4.94</td>
</tr>
<tr>
<td>80.25-80.74</td>
<td>20</td>
<td>24.69</td>
</tr>
<tr>
<td>80.75-81.24</td>
<td>21</td>
<td>25.94</td>
</tr>
<tr>
<td>81.25-81.74</td>
<td>9</td>
<td>11.11</td>
</tr>
<tr>
<td>81.75-82.24</td>
<td>6</td>
<td>7.41</td>
</tr>
<tr>
<td>82.25-82.74</td>
<td>5</td>
<td>6.17</td>
</tr>
<tr>
<td>82.75-83.24</td>
<td>5</td>
<td>6.17</td>
</tr>
<tr>
<td>83.25-83.74</td>
<td>1</td>
<td>1.23</td>
</tr>
<tr>
<td>83.75-83.74</td>
<td>1</td>
<td>1.23</td>
</tr>
<tr>
<td>84.25-84.74</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>84.75-85.24</td>
<td>1</td>
<td>1.23</td>
</tr>
<tr>
<td>Totals</td>
<td>81</td>
<td>100.00</td>
</tr>
</tbody>
</table>
TABLE II  Frequency Distribution of the Densities of Normal Fish Blood

*Lepomis macrochirus*

<table>
<thead>
<tr>
<th>Density Class</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0325</td>
<td>5</td>
<td>6.41</td>
</tr>
<tr>
<td>1.0354</td>
<td>14</td>
<td>17.95</td>
</tr>
<tr>
<td>1.0355</td>
<td>19</td>
<td>24.36</td>
</tr>
<tr>
<td>1.0384</td>
<td>27</td>
<td>34.62</td>
</tr>
<tr>
<td>1.0414</td>
<td>6</td>
<td>7.69</td>
</tr>
<tr>
<td>1.0444</td>
<td>5</td>
<td>6.41</td>
</tr>
<tr>
<td>1.0445</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>1.0474</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>1.0475</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>1.0504</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>Totals</td>
<td>78</td>
<td>100.00</td>
</tr>
</tbody>
</table>
groups indicate a rising density for the same period. (See Table III) The standard deviation of the tissue fluid values of these several groups of fish is 0.150; that of the blood densities is 0.002168.

**Test Groups**

In all experiments, the solution concentration refers to the parts per million of the specific chemical added to the reference water; no account is taken of the same ions which might have been present already in the reference water. The data for all the salt solutions employed are summarized in Table IV. Variations in the numbers of fish tested does not mean that the numbers of fish at the beginning of each test were fewer than the twelve required by the chosen procedure, but rather that the number of observations made was fewer than ten because of mortality among the fish, or accidents occurring while the determinations were being made. In either case the resulting number of determinations was fewer than the intended number.

In a few instances, the values for tissue fluids and for blood densities were not obtained for the same animal, though in most cases these two values were obtained for each fish used.

1. **Sodium Sulfate.** Ten fish were placed in a 17,500 parts per million solution of sodium sulfate in reference
Figure 5. Frequency Distribution Curve of the Percents Tissue Fluid of a Sample of 81 Normal Fish. (Lepomis macrochirus)

Figure 6. Frequency Distribution Curve of the Blood Densities of a Sample of 78 Normal Fish. (Lepomis macrochirus)
<table>
<thead>
<tr>
<th>Date</th>
<th>Blood Density*</th>
<th>Percent Tissue Fluid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>February 29</td>
<td>1.0389</td>
<td>80.37</td>
</tr>
<tr>
<td>March 6</td>
<td>1.0399</td>
<td>80.30</td>
</tr>
<tr>
<td>March 12</td>
<td>1.0441</td>
<td>81.27</td>
</tr>
<tr>
<td>March 16</td>
<td>1.0417</td>
<td>80.92</td>
</tr>
<tr>
<td>March 19</td>
<td>1.0386</td>
<td>81.45</td>
</tr>
<tr>
<td>March 24</td>
<td>1.0410</td>
<td>81.47</td>
</tr>
<tr>
<td>March 28</td>
<td>1.0441</td>
<td>81.22</td>
</tr>
<tr>
<td>April 7</td>
<td>1.0427</td>
<td>81.64</td>
</tr>
</tbody>
</table>

