DECK, James Emerson, 1939-

IN VIVO AND IN VITRO STUDIES ON THE METABOLISM OF THE SWIMBLADDER GAS GLAND OF THE BLUEGILL SUNFISH.

The Ohio State University, Ph.D., 1969
Physiology

University Microfilms, Inc., Ann Arbor, Michigan
IN VIVO AND IN VITRO STUDIES ON THE METABOLISM
OF THE SWIMBLADDER GAS GLAND
OF THE BLUEGILL SUNFISH

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

James Emerson Dock, B.Sc., M.Sc.

* * * * * *

The Ohio State University
1969

Approved by

John
Adviser
Faculty of Zoology
ACKNOWLEDGMENTS

As in all efforts of this sort, the final product represents the combined efforts of many people. I take this opportunity to acknowledge that help and express my sincere gratitude for it.

Dr. John W. Price, my adviser, has generously provided advice when sought, and stimulation when necessary.

Various people have loaned necessary equipment or provided space in which to work. These people include Dr. J. N. Miller, Dr. W. Momot and Dr. W. Parrish.

Aid in mastering techniques was provided by Mrs. Kay Lindsay and also by Dr. W. D. Stull of Ohio Wesleyan University.

Securing of experimental animals was especially difficult during winter months, but by drawing upon the supplies of Miss Elizabeth Wydallis and Mr. Ken Hille my work progressed during that period. My father, Earl Deck, provided much assistance with collections during other periods of the year.

Finally my wife, Martha, helped in this effort in more ways than she realized, the least of which has been in typing the manuscript.
VITA

January 9, 1939 . . . Born - Newark, Ohio

1961. . . . . . . . . B.Sc., The Ohio State University,
Columbus, Ohio

1961-1968 . . . . . Graduate Assistant, Department of Zoology
and Entomology, The Ohio State University
Columbus, Ohio

1963. . . . . . . . M.Sc., The Ohio State University,
Columbus, Ohio

1968- . . . . . . . Visiting Instructor, Department of Zoology,
Ohio Wesleyan University, Delaware, Ohio
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>11</td>
</tr>
<tr>
<td>VITA</td>
<td>111</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi1</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>11</td>
</tr>
<tr>
<td>RESULTS</td>
<td>19</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>39</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>43</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>45</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Glycogen depletion of the swimbladder gas gland in relation to secretion time after one or more withdrawals of 75 per cent of the swimbladder volume of gas.</td>
<td>23</td>
</tr>
<tr>
<td>2.</td>
<td>Glycogen depletion of the swimbladder gas gland after 14 hours of continuous gas secretion after two gas withdrawals at 10 hour intervals of 75 per cent of the swimbladder volume of gas.</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>Total and partial correlations of body weight, gas gland weight and weight of lactic acid produced per 4 hours incubation for times from 4 to 20 hours.</td>
<td>34</td>
</tr>
<tr>
<td>4.</td>
<td>Effects of glucagon on lactic acid production in gas glands incubated in vitro.</td>
<td>35</td>
</tr>
<tr>
<td>5.</td>
<td>Effects of epinephrine on lactic acid production in gas glands incubated in vitro.</td>
<td>37</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>Diagram of the antagonistic innervation of the swimbladder.</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Schematic diagram of a counter-current multiplier system such as is found in the swimbladder.</td>
<td>6</td>
</tr>
<tr>
<td>3.</td>
<td>Relationship of body weight of <em>Lepomis macrochirus</em> to swimbladder volume.</td>
<td>20</td>
</tr>
<tr>
<td>4.</td>
<td>Graphic representation of the events following withdrawal of 75 per cent of the swimbladder volume of gas at time 0 or additional withdrawals at succeeding 10 hour intervals.</td>
<td>21</td>
</tr>
<tr>
<td>5.</td>
<td>Sections of gas gland from control fish (no gas secretion).</td>
<td>26</td>
</tr>
<tr>
<td>6.</td>
<td>Sections of gas gland from experimental fish (16 hours gas secretion).</td>
<td>27</td>
</tr>
<tr>
<td>7.</td>
<td><em>In vitro</em> lactic acid production by isolated swimbladder gas glands of <em>L. macrochirus</em>.</td>
<td>31</td>
</tr>
</tbody>
</table>
INTRODUCTION

The gas-filled swimbladder of fishes may have several functions, depending upon the species considered. Magid (1966) and Dehadrai (1960) among others have demonstrated that the swimbladder may serve as a lung. Tavolga and Wodinsky (1963) suggest that the swimbladder increases the hearing ability of fish that possess one over those that do not. Winn and Marshall (1963) showed that the swimbladder may function in the production of sounds by vibration of the walls of the organ. The usual function of the swimbladder in most fishes, although the ones mentioned above may be accessory, is that of a hydrostatic organ. This function was initially clarified by Moreau (1876) when he stated, "Le rôle de la vessie natatoire est donc de donner au Poisson la densité de l'eau à toutes les pressions et sans efforts musculaires, ..." Alexander (1966) also showed that a fish with neutral buoyancy required less power to swim horizontally at a given speed than did a fish with negative buoyancy.

In those fishes which possess no duct between the swimbladder and the gut, the swimbladder cannot be filled by passing air through the gut and into the swimbladder. Fishes with a closed swimbladder must fill it by an alternate mechanism which involves transport of gases by the blood to be released into the swimbladder. The mechanism by which this feat is accomplished is not fully understood.

The gases in the swimbladder have been analyzed by many workers
and generally in shallow water forms "the gases are the same as
those of the air dissolved in water" (Fange, 1966). Jacobs (1934)
and Fange (1953) demonstrated that the normal swimbladder gas composi-
tion of codfish, Gadus sp., is 7 to 45 per cent oxygen, 0.2 to 1.1
per cent carbon dioxide with the remaining gas being nitrogen. By
puncturing the swimbladder and withdrawing gas, refilling of the
swimbladder can be induced. The major changes in composition of the
gases that refill the swimbladder are the large increases in the
percentage of oxygen and carbon dioxide and the decrease in the per-
centage of nitrogen. In the toadfish, Opsanus tau, Fange and Witten-
berg (1958) reported that newly secreted gas contained an average of
90 per cent oxygen and 4 per cent carbon dioxide.

Although the developmental anatomy of the swimbladder has been
described for two species of the genus Lepomis (Duwe, 1952 and 1955),
a description of the adult anatomy is not available. Since a know-
ledge of the anatomy and physiology of the swimbladder is necessary
for understanding the mechanism of gas entrance into the swimbladder,
the following description deals with literature references to other
species which have been investigated.

