TEMPERATURE ACCLIMATION EFFECTS ON PROTEINS
IN THE EURYTHERMAL FISH GILLICHTHYES MIRABILIS

by

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THE UNIVERSITY OF BRITISH COLUMBIA

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ABSTRACT

The effect of temperature on biochemical adaptation in the eurythermal fish Gillichythes mirabilis, long jaw mudsucker, was studied with respect to two questions: (a) How much restructuring of overall tissue proteins occurs with acclimation to cold and warm temperatures, and (b) how does the nature of protein restructuring relate to eurythermy? Much literature exists showing temperature acclimation induces changes in proteins and enzyme functions, and that these changes may be short term or long term induction effects. However, no statement has been made for the extent of overall protein pattern restructuring that may occur with thermal acclimation.

In the eurythermic species studied, no evidence arises for major changes in protein composition with thermal acclimation. The results are considered in a discussion (a) of some of the environmental limits imposed upon this species, (b) of evolution under eurythermic and stenothermic conditions in selective utilization of gene products for adaptation on the molecular level, and (c) of some characteristics of enzymes important to their functional differences, as found by other investigators.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>List of Graphs</td>
<td>vi</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS</td>
<td>4</td>
</tr>
<tr>
<td>1. List and sources of chemicals used</td>
<td>4</td>
</tr>
<tr>
<td>2. Experimental materials</td>
<td>5</td>
</tr>
<tr>
<td>III. METHODS</td>
<td>6</td>
</tr>
<tr>
<td>1. Acclimation</td>
<td>7</td>
</tr>
<tr>
<td>2. Polyacrylamide gel electrophoresis</td>
<td>7</td>
</tr>
<tr>
<td>a. recrystallization of monomers</td>
<td>7</td>
</tr>
<tr>
<td>b. stock solutions</td>
<td>8</td>
</tr>
<tr>
<td>c. working solutions and preparation of gels</td>
<td>8</td>
</tr>
<tr>
<td>3. Sodium dodecyl sulfate gels</td>
<td>11</td>
</tr>
<tr>
<td>4. Staining and recording of protein bands in SDS and polyacrylamide gels</td>
<td>12</td>
</tr>
<tr>
<td>a. TCA fixation of proteins</td>
<td>12</td>
</tr>
<tr>
<td>b. staining of proteins</td>
<td>12</td>
</tr>
<tr>
<td>c. destaining and storage</td>
<td>12</td>
</tr>
<tr>
<td>d. photography and densitometry graphs of gels</td>
<td>13</td>
</tr>
<tr>
<td>5. Starch gel electrophoresis</td>
<td>13</td>
</tr>
<tr>
<td>a. procedures for a discontinuous buffer system</td>
<td>14</td>
</tr>
<tr>
<td>b. specific enzymes studied</td>
<td>15</td>
</tr>
</tbody>
</table>
6. Tissue preparation and cellular fractionation 17
   a. tissue preparation 17
   b. subcellular fractionation 18
   c. polyribosome isolation 20

IV. RESULTS 21
   1. Acclimation 21
   2. Gel electrophoresis 23
   3. Polyribosome and monoribosome profile 41

V. DISCUSSION 42
   1. Electrophoretic results and tissue comparisons 42
   2. Acclimation adaptations 44
   3. Protein and enzymic functional adaptations 45

APPENDIX A 49
   1. Polyacrylamide gel electrophoresis 49
   2. Sodium dodecyl sulfate acrylamide gel electrophoresis 52

APPENDIX B: Densitometry graphs of acrylamide gels 54

BIBLIOGRAPHY 67
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Surface sea water temperatures 22</td>
</tr>
<tr>
<td>2</td>
<td><em>Gillichythes mirabilis</em> muscle proteins 27</td>
</tr>
<tr>
<td>3</td>
<td><em>Gillichythes mirabilis</em> brain proteins 28</td>
</tr>
<tr>
<td>4</td>
<td>Liver proteins short term freezer storage 29</td>
</tr>
<tr>
<td>5</td>
<td>Liver proteins long term freezer storage 30</td>
</tr>
<tr>
<td>6</td>
<td>Starved 28°C stressed <em>Gillichythes</em> m. tissues 31</td>
</tr>
<tr>
<td>7a</td>
<td>13 days acclimation 32</td>
</tr>
<tr>
<td>7b</td>
<td>20 days acclimation 32</td>
</tr>
<tr>
<td>8a</td>
<td>31 days acclimation 33</td>
</tr>
<tr>
<td>8b</td>
<td>38 days acclimation 33</td>
</tr>
<tr>
<td>9</td>
<td>45 days acclimation 34</td>
</tr>
<tr>
<td>10</td>
<td>52 days acclimation 35</td>
</tr>
<tr>
<td>11a</td>
<td>Low speed v.s. high speed post mitochondrial supernatant 36</td>
</tr>
<tr>
<td>11b</td>
<td>Mitochondria triton-x-100 soluble proteins 36</td>
</tr>
<tr>
<td>12</td>
<td>Acid gels of liver tissue subcellular fractions 37</td>
</tr>
<tr>
<td>13</td>
<td>Sodium dodecyle sulfate acrylamide gels of mitochondria 38</td>
</tr>
<tr>
<td>14</td>
<td>Fresh 24°C trout tissues; liver, muscle, brain 39</td>
</tr>
<tr>
<td>15</td>
<td>Frozen trout tissues; liver, muscle 40</td>
</tr>
</tbody>
</table>
# List of Graphs