* Mean values obtained from the control fish data collected during the experiments conducted on the respective dates mentioned.
Figure 7. Blood Density Means and Percent Tissue Fluid Means of the Several Control Groups of Fish as Plotted Against Their Respective Determination Dates. (*Lepomis macrochirus*)
**Table IV. Solution Characteristics and Statistical Data on Tissue Fluid and Blood Density Values for Test and Control Groups of Fish for Each Chemical Tested.**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>PPM</th>
<th>Sp Resist</th>
<th>Increased Osmotic</th>
<th>pH</th>
<th>Hours Exposed</th>
<th>Number Fish</th>
<th>Mean Tissue Fluid</th>
<th>Standard Deviation</th>
<th>t Value</th>
<th>Number Fish</th>
<th>Mean Control Density</th>
<th>Standard Deviation</th>
<th>t Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaSO₄</td>
<td>17,500</td>
<td>55</td>
<td>2.99</td>
<td>7.9</td>
<td>24</td>
<td>10</td>
<td>81.27</td>
<td>1.4213</td>
<td>N.S.</td>
<td>10</td>
<td>1.0441</td>
<td>0.006860</td>
<td>N.S.</td>
</tr>
<tr>
<td>NaCl</td>
<td>14,100</td>
<td>45</td>
<td>5.85</td>
<td>8.5</td>
<td>7</td>
<td>7</td>
<td>78.13</td>
<td>0.4822</td>
<td>-3.45</td>
<td>7</td>
<td>1.0363</td>
<td>0.003187</td>
<td>-0.516</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>8,400</td>
<td>70</td>
<td>1.84</td>
<td>8.3</td>
<td>24</td>
<td>10</td>
<td>81.45</td>
<td>0.9463</td>
<td>0.02</td>
<td>10</td>
<td>1.0386</td>
<td>0.002966</td>
<td>N.S.</td>
</tr>
<tr>
<td>NaHPO₄</td>
<td>5,800</td>
<td>170</td>
<td>0.99</td>
<td>9.0</td>
<td>24</td>
<td>10</td>
<td>82.42</td>
<td>1.2204</td>
<td>N.S.</td>
<td>10</td>
<td>1.0427</td>
<td>0.003129</td>
<td>N.S.</td>
</tr>
<tr>
<td>KCl</td>
<td>5,500</td>
<td>105</td>
<td>1.76</td>
<td>8.7</td>
<td>24</td>
<td>8</td>
<td>78.86</td>
<td>0.5097</td>
<td>-1.87</td>
<td>8</td>
<td>1.0377</td>
<td>0.004291</td>
<td>N.S.</td>
</tr>
<tr>
<td>Na₄CrO₄</td>
<td>930</td>
<td>520</td>
<td>0.14</td>
<td>8.1</td>
<td>24</td>
<td>10</td>
<td>81.22</td>
<td>0.8267</td>
<td>0.05</td>
<td>10</td>
<td>1.0441</td>
<td>0.001451</td>
<td>N.S.</td>
</tr>
<tr>
<td>Na₄Cr₂O₇</td>
<td>728</td>
<td>105</td>
<td>0.07</td>
<td>5.9</td>
<td>13</td>
<td>11</td>
<td>82.24</td>
<td>1.4419</td>
<td>8.42</td>
<td>9</td>
<td>1.0372</td>
<td>0.003501</td>
<td>-0.375</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>387</td>
<td>1175</td>
<td>0.09</td>
<td>9.6</td>
<td>7</td>
<td>10</td>
<td>81.47</td>
<td>0.8192</td>
<td>0.02</td>
<td>10</td>
<td>1.0410</td>
<td>0.003419</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* Calculated on the basis of the control values equalling 100 percent. N.S. denotes no statistically significant change.
water for 24 hours, and then tested. Statistically the calculated differences obtained for both the tissue fluids and for the blood density determinations were not significant. The mean value obtained for the tissue fluid determinations was 81.27 percent, with a standard deviation of 1.4213 as compared to the control fish which gave 79.88 percent tissue fluids with a standard deviation of 1.7567. The test fish had a mean blood density value of 1.0453 with a standard deviation of 0.005663 as compared with the control fish which had a mean density value of 1.0441 with a standard deviation of 0.006660.

2. Sodium Chloride. Several test fish subjected to a solution of 14,100 parts per million of sodium chloride in reference water were dead several hours after the beginning of the test. Although only seven fish survived long enough to be tested, their blood density and percent tissue fluid mean values were statistically very significant. The test fish gave a mean value of 78.13 percent tissue fluids with a standard deviation of 0.4822 as compared with a mean value of 80.92 percent for the 11 control fish. The standard deviation was 0.8152. The test fish showed a loss of 3.45 percent tissue fluid in seven hours. The blood density values obtained were just as striking: a 0.518 percent drop in density for the group of seven fish tested. The test fish had a mean blood density value of 1.0363 with a standard deviation of 0.003187, compared with the mean value of the
ll control fish of 1.0417 with a standard deviation of 0.003395.

3. Calcium Chloride. Ten determinations were made for each of the two types of testing reported in this experiment. The test fish were subjected to a solution of 8,400 parts per million calcium chloride in reference water. The test group showed a loss of 1.34 percent tissue fluid. The mean value for the test group was 80.36 percent tissue fluid, with a standard deviation of 0.8836, as compared with the control group of 11 fish, which had a mean tissue fluid value of 81.45 percent, with a standard deviation of 0.9436. The change in the percent tissue fluid was statistically significant. Ten test fish had a mean blood density value of 1.0392, with a standard deviation of 0.003567 as compared with ten control fish which had a mean value 1.0386, and a standard deviation of 0.002966. All the fish survived the 24 hour test period. The differences between the means of the two groups was not statistically significant.

4. Sodium Orthophosphate, Dibasic. All but one fish survived the 24 hour test period in a 5,800 parts per million solution of disodium orthophosphate. No significant changes were noted for either the tissue fluid content or for the blood densities. The control group gave a tissue fluid mean value of 81.64 percent, with a standard deviation of 1.0004 as compared with the test group values of 82.42 percent and
a 1.2204 standard deviation. The mean blood density value for the test group was 1.0427, with a standard deviation of 0.003129. The control group yielded a mean value of 1.0428 with a standard deviation of 0.003252.