The oxygen filling the swimbladder has been shown by radioactive
tracer techniques to come from molecular oxygen picked up from the
surrounding water and carried to the swimbladder by the blood (Scho-
lander et al., 1956, and Wittenberg, 1961). In euphysoclistous fish
(Fange, 1953) there is a double blood supply to the swimbladder with
one portion (the rete mirabile) supplying a gas secreting part (the
gas gland) and the other portion supplying the gas reabsorbing part (the oval).

Volume control of the swimbladder is mediated by control over the functionally different areas. The reabsorbing area (oval) is a capillary network overlain by squamous mucosal cells which line most of the swimbladder. The rete mirabile of the secretory area is overlain by one or more layers of cuboidal mucosal cells which make up the gas gland. The combination of the rete mirabile and the gas gland make up the anatomical arrangement for efficient gas secretion.

The physiological mechanisms by which gases may be reabsorbed from the swimbladder are easily understood. Since the partial pressure of gases in the swimbladder is greater than that of the blood, the gases diffuse from the swimbladder to the blood. Control of the amount of blood flowing through the oval determines the loss of gases from the swimbladder. Fänge (1953) demonstrated a vaso-dilatation in the oval during asphyxia, after injection of epinephrine, or during stimulation of the vagus nerve in <i>Ctenolabrus</i>.

The thickness of the mucosa also influences the passage of gases between the swimbladder and the blood with the thin stretched condition being more permeable than the opposite condition. Since the thickness of the mucosa is determined by the degree of contraction of the muscularis mucosae, nervous influences upon this muscle layer are quite important. Although details are not clear, Fänge (1966) suggests that the muscularis mucosae of the oval relaxes under adrenergic stimulation and thus reabsorption of gases from the swimbladder
Figure 1. Diagram of the antagonistic innervation of the swimbladder. The arrows represent the area and direction of gas movement. bv, blood vessels; lu, lumen of the swimbladder; mus, muscularis mucosae; r muc, reabsorbant mucosa; s muc, secretory mucosa.
occurs, while cholinergic stimulation blocks reabsorption by promoting contraction of the muscularis mucosae causing an increase in the thickness of the mucosa (cf. Figure 1).

Filling of the swimbladder requires a different mechanism than reabsorption since gases must seemingly move against a partial pressure gradient from the blood into the swimbladder. However, gases probably do not move against a gradient because of the action of the rete mirabile as a counter-current multiplier system and the acidification of the blood by secretions of the gas gland.

A counter-current multiplier system (see Figure 2 for a diagrammatic representation of the discussion below) is the anatomical arrangement in which efferent and afferent capillaries of the rete mirabile lie adjacent to each other in the form of a hairpin loop. At the apex of the loop is the gas gland, the cells of which produce secretions which enter the capillaries and promote the release of oxygen from hemoglobin in the efferent arm. Since the efferent arm of the capillary loop lies adjacent to the afferent arm, the oxygen freed in the efferent arm may diffuse with the concentration gradient into the afferent arm. Continuation of this process results in an increase in the concentration of oxygen in the capillaries with the greatest concentration near the apex in the efferent arm. The concentration of oxygen at the apex can become sufficiently great to establish a concentration gradient from the capillaries to the swimbladder lumen which will result in the entrance of oxygen into the swimbladder.

During gas secretion it was first noted by Hall (1924) that
Figure 2. Schematic diagram of a counter-current multiplier system such as is found in the swimbladder (modified after Hoar, 1966). Intensity of the stippling indicates the concentration of oxygen.
vasodilatation occurs in the capillaries of the gas gland. Fange (1966) suggests that the secretory mucosa (gas gland) relaxes under cholinergic stimulation, thus promoting passage of gases into the swimbladder while adrenergic stimulation causes contraction of the muscularis mucosae of the gas gland with opposite effects. Mapping the muscularis mucosae of the swimbladder pharmacologically, Fange (1953) found that epinephrine causes relaxation of the reabsorbent mucosa and that both epinephrine and norepinephrine cause contraction of the secretory mucosa (cf. Figure 1).

Although an increased blood supply and a thin mucosa in the secretory region would enhance the movement of gases into the swimbladder, the mechanism by which gases actually leave the blood and become deposited in the swimbladder is not clearly understood. The current theory of gas secretion supported by numerous investigators (Scholander, 1954, Kuhn et al., 1963, Niesel and Roskenbleck, 1963, and Steen, 1963) is that high partial pressures of oxygen are built up by the counter-current multiplier system of the rete capillaries. This oxygen is initially released from the blood by secretions of the gas gland which is at the apex of the capillary loops.

Scholander (1954) found that the action of the counter-current multiplier system is probably sufficient to build up a partial pressure gradient of oxygen from the rete capillaries to the swimbladder lumen. This action depends upon the release of oxygen from hemoglobin in the efferent rete capillaries. It is apparently the function of the gas gland to mediate this oxygen release by secretions into the blood from the gland cells. The major secretion of the gas gland is lactic acid (Steen, 1963), which may accelerate oxygen
deposition by several mechanisms, *viz.*, by salting out, by the Root effect and by the Bohr effect.

A small salting out effect on all gases carried in physical solution may be responsible for deposition of gases, including the inert ones. Kuhn et al. (1963) suggest lactate as one substance which may produce such an effect.

Haetz (1956) suggested that lactic acid and carbon dioxide may act together to release oxygen from hemoglobin. The lactic acid produced in the gas gland cells may dissociate into ions and these lactate ions may enter the bloodstream in exchange for bicarbonate ions. The bicarbonate ions may combine with the hydrogen ions from lactic acid to produce carbonic acid which is dissociated into carbon dioxide and water by carbonic anhydrase. Carbonic anhydrase has been demonstrated to be abundant in the gas gland by Leiner (1940). The carbon dioxide may either enter the lumen of the swimbladder, which accounts for its increased percentage during gas secretion, or it may enter the bloodstream. Root (1931) demonstrated that in some fish an increased carbon dioxide content in the blood, resulting in decreased pH, prevents 100 per cent saturation of the hemoglobin with oxygen. This effect, the Root effect, would cause oxygen to be released from the blood in the efferent rete capillaries. However, in some fish the Root effect seems not to be important (Scholander, 1954).

Species that have more efficient gas secretion also show a marked Bohr effect (Fänge, 1966). As the pH of the blood decreases, the affinity of hemoglobin for oxygen decreases and oxygen is
liberated. The decrease in pH occurs primarily as a result of lactic acid secretion although carbon dioxide may also exert an effect. The lactic acid produced by the gas gland comes, at least in part, from glycogen which is stored in the gas gland cells (Copeland, 1952). However, Ball et al. (1955) demonstrated that glucose added to the medium of gas glands incubated in vitro promotes the formation of additional lactic acid.