<table>
<thead>
<tr>
<th>Graph</th>
<th>Densitometry graphs of polyacrylamide gels:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>Gillichythes mirabilis</em> muscle</td>
<td>55</td>
</tr>
<tr>
<td>II</td>
<td>Liver, non-frozen tissue</td>
<td>56</td>
</tr>
<tr>
<td>III</td>
<td>Liver, one week frozen stored tissue</td>
<td>57</td>
</tr>
<tr>
<td>IV</td>
<td>28°C starved stressed - liver</td>
<td>58</td>
</tr>
<tr>
<td>V</td>
<td>28°C starved stressed - muscle</td>
<td>59</td>
</tr>
<tr>
<td>VI</td>
<td>28°C starved stressed-brain</td>
<td>60</td>
</tr>
<tr>
<td>VII</td>
<td>13 days acclimation</td>
<td>61</td>
</tr>
<tr>
<td>VIII</td>
<td>20 days acclimation</td>
<td>62</td>
</tr>
<tr>
<td>IX</td>
<td>31 days acclimation</td>
<td>63</td>
</tr>
<tr>
<td>X</td>
<td>38 days acclimation</td>
<td>64</td>
</tr>
<tr>
<td>XI</td>
<td>45 days acclimation</td>
<td>65</td>
</tr>
<tr>
<td>XII</td>
<td>SDS gels of mitochondria</td>
<td>66</td>
</tr>
</tbody>
</table>
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I. **INTRODUCTION**

The study of biochemical adaptation to environmental temperature changes in poikilotherms has indicated various mechanisms for maintaining metabolic homeostasis. Discussions of earlier literature have been given by Bulluck (1955), Fry and Hochachka (1970), and more recently by Hochachka and Somero (1971, 1973), and Hazel and Prosser (1974).

The ability to maintain stable metabolic rates over wide ranges of temperature changes, diurnally and seasonally, has been attributed to a number of "strategies" whereby temperature operates as a modulator, affecting enzymic activity either directly or indirectly, and with immediate compensatory or longer seasonal acclimation effects. Direct modulation of enzyme activities during acclimation has been indicated by such phenomena as (a) functional interconversions of one enzymic form (Somero, 1969; Behrisch & Johnson, 1974a), (b) induced changes in isoenzymes variants (Baldwin & Hochachka, 1970; Hochachka & Lewis, 1970; Hochachka & Clayton-Hochachka, 1973), and (c) induction of specific regulatory enzymes resulting in major restructuring of metabolic pathways (Hochachka, 1968).

Temperature may also impose numerous effects indirectly on enzyme activity by affecting cellular ionic environments and enzyme-ligand affinities (Behrisch & Johnson, 1974b; Moon, 1972). All these influences would necessarily have complex effects upon metabolic integrations and cellular energetics.

Studies of protein synthesis have indicated acclimation-induced changes in rates of protein synthesis (Das & Prosser, 1967; Haschemeyer, 1969a & b). These, and results from isoenzyme studies provided ground
for the proposal that economy of cellular energetics would favor production of new variants of an enzyme possessing apparent Km (enzyme activity at substrate concentrations giving one half the maximal reaction velocity) properties more efficient at new acclimation temperatures rather than producing larger quantities of the same enzyme.

A consideration of these facts led to the question: To what extent does restructuring of proteins occur in acclimation? Since a systematic investigation of all regulatory enzymes would have been a ponderous project, the question was approached by subcellular fractionation and studies of overall protein pattern distributions using acrylamide and sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis. Studies were also performed on polysomes and monosomes to determine if any correlations existed between protein synthesis and acclimation temperatures.

The eurythermal fish *Gillichthyes mirabilis* (long jaw mudsucker) was selected for the studies because of its great capacity for metabolic adaptation, which is suggested by the tolerance to wide temperature ranges and other physical factors encountered in its normal estuarine habitat (Todd & Ebeling, 1966; Weisel, 1947).

In attempting to answer the question put forth for this research, the results are discussed with respect to the eurythermic nature of *Gillichthyes*. Considerations of protein and enzymic functional adaptations are also made to try and understand what some of the limiting factors might be which would influence the type of adaptive mechanism for temperature compensations employed
by a species; whether it be by overall protein restructuring, by isoenzymic induction, or by more subtle specific cellular biochemical factors impinging upon protein catalytic functions.
II. MATERIALS

1. List and sources of chemicals used

Acrylamide
Amberlite ion exchange resin MB-3
Amido Schwartz (Buffulo Black/Napthol Blue Black)
Ammonium persulfate - reagent grade
Bromopheno Blue
Coumassie Brilliant Blue R
Dithiothreitol
Glycine (ammonia free)
Hydrochloric acid (HCl) - reagent grade
NN'Methylenebisacrylamide (bisacrylamide)
Riboflavin (B₂)
Sodium dodecyl sulfate (SDS)
Trichloroacetic acid (TCA)
N, N, N'-tetramethylethyldiamine (TEMED)
Triton-x-100 (octyl phenoxy polyethoxyethanol)
Polyacrylamide gel electrophoresis apparatus