5. Potassium Chloride. The concentration of the potassium chloride solution in which the test fish were placed was 5.500 parts per million in reference water. This test ran the full 24 hour period. One test fish died prior to the end of the test and three were lost during the determinative testing. Analysis of the data showed a significant loss of tissue fluids among the test fish. This loss amounted to 1.87 percent of the tissue fluid of the control group. Ten control fish gave a mean value of 80.73 percent tissue fluids, with a standard deviation of 1.7350. The eight test fish gave a mean value of 78.86 percent with a standard deviation of 0.5097. There was no statistically significant difference in the blood densities between the two groups. The eight control fish had a mean blood density value of 1.0389, with a standard deviation of 0.003597, whereas the test group gave a mean value of 1.0377 with a standard deviation of 0.004291.

6. Sodium Chromate. This experiment ran the full 24 hour period. Ten determinations were run on each of the two tissues for both the test and control groups. The mean tissue fluid percentage for the test group was 81.63, with a standard deviation of 0.6610 as compared with the
control group values of 81.22 percent and a standard deviation of 0.8267. The blood density mean value for the test group was 1.0452, with a standard deviation of 0.006406 as compared with the control group which yielded a mean density value of 1.0441 with a standard deviation of 0.001451. The results for both types of determinations indicated no measurable change in the tissues tested.

7. Sodium Dichromate. The solution used in this test was a 728 parts per million concentration of sodium dichromate in reference water. The test period was terminated after 13 hours as the condition of many of the fish indicated that they would not survive the test period of 24 hours. Ten control fish gave a mean value for the percent tissue fluid of 80.30 with a standard deviation of 0.7457. The test group of 11 fish gave a mean value of 82.24 percent tissue fluid with a standard deviation of 1.4409. These figures show a 2.42 percent increase in the fluid content of the tissues. Analysis of this data shows that this increase is statistically very significant. The ten control fish gave a blood density mean value of 1.0339, with a standard deviation of 0.011361. Nine test animals had a blood density mean value of 1.0372 and a standard deviation of 0.003501. The difference between the means shows a 0.375 percent drop in the blood density, a statistically significant difference.

8. Sodium Carbonate. The test fish were subjected
to a solution of 387 parts per million of sodium carbonate in reference water. Determinative testing was begun after seven hours when the fish gave definite indications that they were about to succumb. Ten determinations of the tissue fluid content of the test group gave a mean value of 82.48 percent tissue fluids with a standard deviation of 0.8192. These data show a 1.24 percent increase in the tissue fluid content of the test group over that of the control group. There was no statistically significant difference between the means of the two groups. The two means differed by only 0.0002 gravity units. The mean and standard deviation of the control group was 1.0410 and 0.003419, respectively, compared with the test group which had a mean value of 1.0412 and a standard deviation of 0.003170. Ten determinations were made for each group.

**Phenol Red Experiments**

All fish showed evidence of having ingested water after being placed in solutions containing phenol red. The control fish apparently drank less water than the test fish. In the former, the stomach and other portions of the gastrointestinal tract showed only a pink color as indication of the presence of the dye. In the latter, the liquid which was present had or developed with alkali a decidedly red color indicating a greater quantity of water had been ingested.
An interesting observation was made in regard to the effect of the salt solutions on the mucus of the body. When the control fish were removed from the dye and washed off with tap water all detectable traces of the dye were removed. Fish that had been exposed to salt solutions with phenol red were stained with the dye. The fins, head membranes and ventral surfaces were especially affected.
DISCUSSION

Techniques

Originally it had been hoped to use fish seined from natural bodies of water, but this soon proved to be impractical because of the distances the fish had to be transported and the high mortality such seining and transportation engendered. The University Lake adjacent to the campus contained plentiful supplies of small bream, but the population was highly infected with the bacterium, *Proteus hydrophilus*¹, which causes extensive capillary damage and pyonosis of the red blood cells. This disease is confined to a few families of the poikilothermic animals, and among fishes particularly to the Centrachidae, the family to which bass and bream belong. When stocks of these diseased fish were brought into the laboratory, they were usually reduced to about five percent of their original numbers within a period of ten days. Limited numbers of bream seined from the borrow pits near the west approach to the Mississippi River Bridge, north of Baton Rouge, showed a mortality of less than five percent during the same period of observation. As this source of fish was limited and seasonal in character it was necessary to use hatchery-raised fish. These fish were received in excellent condition and

¹ On several occasions Dr. C. S. McClesky of the Department of Botany, Bacteriology and Plant Pathology of the Louisiana State University was kind enough to identify the bacteria present in these fish.
were relatively free of parasites. No instance of gill parasitism was observed. Quite a number of fish contained platyhelminth cercariae which were usually encysted on the pericardium in the pericardial cavity. These cercariae could usually be scraped aside and afforded no interference with the procurement of blood. In several cases the cardiac musculature was affected by the parasites and difficulty was encountered in piercing the wall of the heart. Neither the literature nor the experience in this laboratory indicated that this parasitism had any effect on the physiology of the animal other than the possible toughening of the heart musculature in a few isolated instances.