Any factor which would modify the amount or rate of lactic acid secreted by the gas gland would have an effect on oxygen release from the blood. This effect on secreting gas into the swimbladder would be additive to the effects on the muscularis mucosae produced by nervous or endocrine stimulation. Initiation of glycolysis is known to occur among vertebrates as a result of hormonal influences, especially of epinephrine from the adrenal medulla and of glucagon from the alpha cells of the pancreatic islets (Turner, 1960). Only two authors have previously suggested any possibility of hormonal influence on the swimbladder other than on the muscularis mucosae. Schreiber (1938) noted a correlation between retic size of Anguilla and the season of the year which he suggested was due to some hormonal influence. Enami (1958) noted negative buoyancy in Carassius auratus deprived of the caudal neurosecretory system. These goldfish gained positive buoyancy after an injection of a caudal neurosecretory system extract from the eel, Anguilla japonica. However, Kawamoto (1962) could not confirm Enami's results concerning loss of buoyancy after destruction of the caudal neurosecretory system.
Nor could Enami's results be duplicated in the same or different species by Lahlou (1966).

The aims of my research have been: (1) to follow glycogen depletion from the gas gland in vivo under controlled conditions to determine the length of time for which glycogen reserves will suffice for gas secretion; (2) to follow in vitro glycogen depletion and lactic acid production of the isolated gas gland; and (3) to investigate the possibility of hormonal influences upon gas gland metabolism, in particular the hormones epinephrine and glucagon. Data on these points will allow a better understanding of the swimbladder function with particular reference to the metabolism of the gas gland during gas secretion.
MATERIALS AND METHODS

The species most thoroughly investigated in this study was the bluegill sunfish, *Lepomis macrochirus*, although some preliminary and other noncritical observations were made using the closely related species *L. cyanellus*, *L. humilis*, *L. microlophus* and various *Lepomis* hybrids. All fish were collected in various places in central Ohio, the bluegills principally from ponds by hook and line. After collection, the fish were transported in minnow buckets to the laboratory where they were placed in a large aquarium. Since fish were typically sacrificed within one week following capture, no food was provided for them. Over the course of one year during which experiments were conducted the usual range of temperature variation in the aquarium was 21 to 23°C with extremes of 16 and 25.5°C.

To determine the amount of gas that is normally present in the swimbladder, a series of 35 bluegills were sacrificed as they were caught in early October. Each fish was rapidly anesthetized in a chlorobutanol solution, weighed to the nearest half gram after blotting with a cloth, decapitated and eviscerated to expose the swimbladder. Using a 5 cc syringe fitted with a #18 needle, the swimbladder was punctured and the gas withdrawn while the fish was held under water to note the possible escape of any gas other than that drawn into the syringe. The volume of gas was determined to the nearest tenth of a cubic centimeter. A regression line relating
swimbladder volume to body weight was calculated for these data by the method of least squares (Figure 3).

A series of experiments was performed between August and February to determine the rate of glycogen depletion from the gas gland in an intact organism. To induce gas secretion in an experimental fish, it was removed from the aquarium and anesthetized with M. S. 222-Sandoz (tricaine methanesulfonate) made up as a 0.1 per cent solution in tap water. Immediately after opercular movement ceased, the fish was removed from the anesthetic solution, weighed to the nearest half gram and placed on a wet paper towel. A 5 cc syringe fitted with a #26 needle was used to puncture the body wall laterally and withdraw gas from the swimbladder. Since complete removal of gas was difficult to effect, a quantity calculated to equal 75 per cent of the gas in the swimbladder was removed. This volume was based on the weight of the fish and calculated from the regression line of body weight versus swimbladder volume (cf. Figure 3). For control fish the same procedure was followed except that after the gas was drawn into the syringe, it was returned to the swimbladder before withdrawing the needle. After puncture the fish was marked for identification purposes by clipping off small portions of the caudal fin in various patterns or by noting various morphological features. After experimental procedures were completed, each fish was returned to the aquarium and allowed to recover. The fish evidently replaced the withdrawn volume of gas in approximately 11 to 12 hours. Therefore, if a gas secretion period longer than this was desired, it was
necessary to perform additional gas withdrawals at ten hour intervals to insure continuous gas secretion.

At varying times after gas withdrawal the fish were sacrificed for histological examination. The control animal in each experiment was sacrificed along with the experimental animal which had been secreting gas for the longest time. Upon sacrifice the gas gland was exposed in the swimbladder and fixed in Gendre's fluid at approximately minus 10° C. for times ranging from 16 to 25 hours. After fixation the tissue was embedded in Paraplast by standard embedding techniques and sectioned at 6 microns. Slides were prepared and subjected to the PAS technique for demonstration of glycogen. An aqueous solution of periodic acid as described by Humason (1967) and the Schiff's reagent of Lillie (1951) were used. Following the suggestion of McManus (1961), excess Schiff's reagent was removed by washing in running water for 10 minutes. The counterstain was a 0.2 per cent solution of light green SF, yellowish, in 95 per cent ethyl alcohol. Control slides for glycogen were subjected to 30 minutes digestion with salivary amylase.

In addition to the gas glands various other parts of different experimental fish were studied. Preparations were made of the liver and subjected to the same fixing and staining procedures as the gas glands, although in some cases Delafield's hematoxylin was used as a nuclear stain. The method of Takasugi and Bern (1962) was used to demonstrate the components of the caudal neurosecretory system. The pancreas was fixed and stained according to the method of Maldonado and San José (1967) except that Delafield's hematoxylin was used
in place of Weigert's hematoxylin. The head kidney was stained for
chromaffin tissue according to the method of Sevki (1934).

All experiments involving in vitro procedures used sterile tech-
nique so far as was possible. To isolate the gas gland the fish was
removed from the holding aquarium, weighed to the nearest half gram
and sacrificed by decapitation without anesthesia. The swimbladder
was exposed by careful removal of the viscera and severance of the
large blood vessels associated with the retia mirabilis. The peri-
toneum and external coat were carefully dissected off the swimbladder
and the gas gland and associated retia mirabilis were exposed and
excised. It is fully recognized that the piece of excised tissue
contains not only the gas gland but also the retia mirabilis. The
anatomical arrangement of these parts is so intimate in this species
that separation is not possible. Hereafter, references to the gas
gland will include the gas gland, retia mirabilis and minimal amounts
of connective tissue. The gas gland after excision was gently spread
on a lens tissue raft approximately 2 cm square which had previously
been weighed. Then the raft and the gas gland were weighed together
from which the wet weight of the gas gland could be determined. The
raft with the gas gland on it was then floated on an incubation medium.
Elapsed time between removal of the fish from the holding aquarium
and tissue floatation on the incubation medium was approximately 20
minutes. Elapsed time between excision of the gas gland and its
floatation was less than 2 minutes.