Eastman
Malinckrodt
Eastman
Mann Res. lab.
Eastman
Eastman
Calbiochem
Allied Chem.
Eastman
Sigman
Buchler Instr. Inc.
2. **Experimental materials**

The eurythermal fish *Gillichthyes mirabilis* was used for the acclimation studies done at Scripps Institute of Oceanography, La Jolla, California. Tissues for trout studies were from frozen stored specimens that had been acclimated in Vancouver by Dr. Peter Hochachka's laboratory at the University of British Columbia. One fresh trout from the San Diego area lake hatcheries was also used in the preliminary studies of trout tissues. This trout had a thermal history of 24°-26°C.

Since the source of *Gillichthyes* (from a local bait shop) was not controlled, size variation occurred with different batches of fish and genetic pools were likewise undetermined. Attempts were made at obtaining uniformity in fish used for acclimation by selecting fish of similar size and from the same batch bought on a particular date.
III. METHODS

1. Acclimation.

*Gillichthyes mirabilis* were cold acclimated at 8°C (6 hr light, 18 hr dark), and warm acclimated at 28°C (15 hr light, 9 hr dark). Controls were held at ambient sea water temperatures (20°-22°C).

Fish were maintained in 2-gallon glass jars, covered with metal screens to prevent fish from escaping especially during warm acclimation. For cold acclimation, fish in ambient sea water were transferred to the cold room directly. Warm acclimation was done more gradually over a two day period to reduce the significantly higher death rate, particularly during the first week of warm acclimation.

To determine if acclimation over a period of time would induce quantitative shifts in protein patterns to become visible qualitative differences, studies were performed at intervals of 13, 20, 31, 38, 45, and 52 days acclimation. One study of extreme condition of stress at 28°C for four months and starving for about two months prior to the experiment, was made to determine what extent protein patterns would be affected by such extremes as compared to long term acclimation only.

Fish were fed chopped frozen squid, also obtained from bait shops, and were transferred into clean temperature-equilibrated sea water once or twice a week.
2. Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis was employed for protein separations because this method gives good separations with reproducibility (Matson, 1965; Dehlinger & Schimke, 1971), and has the advantage of requiring micro-liter amounts of protein. The translucent gels could be stained to give bands that were easily photographed and from which densitometry readings were made.


To ensure purity of acrylamide in its monomeric form and to protect against ion contamination, both acrylamide and bis-acrylamide were recrystallized. Both compounds are exceedingly poisonous and suitable precautions must be taken against contact with the skin or inhalation of the light crystals. 70 grams of acrylamide were dissolved in 1 liter of chloroform at 50°C, filtered hot without suction and allowed to recrystallize at -20°C, after which they were washed with cold chloroform and suction-dried. Bis-acrylamide was similarly purified using acetone (approximately 10 grams per liter).

Recrystallized material was stored under cool, dry, dark conditions to slow spontaneous polymerization and hydrolysis. Separate stock solutions of 15% (w/v) of acrylamide and suitable concentrations of bis-acrylamide in H₂O can be stored for at least one month under refrigeration and in the dark.
b. **Stock solutions** (Davies, 1964)

Separation gel stock solutions (pH 8.9 *):

(A) 1 N HCl *48.0 ml (B) acrylamide 28.0 gm
tris 36.6 gm bis-acrylamide 0.735 gm
TEMED 0.25 ml dH₂O to 100 ml

dH₂O to 100 ml

(C) Ammonium persulfate 0.28 gm/100 ml (10% w/v of acrylamide present) (made fresh on day of use.)

Stacking gel stock solutions (pH 6.7 *):

(D) 1N HCl *48.0 ml (E) acrylamide 10.0 gm
tris 5.98 gm bis-acrylamide 2.5 gm
TEMED 0.46 ml dH₂O to 100 ml

dH₂O to 100 ml

(F) Riboflavin 4 mg/100 ml dH₂O

(G) Sucrose 40% (w/v)

All solutions should be stored cold, and except for (G), stored dark.

c. **Working solutions and preparation of gels**

Working solutions:

Stock solutions were allowed to warm to room temperature prior to use. Separation gels (small pore) were made up with the above stocks at proportions of 2A:1B:1dH₂O, and ammonium persulfate to give 10% w/v of acrylamide present. Stacking gels (large pore) were made with 1D:2E:F:4G.

Chamber electrolyte buffer was made by a 1:10 dilution of stock buffer (29 gm glycine, 6 gm tris, 5 ml N HCl, and 975 ml dH₂O) to give
a final pH of 8.1. It is kept cold at the temperature of electrophoresis (4°C).