The paucity of literature dealing with fresh-water fish physiology and the newness of the techniques employed in the testing program made it essential that this research be limited to the general effects of simple compounds on fish. Inorganic salts are the simplest compounds with which to work, although they are probably the least toxic substances petroleum refineries discharge into streams. The reactions of marine teleosts to inorganic salts have been studied quite extensively, but to what extent the results of experiments on these forms can be used in interpreting data obtained from fresh-water species is questionable. Scott ('10) showed that the blood densities of marine teleosts varied in the same direction as the changes in the concentration of sea salts in the environment.
Westfall ('43) showed that in the goldfish, Carassius auratus, the blood density dropped during rapidly developed anoxia, but that this density reduction could be prevented by placing the fish in a one percent sodium chloride solution. The effects of varying the concentrations of sea water and of sodium chloride on the weight of fish is recorded in numerous papers but in each case the gain or loss was for the whole fish. Although this gain or loss was real it was not assignable to any one tissue. Bert ('71) thought the major tissue involved was the musculature. Smith ('32) postulated that the tissue-water-protein relationship is the fundamental steady state of the vertebrate. It was therefore decided that the changes in the hydration of the muscle tissues of fish subjected to various salt solutions would be a productive line of investigation. It is not certain how much of the water in fish muscle is "free water" but according to Hill ('30) the tissue water of the mammalian muscle is all "free water" as defined by Gortner ('38). For the present study it will be assumed that this same condition obtains in the fish.

The determination of blood densities was first attempted in this laboratory using large-mouthed bass, Micropterus salmoides, weighing between five and ten grams (Abegg, '47b). The data from this study indicated that blood densities would reflect the physiological condition of the fish, but it also showed that this method would require the use of groups
of fish and that the data obtained would have to be treated statistically. The fundamental premise of the testing procedure also pointed to a statistical treatment of the data. The underlying principle of the 24 hour median tolerance limit concentration is that fishes exhibit a range of tolerance to changes in the environment. Therefore, in any test using randomly selected test animals there would be a graded series of responses to the test solutions. Furthermore, as only 50% of the fish die at the 24 hour median tolerance limit concentration, it might be expected that about fifty percent of the tested values of the test animals would be still within the range of the normal fish values. The values for the more sensitive fish would spread out and extend beyond one or the other of the extreme values for the control group. This condition was generally exhibited by all the data, from all the tests and will be discussed below (see Figures 8 and 9 and Table V).

In any experiment the ideal situation is that in which the tissue to be tested would be from the same animal, before and after being subjected to the experimental conditions. In the determination of the changes in the percent tissue fluids this ideal was patently impossible. At the outset of the study, however, it was hoped that a technique could be developed for taking blood samples from the fish before and after the testing periods. Some
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Blood Density</th>
<th>Percent Tissue Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Mean</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>C 1.0330</td>
<td>1.0441</td>
</tr>
<tr>
<td></td>
<td>T 1.0386</td>
<td>1.0453</td>
</tr>
<tr>
<td>NaCl</td>
<td>C 1.0349</td>
<td>1.0417</td>
</tr>
<tr>
<td></td>
<td>T 1.0295</td>
<td>1.0363</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>C 1.0336</td>
<td>1.0386</td>
</tr>
<tr>
<td></td>
<td>T 1.0299</td>
<td>1.0392</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>C 1.0377</td>
<td>1.0427</td>
</tr>
<tr>
<td></td>
<td>T 1.0377</td>
<td>1.0428</td>
</tr>
<tr>
<td>KCl</td>
<td>C 1.0345</td>
<td>1.0389</td>
</tr>
<tr>
<td></td>
<td>T 1.0315</td>
<td>1.0377</td>
</tr>
<tr>
<td>Na$_2$CrO$_4$</td>
<td>C 1.0423</td>
<td>1.0441</td>
</tr>
<tr>
<td></td>
<td>T 1.0428</td>
<td>1.0452</td>
</tr>
<tr>
<td>Na$_2$Cr$_2$O$_7$</td>
<td>C</td>
<td>1.0361</td>
</tr>
<tr>
<td></td>
<td>T 1.0305</td>
<td>1.0372</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>C 1.0368</td>
<td>1.0410</td>
</tr>
<tr>
<td></td>
<td>T 1.0343</td>
<td>1.0412</td>
</tr>
</tbody>
</table>
Figure 8. Blood Density Ranges and Means of the Control and Test Groups of Fish for Each Chemical Solution to Which Fish Were Subjected. (Lepomis macrochirus)

Figure 9. Percent Tissue Fluid Ranges and Means of the Control and Test Groups of Fish for Each Chemical Solution to Which Fish Were Subjected. (Lepomis macrochirus)
exploratory work was done with this in mind and a technique was developed by which the desired drop of blood was obtained without killing the fish. Upon retesting the fish the following day it was found that the blood densities were considerable lower, having dropped as much as $8.8 \times 10^{-3}$. Time did not permit the investigation of this drop in density though it is postulated that the withdrawal of the blood reduced the total blood volume of the fish sufficiently to cause an influx of tissue fluid to restore the original volume. No fish used in this series of tests died during the period of ten days they were observed following the use of this technique; however, the drop in density made the use of this technique inadvisable.