The incubation medium was a buffered physiological saline to
which calcium ions were added in order to provide a fluid more nearly
resembling body fluids. This medium without calcium was originally described by Holmes and Stott (1960) for use with trout tissues. To prepare the saline the following amounts of reagent grade salts were dissolved in 1.0 liter of distilled water:

\[
\begin{align*}
\text{NaCl} & : 7.41 \text{ g} \\
\text{KCl} & : 0.37 \text{ g} \\
\text{CaCl}_2 \cdot 2\text{H}_2\text{O} & : 0.23 \text{ g} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & : 0.31 \text{ g} \\
\text{NaHCO}_3 & : 0.31 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 0.17 \text{ g} \\
\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O} & : 0.40 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : 0.159 \text{ g}
\end{align*}
\]

As measured with a Beckman Model G pH meter at 25° C., the pH of the saline was 7.38. Comparison of histological sections of the gas gland incubated in this medium with gas glands from in vivo procedures revealed barely detectable differences in the appearance of the cell membrane and the general cytoplasmic organisation.

Initial attempts to use the bicarbonate buffered saline of Ball et al. (1955) resulted in cells which appeared in histological sections to be less well organized. In particular cells on the surface of the gas gland were covered with small globules of cytoplasm. These observations indicate that the latter incubation medium was not compatible with the cells of this species either in terms of tonicity or ion balance or both.

To determine the time course of lactic acid production by the isolated gas gland, a series of experiments was performed from April to July. The excised gas gland on its raft was floated on a 5 ml saline substrate in a 60 cm Petri dish. Five replicates for a given time interval were performed on any one day although another set of five replicates was sometimes performed at a later date. The five
covered dishes were incubated in a closed cabinet at room temperature which had a range of 24 to 25°C. After incubation for periods of time varying from 1 to 30 hours, each gland was removed from its dish and individually fixed in ice cold Gendre's fluid and prepared for PAS histochemistry.

The incubation medium was then assayed colorimetrically for lactic acid by the method of Barker and Summerson (1941). In a few instances the medium was also assayed for free glucose by the methods of Nelson and Somogyi as cited by Damm (1965). In all colorimetric assays a Bausch and Lomb Spectronic 20 was used for determining optical density of the samples, standard and blank.

One gas gland was used to determine the approximate base level of lactic acid occurring in the gland. In this case a gas gland was obtained in the usual manner, weighed, and placed in a tissue homogenizer. To the gland was added 1.5 ml of the same saline used for incubation to which was added 10 mg NaF/ml to inhibit glycolysis. After homogenizing the gland, a 1.0 ml sample was deproteinized according to the Somogyi-Shaffer-Hartmann method as described by Hawk et al. (1954). A 1.0 ml sample of the protein-free filtrate was used for lactic acid determination by the method of Barker and Summerson (1941).

To determine what effects hormones might have on the isolated gas gland, the same procedures were followed with certain modifications, primarily the addition of the hormones glucagon or epinephrine to the incubation medium. In these experiments five gas glands were incubated in separate dishes of the hormone-containing medium while
five other glands were incubated at the same time in dishes containing saline only. The excision of the gas gland and the initiation of incubation were alternated between experimental (medium with hormone) and control (medium without hormone) until the total of ten glands were incubating. All glands were allowed to incubate for from 4 to 6 hours after which they were removed and the medium assayed for lactic acid by the method cited above.

L-epinephrine, obtained from Nutritional Biochemicals Corporation, was added to the incubation medium immediately before use to avoid any loss of activity by oxidation. Concentrations of 2.0, 0.2, 0.02 and 0.002 mg epinephrine/100 ml saline were used in different experiments so that the absolute amount of epinephrine to which any glands were exposed during incubation ranged from 0.1 to 0.0001 mg.

Crystalline glucagon was obtained from the Sigma Chemical Company. This material is prepared from a mixture of bovine and porcine pancreas and may contain a trace of insulin (Sigma Chemical Company, 1968). Concentrations of 3.0 or 0.3 mg glucagon/100 ml saline were used. These solutions were refrigerated but allowed to come to incubation temperature before use. I attempted to alter the action of glucagon, which is a rather small polypeptide, by boiling the incubation medium to which it had been added for 5 or 10 minutes. The volume of water which boiled away during this period was added, the solution cooled and then used for incubation. Saline without glucagon was also boiled and reconstituted with distilled water for the control glands. In other experiments I attempted to mimic
the action of glucagon by substituting the protein, gelatin, which is known to lack hormonal activity. Gelatin was added to the saline in the same quantities as was the glucagon.
RESULTS

Inspection of the data points in Figure 3 relating the swim-bladder volume to body weight suggests that these two parameters are linearly related over the range of observed values. Assuming such to be the case, a regression line fitted to these data was calculated to be determined by the formula \( y = 0.10 + 0.07x \) where \( y \) equals swim-bladder volume and \( x \) equals body weight. From this line calculations reveal that the swimbladder volume displaces a quantity of water equal to approximately 7 per cent of the body weight over the range of observed weights. This observation agrees with that of Plattner (1941) who found the swimbladder of freshwater fishes to displace 7 to 10 per cent of the body weight.

Initial withdrawal of 75 per cent of the swimbladder volume of gas resulted in immediate loss of buoyancy. Neutral buoyancy was regained approximately 12 hours later, mediated by gas gland activity. However, if a second gas withdrawal were made 10 hours after the first to induce prolonged gas gland activity, filling was less completely accomplished by 20 hours. If after 20 hours of continuous secretion, another 75 per cent of the original volume were withdrawn, recovery was less complete after 30 hours of continuous secretion than after 20 hours (cf. Figure 4). All these observations were based upon both dissections and behavioral observations. In Fundulus heteroclitus Copeland (1952) found that after repeated gas withdrawals at 12, 24
Figure 3. Relationship of body weight of *Leptomis macrochirus* to swimbladder volume. Each point represents one of the observations from which the regression line was calculated. The calculated value of the line is \( y = 0.10 + 0.07x \).
Repeated Gas Withdrawals at 10 Hour Intervals

Figure 4. Graphic representation of the events following withdrawal of 75 per cent of the swimbladder volume of gas at time 0 or additional withdrawals at succeeding 10 hour intervals. After withdrawal the amount of gas in the swimbladder of approximately 40 fish was judged subjectively, largely by behavioral observations on the buoyancy of the fish as shown by pectoral fin activity and ventral body contact with the bottom of the aquarium.
or 45 hour intervals, the fish refilled the swimbladder only slowly after 7 days of this treatment.