Preparation of gels:
The working solutions were degassed in vacuo for a few seconds prior to use. Gels were made in straight pyrex glass tubing, 80 mm x 5 mm (intern. diam.). Tubes for a set were cut from the same tubing to minimize variations in electrical resistance from column to column during electrophoresis. The glass tubes were acid cleaned, treated with a rinse of 1/200 parts of photo-flo, and dry before use. Treatment with photo-flo facilitates gel removal from the tubes after electrophoresis. Similar result is possible by the use of Plexiglass tubing (Davies, 1964). The tubes were capped and lined up perpendicularly for polymerization of the running gel solution, which was carefully pipetted into the columns to give a volume of 1.5 cm from the top after polymerization. Slight shrinkage occurs during polymerization. The latter takes place in the dark and requires about twenty to thirty minutes. In order to obtain a flat sharp surface after polymerization upon which stacking gel will follow, the running gel solution was immediately overlaid with a small amount of distilled water prior to polymerization, by use of a fine needle micro-syringe. It is very important that surface irregularities be avoided as these affect migration of proteins into the running gel. Similarly, care should be taken to avoid trapping air bubbles when running gel is pipetted into the columns. It has been suggested that polymerization should ideally be at the same temperature as that for electrophoresis to eliminate thermal contraction or expansion of the gel matrix (Chrambach & Rodbard, 1971).

Preparation of the stacking gel required similar precautions for getting uniformity of gel surface and polymerization. The water
above the running gel was removed and residual water soaked up with filter paper. Stacking gel was about 6 cm high after polymerization. For good stacking effect the volume should be greater than 50% of the protein solution volume put on the column for electrophoresis. After being overlaid with water, stacking gel polymerization was initiated by the presence of light by lining the columns at a uniform distance of about two inches from a day-light fluorescent tube. Polymerization takes about ten to fifteen minutes, causing the stacking gel solution to become uniformly opaque when polymerized.  

After polymerization, gel tubes were removed from their caps and inserted uniformly into the electrophoresis apparatus, making sure no air bubbles were trapped, and leaving sufficient column length to allow for observation of protein stacking and migration into the running gels during initial electrophoresis. A few drops of the tracking dye Bromophenol Blue was mixed into the upper buffer compartment, and after temperature of the gels were equilibrated to the 4°C conditions of electrophoresis, protein samples (made heavy by 40% sucrose) were micro-syringed onto the stacking gel for electrophoresis.  

In split-gels, a snugly fitting piece of index card was inserted into the tube and slightly into the stacking gel prior to protein application, and electrophoresis begun immediately after protein samples were applied to the separated sides of the tube (Dunker & Rueckert, 1969).  

Electrophoresis was carried out with an initial current of 2 ma per tube. After protein entered the separation gel, current was increased to 5 ma per tube. Current should not exceed 5 ma per tube to avoid excessive ohmic heating, which results in protein migration pattern artifacts. Initial low current is a precaution against
convective losses of the sample into the upper reservoir (Davies, 1964). Electrophoresis was terminated when the tracking dye was about 3 mm from the bottom of the gel (in about 1.5 hrs). A variable voltage regulated power supply was used (Heath Kit Inc.).

3. **Sodium dodecyl sulfate gels**

Proteins of mitochondria, post mitochondrial supernatants, and microsomal fractions were also studied using SDS polyacrylamide gels, which separates proteins based upon their size differences alone and not on charge. See appendix A for further details of the principles operating in this system.

Electrophoresis techniques were similar to those described for polyacrylamide gels, and were adapted from the methods of Weber and Osborn (1969), and from Kiehn and Holland (1970). 7% gels were prepared without stacking gels, using the solutions given for polyacrylamide gels, but with the addition of 0.1% SDS, and 0.1% 2-MeOH and 15 mg/ml ammonium persulfate added immediately before use. Electrophoresis chamber buffers likewise contained an addition of 0.1% SDS. Tissue homogenated for SDS electrophoresis were treated with 0.1% SDS and 2-MeOH, and incubated at 37°C for thirty minutes, then made heavy with 40% sucrose and mixed with about 5 ul of tracking dye (0.05% Bromophenol Blue in water). Dialysis, which is often recommended, was omitted as in Weber and Osborn. Mitochondria were freeze-thawed before the SDS mercaptoethanol treatment to disrupt the particals.

Electrophoresis at 8 ma per tube took about 3-4 hours for the 8 cm gels used. The gels were then stained with Coumassie Blue as described in section 4 of the methods.
4. Staining and recording of protein bands in SDS and polyacrylamide gels

Amido Schwartz and Coumassie Blue dyes were used. Comparisons of the dyes showed better resolution of minor protein bands by Coumassie Blue, which was the dye utilized in the majority of the experiments. Staining procedures were modified from Chrambach et al. (1967), and destaining procedures were from those used by Weber and Osborn (1969).

a. TCA fixation of proteins

Gels were removed from their tubing for protein fixation and staining by careful rimming under water with a long fine blunt needle syringe and by slight pressure of pipette bulb. The gels were immediately put into test tubes containing 12-15% trichloroacetic acid (TCA) for fixation of the protein bands separated during electrophoresis. Maximum protein band sharpness is obtained by quick fixation at these somewhat higher concentrations of TCA than the 10% normally used (Chrambach et al., 1967).

b. Staining of proteins

Staining was done separately from protein fixation since the dye is somewhat insoluble in TCA, and especially with the higher concentrations utilized in these methods. The staining solution was made up in 454 ml 50% MeOH, 46 ml acetic acid (9%), and 0.125 g Coumassie Blue (0.025% final conc.). A stock solution of 2% Coumassie in 50% MeOH is stable. Staining time was 1-2 hours or longer, depending on the intensity of stain required.

c. Destaining and storage

This was accomplished by soaking gels in washes of the destaining
solution which was made of 7% acetic acid and 5% methanol.