The use of the falling-drop method for determining specific gravities combined the merits of small sample size, 0.01 cubic millimeter, with relatively great speed (Barbour and Hamilton, '26). The Hammerschlag method used by Scott ('10) is cumbersome and time consuming, while the usual copper sulfate method requires too large a sample.

The variability of the density of the blood is evident in the data presented in Table III and in Figure 7 which show the means for the several control groups plotted against time. The causes of this variability might be attributable to several factors which it was not practical to test at the time the determinations were made. Some of
the possible causes suggested are: poor acclimatization of the fish to the reference water, a variation in the numbers of blood cells, differences due to technique or seasonal variation as pointed out on page 7. The first possibility is the most reasonable explanation from observations of the reactions of the fish in reference water. Other tests on the blood would have been highly desirable but the procedures necessitated the use of uncoagulated blood.

Failure to find suitable methods of preventing the coagulation of the blood without altering its density precluded the obvious use of plasma density determinations which would have shown the changes in water balance much more accurately than the whole blood determinations. Furthermore, the protein content of the blood could have been determined which would also have aided in the interpretation of the physiological changes induced by the experimental procedures. The small quantities of blood obtainable from the size fish used made it impossible to heparinize each sample quantitatively and this variable would have introduced a considerable margin of error in the density values. Copely and Whitney ('43) showed that the density of sheep, dog, and human blood increased with the addition of heparin and that this increase was not in linear

proportion. Barbour and Hamilton ('26) showed that the exhalation of blood caused considerable changes in the density of the blood.

As stated earlier the type of reference water used in this study is in need of further investigation before its use as a standard laboratory-reagent may be recommended. Early exploratory tests on reference water formulae indicated that the concentration of ammonia compounds built up in the water quite rapidly during the first few days following the introduction of fish which had been held in tap water. It is suggested that the sudden change of water composition might have upset the normal metabolic processes of the fish causing a greatly increased production of these ammonia compounds, such as urea, and that after several days the fish might have developed a uremic condition.

The sudden change from a high bicarbonate water to a mineral balanced water is also suggested as the cause of the initial hyper-excitability manifested by the fish.

The Normal Fish

Normal fish as defined herein are those fish which were used and tested as control animals. This data has been analysed to show more clearly the relationships which normally exist between the tissue fluid and the blood density of the bream under laboratory conditions.

The frequency distribution curve for the percent
tissue fluid values of the normal fish is peaked and exhibits a slight skewness to the right, in the direction of hydration (see Table I and Figure 5). This skewness is explainable by the progressive hydration of the tissues during the period from February 29 through April 7, 1948, as shown in Figure 7 and Table III.

The blood density values of the control fish also present a frequency distribution curve which is peaked and slightly skewed to the right (see Table II and Figure 6). The skewness toward a greater blood density would indicate an inverse relationship between the percent tissue fluid and the blood density.

The tissue fluid data for the normal laboratory fish seems to be in agreement with the condition reported by Westfall ('43) for goldfish kept in his laboratory in tap water. The data presented in Table III and Figure 7 indicate a definite trend on the part of the fish toward hydration during the period between February 29 and April 7, 1948. However, Westfall ('43) also states that the blood density decreased in the fish he tested. This observation is at variance with the results of the present study. It is not possible to make direct comparisons between that work and the work reported here, as the data of his study were not published but only referred to incidentally. No reference was made to the time of year or the period of time involved. It is possible that the water in which the
fish were kept and the diet they were fed produced the results reported by Westfall. The seasonal cycle of the total numbers of blood cells reported by Schaeffer ('25) for the sunfish, *Eupomotis gibbosus*, would seem to support the data presented here. Schaeffer showed a rise in blood cell counts from 400,000 in December to 2,200,000 at the end of April. This would indicate that there was probably a coincident rise in blood density during this time although no values were reported on this point. The means of eight control groups used during the period from February 29, through April 7 are plotted against time in Figure 7 and indicate a tendency toward an increased density of the blood. Figures 5, 6 and 7 then support the suggestion of a seasonal change in tissue fluid content and blood density. The evidence also suggests that perhaps the changes result from an internal shift of water from the blood into the tissues during the spring months. More data is necessary before any conclusions can be arrived at on this interesting problem.

**The Test Results**

The data presented in Table IV show that the concentrations used which were based on the twenty-four hour median tolerance limits of small bream, Abegg ('47) and Williams ('48), did not behave in a uniform manner in their toxicity to the large bream used in the present study. In
some of the solutions the fish survived the 24 hour period of the test while in others the fish were dying several hours after being placed in the solutions.

The reactions of the fish to the several salt solutions is difficult to explain except if one assumes that the ions enter the fish's body. There are several possible pathways by which this entrance could be effected. The skin, gills, oral membranes and by ingestion. It has been shown above that the salt solutions used in this study altered the mucous covering of the body and thus the ions might then be able to penetrate the skin but it is doubtful how effective this would be in causing the reported changes. The extent of permeability of the gills and oral membranes to the various ions is also in doubt though Krogh (‘39) states that there is absorption of some sodium and chloride ions through the oral membranes. The phenol red experiments to be discussed below show that the ions can enter the body by the ingestion of the solution by the fish.