Completeness of recovery was judged subjectively by behavioral observations. Immediately after gas withdrawal, while still under anesthesia, a fish returned to the aquarium would sink rapidly and fall heavily on the bottom. The control fish, from which gas had been withdrawn and replaced, would fall more slowly to the bottom and rest lightly there. Upon recovery from anesthesia the control fish would swim in the usual manner or rest near the bottom with the ventral portions of the caudal, anal and pelvic fins barely touching the bottom. The pectoral fins moved slowly. Experimental fish showed quite different activity after recovery from anesthesia in that they did not swim about unless they were disturbed. They rested heavily on the bottom with the ventral body surface having much contact with the bottom rather than resting lightly with only the fins in contact with the bottom. The pectoral fins showed exaggerated movement both in rate and length of stroke. As refilling of the swimbladder occurred, the ventral body or fin contact became progressively less until at approximately 10 hours following gas withdrawal the experimental fish appeared similar to control fish. However, pectoral fin activity was still exaggerated and this condition of normal body contact with the bottom, but exaggerated pectoral fin movement, was regarded as the terminal but not quite complete stage of refilling. Normal pectoral fin movement returned approximately 12 hours after the initial gas withdrawal.

When the gas gland cells of animals that had been secreting gas
into the swimbladder for varying lengths of time were examined histo-
chemically for the presence of glycogen, a steady but somewhat
irregular decrease of glycogen could be noted with increase in secre-
tion time. This decrease presumably results from the lactic acid
production as a result of glycolysis. In a series of experiments the
earliest time at which complete glycogen depletion could be detected
was 15 hours of continuous secretion (cf. Figure 5a), while by 20
hours of continuous secretion, depletion was always complete. The
data in Table 1 show the relationship of the duration of gas secretion
and glycogen depletion in five fish selected as representative of
results in two experimental groups.

<table>
<thead>
<tr>
<th>Secretion Time</th>
<th>Body Weight</th>
<th>Degree of Depletion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td>Grams</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.5</td>
<td>2/3</td>
</tr>
<tr>
<td>13</td>
<td>3.0</td>
<td>3/4</td>
</tr>
<tr>
<td>16</td>
<td>3.5</td>
<td>complete</td>
</tr>
<tr>
<td>20</td>
<td>5.5</td>
<td>complete</td>
</tr>
<tr>
<td>30</td>
<td>5.5</td>
<td>complete</td>
</tr>
</tbody>
</table>

* Fractions represent subjective judgments based upon
comparisons with control specimens. Degree of depletion
is slightly less than indicated due to experimental
error introduced by method of fixation and PAS technique.

To determine the extent of variation that was possible, a
single experiment was conducted in which five fish were sacrificed after
14 hours of gas secretion. The data in Table 2 show that for the first
listed individuals of similar size the rate of glycogen depletion was
fairly constant. The single larger fish in this experiment showed much
less glycogen depletion, a result which is consistent with my other experiments in which glycogen depletion occurred at a slower rate in larger fish than in smaller fish.

Table 2. Glycogen depletion of the swim-bladder gas gland after 14 hours of continuous gas secretion after two gas withdrawals at 10 hour intervals of 75 per cent of the swimbladder volume of gas

<table>
<thead>
<tr>
<th>Body Weight</th>
<th>Degree of Depletion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>almost complete</td>
</tr>
<tr>
<td>4.5</td>
<td>almost complete</td>
</tr>
<tr>
<td>5.5</td>
<td>3/4</td>
</tr>
<tr>
<td>5.5</td>
<td>3/4</td>
</tr>
<tr>
<td>9.5</td>
<td>1/3</td>
</tr>
</tbody>
</table>

*Judged in same manner as in Table 1.

Preliminary in vitro tests to determine whether glycogen depletion may occur anaerobically gave negative results. Equally large amounts of glycogen remained in the gas gland cells incubated for 4 and 24 hours when they were submerged in the incubation medium. Gas glands floated on lens tissue rafts open to the air underwent progressive glycogen depletion expressed as lactic acid production as recorded below (Figure 7). Hence, in *Lepomis* oxygen is regarded as being essential to effective glycolysis in the gas gland. O'Hara (1968) showed in two species of *Lepomis* that smaller fish consume more oxygen per gram body weight than larger fish. This implies that tissues of smaller fish have a richer supply of oxygen which in turn will support a more rapid rate of glycolysis than will occur in larger fish, a result consistent with the findings in this study (Table 2).
As glycogen depletion occurred in experimental animals, no consistent pattern could be observed. Copeland (1952) observed that glycogen was initially depleted from the region of the cell adjacent to the rete capillaries. In my work some gas gland cells would be completely depleted of glycogen, some completely full and others in intermediate stages of depletion and seemingly without relation to the distribution of rete capillaries.

Since the control animal in each experiment was not filling the swimbladder with gas, the gas gland cells were always strongly PAS positive, indicating an abundance of glycogen. This condition is illustrated by Figure 5a. Figure 6a shows the absence of PAS positive material from a gas gland which secreted gas for 16 hours. That the PAS positive material was glycogen was demonstrated by salivary amylase digestion prior to the oxidation step of the PAS technique. Sections treated with salivary amylase showed virtually no PAS positive material within the gas gland cells (see Figure 5b).

When comparing tissue sections treated with salivary amylase from the control and experimental animals, a distinct difference in appearance can be noted. Gas gland cells from control animals show a solid cytoplasmic matrix of the color of the counterstain, while gas gland cells from the experimental animals show a reticulate or vacant cytoplasmic area which takes up little of the counterstain. Comparison of Figures 5b and 6b illustrate this point. In addition the PAS positive material which is present in the blood vessels of the control animal gas glands is absent from the blood vessels of the experimental animal gas glands.
Figure 5. Sections of gas gland from control fish (no gas secretion) subjected to PAS technique and light green SF, yellowish, counterstain. 400X. a: Cells of the gas gland (upper layer) are filled with PAS+ material which is glycogen. b: Same as a except that the section has been treated with salivary amylase for 30 minutes before PAS technique to digest glycogen. Only blood vessels show PAS+ material. The lower layer of connective tissue shown in a is not present in this section.
Figure 6. Sections of gas gland from experimental fish (16 hours gas secretion) subjected to PAS technique and light green SF, yellowish, counterstain. 400x. a: Cells of the gas gland (upper layer) show no PAS+ material indicating the depletion of glycogen. b: Same as a except that section has been treated with salivary amylase for 30 minutes before PAS technique to digest glycogen. Note that in neither section do the blood vessels show a PAS+ reaction.
To relate the findings concerning the gas gland to other events that might occur in the body, various other organs (liver, head kidney, pancreas and caudal neurosecretory system) in the fish were examined histologically. In general no relationship was found between gas gland activity and the activity of any of the organs studied.