Gels were stored in 7% acetic acid and in the dark to prevent fading. No visible shrinkage of gels was found when stored this way, as compared to storage in TCA, which is often recommended.

d. Photography and densitometry graphs of gels

Gels were put in a clear plastic dish, covered with distilled water and photographed by transmitted light using Polaroid black and white 4 x 5 55P/N type film. Increased photographic sensitivity to protein stained bands was obtained by use of a dark green filter (No. 74). Red filter caused dense blue stained bands to become too black and lighter bands to be cut off. Timing was varied for optimal exposure at f32. Negatives were prepared for printing by treatment in 18% sodium sulfite as directed from Kodak, and rinsed in photo-flo.

Densitometry graphs of the negatives were made from a double beam recording microdensitometer, MK IIIB from Joyce, Loebl and Co.. Recordings were also done directly from the gels for comparison. Absorption spectrum studies of the gels were done at 325 mu for Amido Schwartz and at 560 mu for Coumassie Blue. Results from these methods were comparable and the densitometer method was selected for its convenience. Radioactive labeled nuclear proteins separated with sodium dodecyl sulfate polyacrylamide gels have been shown to give densitometry readings that correlate with P32 radioactivity for gel slices (Johnson, et al., 1974).

5. Starch gel electrophoresis

Starch gel electrophoresis was selected for studies of specific enzymes because the gel slab could be stained in one step. A system
of discontinous buffers was used which reduces electrophoresis time (Poulik, 1957), and therefore diffusion; factors that contribute to better protein separations. Buffer for the starch gels contained 0.076 M tris and 0.005 M citric acid (pH 8.6), and buffer for electrode chambers contained 0.3 M boric acid and 0.05 M sodium hydroxide. Electrophoresis was followed by the visible brown colored boundary which increased in intensity as the boundary moved along the gel towards the cathode. This boundary is probably caused by the replacement of the citrate ions in the gel by borate ions from the electrode vessels according to Poulik (ibid., 1957). The migration of proteins in this system gave good results in 3-4 hours using a potential gradient of 6 V/cm and at 4°C.

The basic procedures for starch gels followed the method introduced by Smithies (1955). Partially hydrolyzed starch obtained commercially was made up in appropriate buffer to make a 10% gel, using an erlenmeyer flask. The starch solution was heated slowly with continuous swirling and brought to the boiling point for 1-2 seconds. The hot solution was degassed and poured into the electrophoretic tray, covered securely with a sheet of heavy plexiglass making sure no air bubbles were caught between, and left to solidify at room temperature. After careful removal of the glass plate the gel was covered at all times with seran wrap to maintain stable moisture content. The gel was cooled to 4°C before protein was applied for electrophoresis. This was done by saturating pieces of Whatman filter paper with the protein solution and then inserting these into slits cut perpendicularly to the gel at the origin and set a few centimeters in from the end of the gel. Contact between electrolytic vessels and the gel was made by several sheets of filter paper saturated with electrode buffer.
b. **Specific enzymes studied**

A preliminary study was made of some regulatory enzymes in *Gillichthyes*. A more comprehensive list of enzymes have subsequently been studied by Dr. G. Somero, which provided similar indications. This list is given in table I, and was graciously supplied by Dr. Somero, to whom I am greatly indebted.
TABLE I. PROTEINS EXAMINED ELECTROPHORETICALLY

Dehydrogenases:
- \(\alpha\)-glycerophosphate dehydrogenase
- ethanol dehydrogenase
- glucose-6-phosphate dehydrogenase
- isocitrate dehydrogenase
- lactate dehydrogenase
- malate dehydrogenase
- octanol dehydrogenase
- 6-phosphogluconate dehydrogenase
- xanthine dehydrogenase

Other:
- acid phosphatase
- esterases
- fumarase
- glutamate-oxaloacetate transaminase
- leucine aminopeptidase
- peptidases
- phosphoglucomutase
- phosphohexose isomerase
- general protein

* grateful thanks are given to Dr. George Somero for this table.
6. **Tissue preparation and cellular fractionation**

   a. **Tissue preparation**

   Fish were killed by decapitation. Liver and brain tissues were quickly excised and homogenized each in 5 volumes of cold TMK-S (0.25 M sucrose, 0.05 M tris-HCl, pH 7.6, 0.025 M KCl, and 0.005 M MgCl₂), based upon the methods of Haschemeyer (1967), and with modifications from Schnaitman (1969), and Kiehn and Holland (1970) for tissue preparations for SDS gel electrophoresis. All procedures were done on ice and centrifugation was at 2°-4°C. In studies comparing individual variations of animals from the different acclimation temperatures, tissues from each animal were homogenized separately. Attempt at uniformity in all procedures was made to minimize variations due to mechanical manipulations.