The methods whereby fishes regulate the internal environment has not received a great deal of attention from physiologists particularly in the case of fresh-water fishes. The work of Smith (‘30 and ‘32) and Keys (‘33) and Keys and Willmer (‘32) have done more to explain the osmotic regulation of fishes than any other work reported in the literature. However, these papers pertain almost exclusively to the eel, Anguilla.
These investigators were led to the conclusion that the eel behaves like a fresh-water teleost in fresh water and like a typical marine teleost in sea water. They agree that sodium, potassium and chloride ions and water are excreted largely by the gills and that calcium, magnesium, inorganic phosphate and sulfate ions plus small quantities of sodium and chloride ions and water are excreted by the kidneys.

Keys ('37), investigating the properties of the gill membranes of fishes, indicated that the number of chloride secreting cells varies with the species of fish and the environment. Fresh-water teleosts have considerably fewer cells of this type than marine species. He found that these cells are difficult to detect in fresh-water species due to the presence of large mucous cells at the base of gill leaflets where the chloride secreting cells are located. These two types of cells are morphologically very similar. A slight eosinophilia of the chloride secreting cells is the only characteristic by which they can be separated from the mucous cells.

The eel drinks great quantities of water when in a marine environment but only sparingly when in fresh water. This is stated to be characteristic of the stenohaline teleosts in their respective environments (Smith '30). On the basis of the eel studies it has been assumed by subsequent
investigators of fresh-water fish physiology that fresh-water teleosts do not drink water but obtain their required water by endosmosis through the oral membranes. Experiments in this laboratory, using Smith's ('30) phenol red technique, have shown that the fresh-water stenohaline teleost, *Lepomis macrochirus*, does drink water under normal conditions as well as under high osmotic pressures, although the quantity ingested seems to be less under the former condition. The test fish in solutions of various salts are therefore subjected to external and internal environmental effects.

The ions which have entered the body of the fish must be excreted either renally or extrarenally. Calcium carbonate is the only salt which Smith ('30) found to be excreted by way of the alimentary tract. Whatever the mode of excretion the ions must enter the circulatory system or tissue fluids of the fish. With a greatly reduced number of chloride secreting cells the chlorides must be excreted very largely by the kidneys. All fishes are limited to the excretion of a urine which is hypotonic or isotonic with the blood. This requires the fish to obtain large quantities of water for dilution purposes. Although the fish attempt to get this water by drinking, as reported above, the effect is only to aggravate the condition the fish is attempting to overcome and in these cases the tissues would be required to supply this water.
Under normal conditions the fish is protected from losing ions to, or gaining water from, the environment by the mucus covering the body. The phenol red experiments used in determining the entrance of water into the body by ingestion also showed that, in the salt solutions used, the mucus covering of the body was destroyed. This might permit some water to be gained or lost through the skin. In solutions of high osmotic pressures water was lost; in solutions of low osmotic pressures water in the tissues increased. This is in agreement with the results of Summer ('06b). Carp were placed in a divided chamber so that the head and body would be in different solutions. When sea water was in the head chamber and fresh water in the body chamber the fish lost weight. When the conditions were reversed the fish did not lose weight. The fish was therefore presumed to have lost water only through the oral membranes or the gills.

His data did not necessarily prove the impermeability of the skin when exposed to salt solutions. In his divided-chamber experiments the body mucous film was very probably dissolved by the salt solution. This might then have permitted the osmotic loss of water through the body surface. The fact that there was no loss of weight (i.e., water) can be explained by the fact that the fish had its head in fresh water. By drinking or by the endosmosis of water through the oral membranes it was quite easy for the fish to
replace the body water as rapidly as it was lost through the body skin. The results obtained by Portier and Duval ('22) also attest to the permeability of the mucous-free body surface. When the body mucus had been removed from eels, Anguilla, there was a marked change in the osmotic pressure of the blood.

Before proceeding to an analysis of the data it is necessary to state that the interpretation of the results does not strictly conform to the statistical implications. Although the analysis of the data from some of the tests showed no statistical differences between the means of the test and control groups of fish, other methods of analyzing the data indicated significant trends. Plotting the ranges of the blood density and percent tissue fluid values for each test and control group indicates that even when the means show no significant change there is a tendency of the whole test group range to shift in relation to the control group range (see Figures 8 and 9). It is suggested that these shifts, presented in Figures 8 and 9, indicate incipient changes in the water balance of the fish which failed to proceed to statistical significance in the time the fish were exposed to the test solutions (see Table V).

The discussion of the effects of the various salt solutions on the fish will be limited to the changes in the osmotic balance of the tissues and blood. Although
changes in the osmotic balance of the tissues may not be effective per se in causing the death of the fishes, they may work in conjunction with several other factors, i.e., pH, specific ion effects, acid-base balance, permeability of the membranes to specific ions and the rates of their excretion in relation to the rate of their absorption or penetration. All these factors are important and recognition of them is requisite to an understanding of the complete effects produced by each chemical. The data for such a complete treatment of the subject are not available.