Since the liver represents the greatest available carbohydrate store in the body, the liver was also treated histochemically to reveal glycogen. The maximum time period of gas secretion of any fish used was 30 hours which represents by any measure sufficient time for complete glycogen depletion of the gas gland cells. Since Ball et al. (1955) have demonstrated that the gas gland can metabolize supplied glucose in vitro, presumably the same possibility exists in vivo. With the liver as the likely source of this glucose, one might expect a decrease in the liver glycogen. However, no obvious difference between the control and experimental animals could be recognized.

The action of epinephrine in stimulating glycolysis led to a brief investigation of the head kidney which is the site of cells comparable to the mammalian adrenal medulla. This action of epinephrine could lead to secretion of epinephrine from the chromaffin tissue during gas secretion. The chromaffin cells are randomly distributed throughout the tissue of the head kidney as individual cells or as small groups of cells. Although in one experiment there was a distinct general difference in intensity of staining between control and most experimental animals, one experimental animal having been deflated longer than any other animal was not different from the
control animal. Even among the other experimental animals some chromaffin cells or groups of cells appeared quite similar to the cells of the control animals.

The effect of glucagon in stimulating glycolysis suggested the pancreas as another possible area of investigation. Two kinds of cells could be distinguished in the pancreatic islets presumably corresponding to the alpha and beta cells. Comparisons between experimental and control fish reveal no consistent differences in the appearance of either cell type.

Enami's suggestion that the caudal neurosecretory system may be involved in buoyancy control led to a brief investigation of this system. Neither in the neurosecretory cell bodies nor in the urophysis from which the neurosecretory material is released could any differences be noted in comparisons between experimental and control fish. No differences in the amount of neurosecretory material present could be noted.

The data gathered on lactic acid production in vitro by the swimbladder gas gland are presented in Figure 7. After one and two hours of incubation there was an average of 51 mg per cent and 54 mg per cent of lactic acid respectively. These quantities may reflect the basal level of lactic acid in the gas gland tissues. A single measurement of the lactic acid content of a gas gland which had been treated with fluoride ions to inhibit glycolysis gave a value of 15 mg per cent lactic acid. This latter value is somewhat lower than the minimum value of 24 mg per cent lactic acid for one hour of incubation in buffered saline. Whether there is significant glycolysis occurring in the isolated gas gland during the first two
hours of incubation is doubtful, although Heath and Pritchard (1962) report the normal blood lactic acid level in the bluegill to be 10 mg per cent, a value close to the single value for the fluoride inhibited gas gland. Blood, however, is not the main constituent of the gas gland. Ball et al. (1955) found the initial level of lactic acid in isolated gas glands of Stonotomus to be 60 mg per cent, a value which more nearly agrees with my observations on one and two hours of incubation. Until more data are gathered for the normal level of lactic acid present in the gas gland, no definite statement can be made concerning the amount present or its source.

After incubation for 4 to 20 hours, lactic acid is found in increasing amounts in the incubation medium. The methods used do not show if this lactic acid represents the total of lactic acid produced by the gas gland cells or whether it merely represents the amount of lactic acid produced in excess of what glycolytic products are further metabolized through the citric acid cycle or some alternate metabolic pathway. From the data of other workers, however, it is likely that the latter alternative is true as is discussed below.

After 22 hours of incubation there is a sharp increase in lactic acid production following a decrease at 21 hours. It seems possible that at 20 to 21 hours of incubation glycogen is depleted from the gas gland cells and the maximum lactic acid production at 22 hours may represent the catabolism of the pools of intermediate products in the glycolytic pathway.

The amount of lactic acid declines steadily after 22 hours of
Figure 7. *In vitro* lactic acid production by isolated swimbladder gas glands of *L. macrochirus* incubated in saline buffered to pH 7.4 at 24-25°C. Vertical lines represent the range of observed values, vertical bars represent ± the S. E. of the mean and the joined horizontal lines represent the means. Number in parentheses above each vertical line is the number of observations for that incubation time.
incubation until by 26 hours or more none or almost none can be
detected. Two single measurements after very long incubation periods
(56 and 74 hours) reveal no lactic acid in the incubation medium.
It is possible that this lactic acid may be catabolized by the citric acid cycle or converted to some other metabolic product by the gas
gland. Although the gas gland cells have few mitochondria (Copeland, 1960 and Dorn, 1961) and the NADI reaction is weak (Fänge, 1966),
cellular respiration is stimulated by succinate (Ball et al., 1955)
and inhibited by malonate (Fänge, 1953). Thus the possibility exists
that lactic acid could be catabolized by the citric acid cycle in
the gas gland cells, although no data other than the disappearance
of lactic acid from the incubation medium are available on this point
for the species with which I worked. Information on possible alternate
pathways of metabolism seem to be lacking for any species. Steen
(1963) also suggests that lactic acid may be catabolized by the gas
gland cells, but Fänge (1966) suggests that blood cells and the
vascular endothelial cells may be responsible. In my work the sub-
mucosal connective tissue could never be completely removed from the
gas gland before incubation. Perhaps the cells present there could
also account for some portion of the lactic acid catabolism.

Apparently free glucose is not released into the incubation
medium from the gas gland cells as a result of glycolysis. The
incubation medium for each of five gas glands incubated for 4 hours
was assayed for free glucose, but none could be detected. The same
observation was made for sets of five gas glands incubated for 12
and for 24 hours.
Since the in vivo results suggested an influence of body size on the rate of glycolysis in the gas gland, the data gathered in vitro were analyzed statistically to see if the same relationship held true. The rate of lactic acid production by gas glands from 4 to 20 hours of incubation is nearly linear (see Figure 7). The absolute amount of lactic acid produced by each gland was divided by the appropriate factor to reduce the quantity to the amount produced in 4 hours. This quantity, the weight of the gas gland that produced the lactic acid and the weight of the fish from which the gas gland was taken were treated by the method of partial correlation as outlined by Simpson et al. (1960). This method allows the comparison of correlation among more than two variable factors and determination of how much of the correlation value results from the relationship between any two of the variable factors. The results of this analysis are presented in Table 3.

From the results in Table 3, it may be deduced that the weight of the gas gland influences the production of lactic acid when the influence of body weight is eliminated. Conversely, body weight is not correlated with lactic acid production, at least not in a rectilinear fashion, when the influence of gas gland weight is eliminated. Even though the total correlation between body weight and lactic acid production is significant, the high total correlation between body weight and gas gland weight is responsible for this situation. Apparently for the in vitro procedure the size of the gas gland itself rather than the size of the fish from which it was taken is the factor determining the rate of lactic acid production. This finding may not,
however, be comparable to the results from the in vivo procedures
where body size seems to be important.

Table 3. Total and partial correlations of body weight, gas gland
weight and weight of lactic acid produced per 4 hours incubation for
times from 4 to 20 hours. N = 45.