   Brain tissue homogenization was accomplished by 10 strokes of a close fitting Dounce B homogenizer. Liver tissue was minced and homogenized using 5 strokes of the loose fitting Dounce A homogenizer, filtered through a nylon mesh to remove connective tissue, then homogenized with 15-20 strokes of the Dounce B homogenizer.

   Muscle tissue was homogenized in 10 volumes of distilled water for isolation of glycolysis proteins only (White, Handler, & Smith, 1964). Muscle tissue was obtained by removal of the epaxial muscle mass and stripping it of skin, mincing it finely with a Waring blender and then filtering with nylon mesh. The filtrate was homogenized with a ground glass homogenizer, centrifuged briefly at low speed to remove fibrillar material and the supernatant homogenized with the Dounce B homogenizer.
b. **Subcellular fractionation**

Subcellular fractionation followed the scheme shown below, which was modified from the composite flow chart in Mahlar and Cordes (1966), and from the authors cited in the preceding section for tissue preparation:

1. **Homogenize minced tissue (0.2g/ml) in TMK-S, with Dounce A, filter, Dounce B**
2. **400 x g, 10 min (twice)**
   - RBC
   - unbroken cells
   - connective tissue
3. **750 x g, 10 min (twice)**
   - crude nuclei
   - large membrane fragments
4. **Post nuclear supernatant**
5. **7,700 x g, 15 min (twice)**
   - crude mitochondria
   - post mitochondrial supernatant I (PMS I)
6. **14,800 x g, 30 min**
   - low speed microsomal fraction
   - rough and smooth vesicles
   - membrane fragments
   - (treatment with SDS MeOH for electrophoresis)
7. **PMS II (+DTT for electrophor.)**
8. **Soluble insoluble**
   - (+ DTT for electrophoresis)
9. **105,000 x g, 90 min**
   - ribosomal pellet
The fat layer that forms at the meniscus should be removed after centrifugation, otherwise lipid interference with clean fractionation occurs. This is especially important in polyribosomal isolation (Haschemeyer, 1967).

The initial centrifugation at 400 g was omitted in studies that did not include nuclei isolation. Likewise, the 750 g centrifugation step was omitted in studies limited to post mitochondrial fractions. The homogenates were then directly centrifuged at 7,700 g following the scheme shown.

Crude nuclei and mitochondrial pellets were made more pure by three washes with tris-sucrose (0.05M tris, pH 6.7, 0.25M sucrose). The washed pellets were then resuspended in 20 x volume of tris-sucrose and centrifuged at 14,000 g for 20 minutes. The particles were resuspended in TMK-S containing 2% triton-x-100 and 0.005 M dithiothreitol (DTT) for acrylamide electrophoresis. Freeze thawing was employed to promote lysis. In sodium dodecyl sulfate acrylamide gel electrophoresis, treatment with SDS and mercaptoethanol and incubation at 37°C was performed prior to electrophoresis, as described in section 3 of the methods.

Post mitochondrial supernatant was treated with triton-x-100 and DTT for electrophoresis, or alternatively treated with 2% triton-x-100 only and centrifuged at high speed to give triton-x soluble and insoluble fractions for electrophoresis.

Microsomal fractions were isolated by centrifugation of the untreated post mitochondrial supernatant (PMS I) at 14,800 for 30 minutes. The supernatant from this was layered over 40% sucrose made up in tris, and centrifuged at 105,000 g for 90 minutes to give a ribosomal pellet which was used for urea gel electrophoresis (Haschemeyer,

Polyribosomal isolation was obtained by homogenation of liver in 3 volumes of TMK-S pH 7.6, using 3 strokes of Dounce A, filtering with nylon mesh, and homogenating gently with 5 strokes of the Dounce B homogenizer. The homogenate was spun at 7,700 g for 20 minutes and the post mitochondrial supernatant treated with 2% triton-x-100 for 10 minutes. The PMS was then divided into two fractions; one for control was treated with 2 ug/ml ribonuclease. Equal volumes of control, PMS, and TMK-S with 2% triton-x were carefully layered onto three linear sucrose gradients (10-35% w/v at 4°C) containing 0.01 M tris-HCl pH 7.6, 0.02 M KCl and 0.005 M MgCl$_2$. Centrifugation was done in SW25 or SW27 Spinco rotors at 25,000 or 26,000 rpm for 0.5 to 2 hours.

The centrifuged gradients were punctured at the bottom with a fine needle and equal volume fractions were collected for analysis of absorbance at 260 um. All procedures prior to this last step must be done on ice. To protect against RNase contamination, all apparatus for homogenation was acid washed, and handling of apparatus parts used in direct contact with the tissue homogenate was avoided. Ribonuclease-free sucrose was prepared by boiling sucrose in a double boiler for one hour.
IV. RESULTS

1. Acclimation

The results presented here are selected from three batches of fish that were acclimated over the periods July 2 through October 27. (See chart below.) During this time sea water controls experienced varying ambient temperatures as shown here and in figure 1.