The phenol red experiments reported in this study indicate that fresh-water fish drink water to some extent under normal conditions but when they are in an environment of a strong salt solution the rate of intake seems to be greater. Furthermore, the alteration of the mucous covering of the body reported in the previous chapter could affect the permeability of the skin and produce changes in the water balance of the body. However, it was pointed out that the effectiveness of skin in this connection was probably secondary to the ingestion of the salt solutions. The gills and the gut are suggested as the sites of the penetration of the chlorides into the blood with the resulting rise in the blood chlorides.

Kaplanski ("34 and '37) showed that carp subjected to a 1-1½ percent salt solution over a long period of time had an increase in the sodium content of the muscle but
that the chlorides remained constant. The reverse situation was found in the blood. Phillips ('44) showed that brown trout when fed gelatine capsules containing sodium chloride or when bathed in sodium chloride solutions had higher levels of blood chlorides than the normal fish. He further showed that the rate of absorption was curvilinear when plotted against time and that the ability of the fish to handle excessive amounts of the salt was dependent upon its concentration in the gastrointestinal tract. He was not able to determine whether this rise in blood chlorides was a true increase or merely a concentration through the exchange of water. In the present study a very significant decrease in blood density was observed in the fish subjected to a 14,100 p.p.m. solution of sodium chloride. This indicates that the rise in blood chlorides reported by Phillips is probably real.

It would be expected that the body would attempt to counteract the rise in blood chlorides in two ways: by excreting chlorides or by diluting the blood. The limited ability of fresh-water fishes to control the high chloride levels of the blood through the chloride secreting cells of the gills places the burden of excretion on the kidneys. However, since fishes are limited to the excretion of a urine which is hypotonic to or isotonic with the blood, great quantities of water would be required to dilute the urine and effect the excretion of the chlorides. The test
fish are not able to obtain this water from their environment which is already high in chlorides. The more water ingested the greater the quantity of chlorides to be excreted. Fresh-water teleosts do not have the ability to produce water for renal excretion like marine fishes. Marine teleosts are able to ingest sea water and effect a separation of the water from the salts leaving some water for metabolic purposes. The means by which this is accomplished depends upon the presence of the chloride secreting cells described by Keys and Willmer ('32). These cells have the ability to excrete the chlorides of sodium and potassium in concentrations hypertonic to the blood. The other ions, which are present in relatively small quantities as compared to those mentioned above, are excreted renally in a urine which is hypo- or isotonic with the blood. The lack of a sufficient number of chloride secreting cells in the fresh-water fishes leaves them with no source of water other than the tissue fluids of the body. Loss of tissue fluids of the body and decreased density of the blood has been demonstrated by the data of this study suggesting an internal shift of water from the tissue to the blood.

Sodium sulfate also affects the water balance of the fish through osmotic means. Here the situation is different from that in the case of the chlorides due to the differences in the permeabilities of the membranes to the chloride ion and the sulfate ion. The membranes are not
very permeable to the sulfate ion and it is excreted with
greater difficulty than any other inorganic radical normal-
ly found in the (human?) body (Hawk, Oser, and Summerson '47).
This also is assumed to be the case in teleost fish. The
sodium sulfate solution is ingested by the fish but is ab-
sorbed very slowly by the blood. The osmotic effects would
then be, a loss of water from the blood to the intestinal
tract and in turn there would be a loss of water from the
tissues to the blood. This is in conformity with the data.
The test group of fish showed a loss of tissue fluids and
an increased density of the blood.

Sodium carbonate and sodium dichromate were similar
to each other effected the same general changes in the water
balance of the bream; the tissue water increased and the
blood density decreased. The mode of action of these two
salts is probably very different in accomplishing these
changes, though they were functionally similar in that they
both effected the coagulation of the mucus. In the case of
the sodium carbonate solution the effect of the high pH,
9.6, due to the excess hydroxyl ions from the dissociation
of the salt, could be a contributing factor in the precipi-
tation of the mucus. The ingestion of the solution would
have no direct osmotic effects because of the low concentra-
tions of the salt used. It is suggested that the acid-base
balance of the blood could have been affected but no data
is available on this point. The general hydration of the
tissues suggests an effect on the permeability of the membranes, permitting an influx of water.

The sodium dichromate solution was slightly acid (pH 5.9), and although this was probably active in the total effect of the solution on the fish, it was probably only a contributing factor. This salt, a very powerful oxidizing agent, inactivates proteins and thus probably altered the permeability of the membranes. The ingestion of this salt would be certain to have serious effects on the internal organs if its actions on them is similar to that found in man where it causes intense irritation of the gastrointestinal tract. Upon absorption, the ions are toxic to the blood capillaries and to the kidneys and therefore oliguria and circulatory shock are the prominent symptoms (Goodman and Gilman, '41). It is suggested that the ions affect the capillaries of the gills without the ingestion of the salts or that the capillaries are affected after ingestion. In either case the capillaries would become damaged and their permeability altered. If kidney damage followed the water would have no means of escape and consequently the condition as actually found in the fish might occur: hydration of the tissues and decreased density of the blood.

Sodium chromate solutions did not give clear cut hydration effects on the tissues as did sodium dichromate solutions. The difference perhaps can be associated with
the ionization of the salt which yields hydroxyl ions.

Furthermore, the oxidative powers associated with the di-
ochromate ion are lacking in the chromate ion which reduces its overall effectiveness in damaging membranes. Thus the influx of water would not be as rapid, and the rate of excretion would not be affected to the same degree.