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r</th>
<th>Degrees of Freedom</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>body wt. vs. gas gland wt.</td>
<td>.64</td>
<td>43</td>
<td>.001</td>
</tr>
<tr>
<td>body wt. vs. wt. lactic acid/4 hr.</td>
<td>.41</td>
<td>43</td>
<td>.01</td>
</tr>
<tr>
<td>gas gland wt. vs. wt. lactic acid/4 hr.</td>
<td>.59</td>
<td>43</td>
<td>.001</td>
</tr>
<tr>
<td>body wt. vs. gas gland wt. excluding wt. lactic acid/4 hr.</td>
<td>.54</td>
<td>42</td>
<td>.001</td>
</tr>
<tr>
<td>body wt. vs. wt. lactic acid/4 hr. excluding gas gland wt.</td>
<td>.05</td>
<td>42</td>
<td>not significant</td>
</tr>
<tr>
<td>gas gland wt. vs. wt. lactic acid/4 hr. excluding body wt.</td>
<td>.47</td>
<td>42</td>
<td>.001</td>
</tr>
</tbody>
</table>

The in vitro incubation method was also used to test the effects
of glucagon and epinephrine on gas gland metabolism. Student's t
test modified for small samples was used to determine if a signifi-
cant difference existed between the mean values of lactic acid pro-
duction for experimentals and controls. The results obtained by these
procedures were presented in Table 4 and Table 5.

The results in Table 4 show that glucagon promotes the rate of
glycolysis resulting in an increased rate of lactic acid production.
The amount of glucagon was diluted by a factor of 10 in one experiment
to determine a dosage response effect. With this minimum concentra-
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature</th>
<th>Incub. Time</th>
<th>Range lactic acid</th>
<th>Mean lactic acid</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mg glucagon/100 ml saline</td>
<td>27.0</td>
<td>5</td>
<td>91 - 180</td>
<td>128</td>
<td>0.28</td>
<td>n.s.</td>
</tr>
<tr>
<td>control (no glucagon)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg glucagon/100 ml saline</td>
<td>27.5</td>
<td>5</td>
<td>86 - 169</td>
<td>131</td>
<td>3.28</td>
<td>0.025</td>
</tr>
<tr>
<td>control (no glucagon)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg glucagon/100 ml saline</td>
<td>28.5</td>
<td>5</td>
<td>127 - 202</td>
<td>161</td>
<td>3.91</td>
<td>0.01</td>
</tr>
<tr>
<td>control* (no glucagon)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg glucagon/100 ml saline, boiled 5 min.</td>
<td>23.5</td>
<td>5</td>
<td>93 - 172</td>
<td>142</td>
<td>1.98</td>
<td>0.10</td>
</tr>
<tr>
<td>control (no glucagon, boiled)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg glucagon/100 ml saline, boiled 10 min.</td>
<td>22.0</td>
<td>5</td>
<td>99 - 158</td>
<td>125</td>
<td>2.89</td>
<td>0.025</td>
</tr>
<tr>
<td>control* (no glucagon, boiled)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg gelatin/100 ml saline</td>
<td>24.5</td>
<td>5</td>
<td>116 - 172</td>
<td>142</td>
<td>0.40</td>
<td>n.s.</td>
</tr>
<tr>
<td>control (no gelatin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*These samples have 4 replicates while all others have 5.
tion of glucagon there was an absolute amount of 0.015 mg in the 5 ml of incubation medium, an amount which produced no increase in lactic acid production. At 10 times this concentration a statistically significant difference could be noted but greater concentrations were not used. Glucagon caused the production of 1.3 to 1.7 times more lactic acid by the gas gland than saline alone.

To rule out other possible effects of glucagon, I attempted to destroy its structure by boiling for brief periods. These attempts were unsuccessful probably because bovine glucagon is a rather small polypeptide composed of 29 amino acids and thus probably without appreciable secondary or tertiary structure which would be affected by boiling. Chemical or enzymatic hydrolysis would likely be more effective for inactivation. Another kind of control experiment consisted of adding gelatin to the incubation medium. If the added glucagon were producing some osmotic effect or if it were being hydrolyzed and the constituent amino acids were being metabolized to lactic acid, the addition of a protein which is not a hormone could produce similar effects. Gelatin addition, however, showed no effect on lactic acid production, thus supporting the hypothesis that the effects of the addition of glucagon are truly hormonal in nature.

Young and Chavin (1965) report that injection of glucagon into the goldfish produces a hyperglycemic effect. At a dosage of 100 micrograms/kilogram, serum glucose levels were higher than controls after 30 minutes. In this case hyperglycemia must have resulted from mobilization of hepatic glycogen. The dosage used by these investigators is approximately five orders of magnitude smaller than the minimal dose which I found to be necessary for stimulation of
Table 5. Effects of epinephrine on lactic acid production in gas glands incubated in vitro.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature degrees C.</th>
<th>Incub. Time hr.</th>
<th>Range mg per cent lactic acid</th>
<th>Mean mg per cent lactic acid</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mg epinephrine/100 ml saline</td>
<td>24</td>
<td>6</td>
<td>134 - 218</td>
<td>164</td>
<td>1.43</td>
<td>0.2</td>
</tr>
<tr>
<td>control (no epinephrine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg epinephrine/100 ml saline</td>
<td>26</td>
<td>4</td>
<td>112 - 135</td>
<td>121</td>
<td>1.46</td>
<td>0.2</td>
</tr>
<tr>
<td>control (no epinephrine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg epinephrine/100 ml saline</td>
<td>23</td>
<td>5</td>
<td>28 - 82</td>
<td>55</td>
<td>1.50</td>
<td>0.2</td>
</tr>
<tr>
<td>control (no epinephrine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
glycolysis in the gas gland in vitro. Whether this difference in dosage response results from a true difference in responsiveness between liver cells and gas gland cells or to a difference between in vivo and in vitro responsiveness or to some additional factor or factors cannot be stated with the available data.

Six preliminary experiments using five gas glands each consisted of incubation for 4 hours in saline with epinephrine but without accompanying controls. In every case the mean value of lactic acid produced was slightly lower than or equal to the mean value for 4 hours of incubation in saline as shown in Figure 7. The four concentrations of epinephrine used ranged from 2.0 to 0.002 mg/100 ml saline. To be sure that epinephrine was not influencing the gas glands the three additional experiments in Table 5 were conducted with accompanying controls for statistical comparisons like those for glucagon. Since the mean value in all comparisons is not statistically significant, epinephrine is assumed not to affect the production of lactic acid by stimulation of glycolysis in the gas gland cells.
DISCUSSION

That glycogen depletion of the gas gland cells occurs as the swimbladder fills with gas has been confirmed for *Lepomis macrochirus*. There is a sufficient glycogen reserve in the gas gland cells of this species to allow complete refilling of the swimbladder with gas at least one time and probably nearly two times. It is, of course, quite unlikely that a fish would have its swimbladder deflated under natural conditions, but there would be the necessity of secreting gas into the swimbladder as the fish swims downward in water. As the depth increases, the atmospheric pressure becomes greater at a rate of approximately one atmosphere of pressure for every ten meters of depth. This set of conditions would mean that for every ten meters of descent, the gas gland would have to secrete a volume of gas equal to one-half of the volume present at the lesser depth in order to maintain neutral buoyancy.