<table>
<thead>
<tr>
<th>ACCLIMATION DAYS</th>
<th>DAY OF FISH</th>
<th>AMBIENT SEA WATER CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>(8 and 28 C)</td>
<td>EXPERIMENT</td>
<td>BATCH</td>
</tr>
<tr>
<td>13</td>
<td>7/16</td>
<td>I</td>
</tr>
<tr>
<td>20</td>
<td>8/2</td>
<td>II</td>
</tr>
<tr>
<td>31</td>
<td>7/3</td>
<td>I</td>
</tr>
<tr>
<td>38</td>
<td>9/24</td>
<td>III</td>
</tr>
<tr>
<td>45</td>
<td>8/16</td>
<td>II</td>
</tr>
<tr>
<td>52</td>
<td>10/27</td>
<td>II</td>
</tr>
</tbody>
</table>

Fish survival during cold acclimation was significantly better than in warm acclimation, especially during the initial period of acclimation. The precaution for gradual acclimation described in the methods reduced this difference. Fish in cold acclimation maintained good appetite although their food consumption decreased compared to warm fish. Swimming activity in cold acclimation fish was also reduced and the fish tended to remain at the bottom of their jars. Warm acclimation fish were more active and tended to try to escape from their containers; they also exhibited surface air gulping behavior. (See discussion V-2.)

Body weight changes over the acclimation periods were also different under the two acclimation temperatures. In general, cold
Fig. 1. Surface sea water temperatures--1971.

From off the pier at Scripps Institute of Oceanography, University of California, La Jolla: Temperatures experienced by ambient sea water controls in the studies of Gillichthyes m., as indicated in the text on page 21.
acclimation fish tissues had greater fat content; their livers were especially high in lipids and were large and pale. Warm acclimation fish tended to lose weight over long term holding at 28°C. The decrease in protein stain obtained in polyacrylamide gel electrophoresis may be due to this loss of weight being reflected at the tissue biochemistry level as discussed in section V. Livers from long term acclimated fish were dark and tended to atrophy after about six weeks. Starvation at 28°C accentuated these abnormalities.

2. Gel electrophoresis

Results from acrylamide gel electrophoresis for Gillichthyes are shown in figures 2 to 13, and for trout in figures 14 to 15. Graphs for some of these figures are to be found in appendix B if more careful comparisons are wanted in reference to the differences given in the following results.

Acclimation effects on Gillichthyes were greater in liver tissue than in muscle or brain. Muscle (figure 2) and brain (figure 3) both showed no apparent changes in protein pattern distribution using acrylamide gel electrophoresis. Liver tissue was studied in greater detail. Preliminary studies were made to determine the effects that freeze storage of tissue would have on electrophoresis results. Proteins were stable with overnight freeze storage but longer storage increased denaturation of the slower migration species, affecting the resolution of minor protein bands with one week storage (figure 4b vs. 4a), and producing greater smearing and aggregation effects with storage over months (figure 5). It was found that starvation at high acclimation temperature changed the distribution pattern of proteins more than short term freeze storage, but that resolution of the protein bands were good;
liver tissues showed a greater number of fast migration protein bands around band 13 and 14, and a greatly increased peak for band 18 (figure 6). Starvation and high temperature conditions also caused major protein pattern changes in muscle tissue (figure 1 vs. 6; graph 1 vs. 5).

Acrylamide gel electrophoresis of liver post mitochondrial supernatant (7,700 x g, 20 mins) in general produced 20 clearly visible protein bands:

13 days acclimation (figure 7a; graph 7). No significant differences in protein distribution patterns were observed for short term acclimation of 13 days. The slight reduction in the intensity of the faster migration bands 18-20 in warm acclimation may be due to dilution effects from a slightly faster lead in migration rate. A discussion of factors affecting migration rates in gel electrophoresis is given in appendix A. The resolution of bands 3, 4, and 5 was better in cold than in controls, or warm fish. These results were duplicated in repeat electrophoretic runs of the same tissue homogenates (figure 4a; graph 2).

20 days acclimation (figure 7b; graph 7). A longer electrophoretic run was performed in this experiment to attempt better separation of the slower moving proteins. There was again a somewhat more intense staining of bands 3, 4, and 5 from 4°C fish, relative to 28°C or sea water control fish. Since the similar results were obtained from fish of two different batches, these minor variations between acclimation temperatures may be significant differences.

31 days acclimation (figure 8a; graph 8). Results from this experiment were poor, but within the range of resolution good reproducibility of protein banding pattern was obtained with duplicate gels. No apparent differences between 8°C and sea water controls were
indicated.

38 days acclimation (figure 8b; graph 9). Batch III fish used here exhibited greater number of protein bands, and these bands contained many variations in the regions of protein bands 3-13. Variations between tissues from individual fish were as great as between fish from different acclimation temperatures. This batch of fish exhibited a similar range of variations in a 20 days acclimation electrophoresis study. Possible polymorphic contributions to these variations are discussed in section V.