Sodium orthophosphate, dibasic, had very little effect on the tissue fluid volume and blood densities of the fish. The data suggest that the water balance of the fish was not affected.
CONCLUSIONS

1. Statistically significant changes in the percent tissue fluids of the body musculature of the bream, *Lepomis macrochirus*, are effected by the solutions of sodium chloride, potassium chloride, sodium dichromate, calcium chloride and sodium carbonate at the concentrations used in this study and in the length of time the fish were exposed to these solutions.
   
   (a) Sodium chloride, potassium chloride, and calcium chloride solutions cause a decrease in the percent tissue fluids.
   
   (b) Sodium dichromate and sodium carbonate solutions cause an increase in the percent tissue fluids.

2. The density of the blood of the test fish is decreased in the solutions of sodium dichromate and sodium chloride at the concentrations used and in the lengths of time the fish were exposed to these solutions. The changes in density effected by these two solutions are significant.

3. The size of fish used in any toxicity testing program is an important factor in the interpretation of the data. The large test fish used in this study survived the twenty-four hour test period in solutions of sodium sulfate, sodium chromate, sodium orthophosphate, dibasic, and calcium chloride. The concentrations of these
solutions were those which had been shown to yield a twenty-four hour median tolerance limit value for small bream (4-10 cm.). The large fish therefore exhibited an increased tolerance to the aforementioned solutions.

4. Fresh-water fish drink some water normally but when in solutions which are markedly hypertonic to their normal environment they drink greater quantities of water.

5. Ingestion of the salt solutions subjects fish to modifications of the chemical and osmotic environment, internally and externally.

6. The mucous covering of the body is affected by salt solutions used in this study. Sodium dichromate and sodium carbonate precipitate the mucus; whereas, the solutions of the other salts used in this study cause its dissolution.

7. The frequency distribution curves for the blood density and tissue fluid values of the control sample are peaked indicating that a majority of the values lie at or near the mean.

8. Sodium orthophosphate dibasic solutions at the concentration used in this study has no effect on the water balance of the fish after an exposure of fish to this solution for twenty-four hours.

9. The reference water used in this study was not deemed wholly satisfactory as a dilution water for studies pertaining to the effects of chemical compounds on the
physiology of fishes.

10. The blood density and tissue fluid mean values of the several normal fish samples tested between February 29, and April 7, 1948 indicate an increasing hydration of the tissues and a rising density of the blood during this period of testing.

11. Other conclusions as to the physiological effects of the various salt solutions on fish are not warranted by the data presented in this study. However certain explanatory postulations are offered to explain the results obtained.

(a) The decreased blood densities associated with the chlorides were probably due to the influx of tissue fluids in an attempt to keep the chloride level of the blood within the tolerance of the fish.

(b) The rise in blood density and the decreased tissue fluids caused by the sodium sulfate solution may be due to the internal osmotic effects of the solution.

(c) The general hydration of fish placed in solutions of sodium dichromate and sodium carbonate may be due to a change in the permeability of the skin and gills.

(d) The dissolution of the mucous covering of the body caused by the other chemicals (except chromate)
used in this study may affect the permeability of the general body surface and oral membranes as well as the gills.

(e) The changes in blood density and tissue fluids of the several control groups of fish during the testing period may be due to a seasonal change associated with a shift in body fluids.
SELECTED BIBLIOGRAPHY


____. 1908b. Variations in the osmotic concentration of the blood and coelomic fluids of aquatic animals caused by changes in the external medium. Biochem. Jour. 3:473.


Smith, E. 1912. Fundulus and fresh water. Sci. n.s. 25:144.


Roland Abegg was born on December 14, 1914 at Perth Amboy, New Jersey. After graduation from the local high school in 1932 he attended Thornton Township Junior College, Harvey, Illinois, receiving his diploma from that institution in 1934. The next two years were spent at the University of Michigan where he received the degree of Bachelor of Arts in 1936. The following summer he was employed by the University of Michigan Museums, Division of Mammals, as research assistant at the Edwin S. George Reserve. In the fall of the same year he accepted a fellowship in the Department of Zoology of the Louisiana State University. In July 1937 the Louisiana State Department of Conservation engaged him as superintendent of the Louisiana State University Quail Farm where he remained until the fall of 1942. During this time he completed the requirements for the degree of Master of Science which he received in 1939 from the Louisiana State University, Department of Zoology. He returned to the department in the fall of 1942 as a graduate assistant, retaining this appointment through the fall quarter of 1943 with the exception of the summer of 1943 during which time he served as an assistant in the department. Two years, 1944-1945, were spent in industrial laboratories as a chemical analyst. In 1936 he returned to the
Louisiana State University as the senior fellow on an industrial research project sponsored by the Louisiana Petroleum Refiners' Waste Control Council and administered by the Louisiana State University Department of Zoology. He is at present engaged on this project while completing the requirements for the degree of doctor of philosophy.
Candidate: Roland Abegg

Major Field: Zoology

Title of Thesis: "Some Effects of Inorganic Salts on the Blood Density and Tissue Fluids of the Bluegill, Lepomis macrochirus"

Approved:

George H. Mickey
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination: May 31, 1948