Since this species can replace 75 per cent of its swimbladder volume in approximately 12 hours, 8 hours of gas secretion would be necessary to regain neutral buoyancy if a bluegill descended ten meters. Eight hours of gas gland activity would deplete more than one-third of the glycogen reserves if the data gained *in vitro* were comparable (see below). Thus, this species could descend to a maximum depth of less than 30 meters below its original depth and still be able to regain neutral buoyancy by using its gas gland glycogen reserves for the production of lactic acid. Although my
data do not demonstrate it, other sources of glucose such as the liver could probably be used by the gas gland for lactic acid production. This situation seems to be less efficient, however, since fish deflated for relatively long periods were slower in refilling the swimbladder. In its natural habitat it is unlikely that a bluegill would ever descend much more than 10 meters and probably 3 to 5 meters would cover the maximum descent of most individuals.

Although filling of the swimbladder is known to be integrated by nervous stimulation and hormonal influences on the muscularis mucosae and the vascular supply, it is not surprising that a hormonal influence on metabolism of the gas gland should also exist. That hormonal control is exerted over many metabolic functions is well recognized today.

Enami (1958) suggested an involvement of the caudal neurosecretory system in buoyancy regulation. My results with the caudal neurosecretory system along with those of the two authors previously cited do not support this theory nor do the unpublished results of three other investigators cited by Fridberg and Bern (1968). Fridberg (1966) also states that many workers have repeated Enami's buoyancy experiments without confirmation of his results. Until additional positive evidence becomes available, an influence of the caudal neurosecretory system on the swimbladder may be regarded with skepticism.

Glucagon, however, has been shown in these experiments to stimulate glycolysis in the gas gland while epinephrine has been shown to lack this action, at least in vitro. However, in other circumstances among vertebrates both of these hormones promote glycolysis by causing activation of adenyl cyclase which by a series of steps causes
the activation of phosphorylase, the enzyme responsible for phosphorylation of the glucose units of glycogen. Both hormones promote glycolysis in the liver, but only epinephrine is effective in skeletal muscle. From the experiments on \textit{Lepomis} it is probable that only glucagon is effective in gas gland tissue. Even though both hormones operate by the same pathway, there is apparently some difference in the starting point. Bitensky \textit{et al.} (1968) suggest that separate adenyl cyclase systems exist in rat liver which respond to epinephrine but not to glucagon and \textit{vice versa}. Thus the ability of the gas gland to respond to glucagon but not to epinephrine may reflect the presence of a glucagon responsive adenyl cyclase system.

If the findings on hormonal influence are applied to fish living under natural conditions, glucagon can be assumed to accelerate the rate at which neutral buoyancy can be regained after a descent. If the fish descended 10 meters and secretion of gas to regain neutral buoyancy takes 8 hours, it might be assumed that this secretion would occur under the influence of glucagon. Thus \textit{in vitro} incubation is not strictly comparable to \textit{in vivo} experiments with reference to the time course of glycogen depletion. Glucagon accelerates lactic acid production approximately 1.5 times \textit{in vitro}. If the same effect is present in vivo, then approximately 12 hours of gas secretion time by the gas gland would be necessary for regaining neutral buoyancy if glucagon were not secreted. Twelve hours incubation \textit{in vitro} depletes over half of the glycogen present. Therefore the maximum descent possible for this species with complete depletion of its gas gland glycogen would be less than 20 meters. The probable maximum descent
as suggested above of 3 to 5 meters would therefore consume 15 to 25 per cent of the gas gland glycogen store which would give the animal an abundant reserve.

In summary, the gas gland of *L. macrochirus* contains an abundant glycogen store that is catabolized to lactic acid when gas is being secreted into the swimbladder. The swimbladder is under control of both the nervous system and the endocrine system. Epinephrine is active in regulation of the swimbladder volume by influencing contraction of the muscularis mucosae but it does not exert any apparent influence on gas gland metabolism. Since Fange's experiments (Fange, 1953) showed that epinephrine put the swimbladder in a condition to reabsorb rather than to secrete gas, it would be biologically contradictory for epinephrine to promote glycolysis in the gas gland cells. In addition to acetylcholine, glucagon may now be considered as antagonistic to the action of epinephrine in that glucagon promotes secretion of gas into the swimbladder by accelerating gas gland metabolism.
SUMMARY

1. The swimbladder of *Lepomis macrochirus*, a euphysoclistous fish, was studied by both *in vivo* and *in vitro* techniques.

2. The volume of the swimbladder in this species displaces a volume of water equal to 7 per cent of the body weight as is characteristic of freshwater fish.

3. To experimentally induce gas secretion and therefore gas gland activity, 75 per cent of the swimbladder volume of gas was withdrawn by puncture techniques. Continuous secretion was induced by one or more additional withdrawals at 10 hour intervals.

4. The glycogen stores of the gas gland were depleted after 15 to 20 hours of gas secretion as revealed by PAS histochemistry.

5. Attempts to relate these findings on glycogen depletion in the gas gland to events in the liver, pancreas, head kidney and caudal neurosecretory system as shown by histological techniques were unsuccessful.

6. Isolated gas glands were incubated in vitro on a buffered saline for periods of time ranging from 1 to 30 hours. After incubation the saline was colorimetrically assayed for lactic acid produced by glycolysis in the gas gland.

7. Lactic acid was present at an average level of 50 mg per cent after incubation periods of 1 or 2 hours and rose to an average maximum of 230 mg per cent after 22 hours. Thereafter the lactic acid level of the medium declined reaching zero at 26 hours of incubation.
8. The addition of glucagon to the incubation medium, even after boiling, at a concentration of 3 mg/100 ml saline accelerated the rate of lactic acid production approximately 1.5 times.

9. The addition of glucagon at a concentration of 0.3 mg/100 ml saline, gelatin at concentrations of 3.0 and 0.3 mg/100 ml saline or epinephrine at concentrations of 2.0, 0.2, 0.02 and 0.002 mg/100 ml saline caused no acceleration in lactic acid production.

10. The significance of these findings is discussed with reference to the ability of this species to move deeper in water and regain neutral buoyancy.


Fridborg, G. 1966. Personal communication.


Sigma Chemical Company. 1960. Personal communication.