45 days and 52 days acclimation (figure 9, graph 10; figure 10). Warm acclimation tissues in general appeared to have poorer protein resolution in the slower migration species (bands 3, 4, and 5), compared to cold to controls, and poorer separation of some bands (7, 8) compared to controls. It might be that these apparent differences are accentuations of the slight variation indicated earlier for 13 and 20 day acclimations.

Miscellaneous studies

Comparisons of low speed and high speed supernatant proteins (figure 11a) suggests that sea water controls have somewhat higher concentrations of slower migration larger protein species in the slow speed supernatant. The reverse, a reduction of larger slower migration protein species, may be indicated for warm acclimation. More careful quantitative studies would have to be done to support these preliminary indications.

Mitochondria trition-x-100 soluble proteins (figure 11b) revealed differences in protein pattern which were also apparent in split gels. However, insufficient study was made in order to conclude
if the differences were real or were due to individual fish variations, as was found in the liver tissues of batch III fish.

Acid gel electrophoresis of various subcellular fractions (figure 12) gave poor resolution and basic gels were therefore utilized for the studies given. A discussion of factors necessary for good electrophoretic resolution, which have to be worked out for each system of proteins under study, is given in appendix A.

Mitochondrial SDS (sodium dodecyl sulfate) acrylamide gel electrophoresis results were very similar for all temperatures, with no apparent differences revealed by split gels (figures 13a, 13b; graph 11). Variations in the number of minor bands in the upper portions of the gels ('a' on graph) may be due to concentration effects and other factors affecting this upper region of the gel as discussed in appendix A.

**Trout tissues**

Long term storage of trout livers rendered them useless for electrophoretic study, as seen by the great loss in protein band resolutions (figure 15) relative to results from fresh tissues (figure 14). The greater number of protein bands found in the intermediate temperature (12°C) muscle was more similar to the fresh 28°C trout tissue (figure 14) than to stored 4° or 17°C tissues. This could suggest that acclimation conditions may affect the nature of protein structural stability and especially of the larger slower migration species, which similarly appeared to be more labile in warm acclimated *Gillichthyes* liver proteins.
Acrylamide gel electrophoresis results:

Fig. 2 to 13, *Gillichthyes mirabilis*.

Fig. 13 to 15, trout.

Abbreviations: AS = Amido Black general protein dye. b = bromophenol blue tracking dye. BSA = bovine serum albumin. CBB = Coumassie Brilliant Blue general protein stain. C = control, held at ambient sea water temperatures. Numbers on side of columns refer to protein bands. For comparison with densitometry graphs, see Appendix B, for those gels numbered with brackets around their numbers.

Acclimation temperatures: 8°C, cold; 28°C, warm; controls, as indicated or see table on page 21.

Proteins: Liver and brain tissues were prepared for electrophoresis by homogenization in 5 volumes of cold TMK-S (0.25 M sucrose, 0.05 M Tris-HCl, pH 7.6, 0.025 M KCl, 0.005 M MgCl₂) using the dounce B homogenizer as described in the methods (p.17). Proteins shown in all figures, except where indicated, are from post mitochondrial fractions (7,700-x g; 20 min.) treated with 2% triton-x-100 and 0.005% dithiothretol for electrophoresis. Mitochondrial fractions were washed with 0.05M sucrose and 0.05M tris-HCl, pH 7.6 and treated with 0.1% SDS and 0.1% mercaptoethanol at 37°C for 30 min, then made heavy with 40% sucrose and tracking dye added, prior to electrophoresis (methods, p. 11)

Fig. 2. *Gillichthyes m.* muscle: 13 days acclimation, glycolysis proteins from water soluble, 17,000 g, 20 min. supernatant. Duplicate gels for each temperature are from pooled tissues of two or three fish.
Fig. 3. *Gillichythes m.* brain:

(a) 21 days acclimation. Intensity differences are due to 50λ, 100λ, 150λ, of proteins applied to columns. Gels for each temperatures are from pooled tissues of 2 fish. Gel 1-3, 8°C; gels 4-6, 22°C; gels 8-10, 28°C.

(b) 45 days acclimation. AS, amido black dye duplicates of gels 1-6. Gels for each temperature are duplicates using the same tissue homogenate.
Fig. 4. Liver proteins short term freeze storage. Tissues quick frozen in dry ice - ethanol. See Appendix B, graph II, III.

(a) Fresh tissue; 13 days acclimation. Compare with fig. 7a; overnight freeze - storage. 8°C, gel 1,2; 19°C-22°C, gels 3, 4; 28°C, gels 5, 6.

(b) Frozen 1 week; same tissue homogenates from (a).
Fig. 5. Liver proteins long term freeze storage.

Gels 1,2, fresh ambient sea water fish. Gels 4-6, tissues frozen for months.
Fig. 6. Starved stressed 28°C *Gillichthyes m.* tissues.

Duplicate gels 1,2 = liver; 3,4 = muscle; 5,6 = brain.

Gels 7,8,9, same tissues but gels dyed with amido black.

See Appendix B, graph IV, V, VI, respectively for liver, muscle, brain.
Fig. 7.  

(a) **13 days acclimation**

8°C, gels 1, 2; 19°C-20°C, gels 3,4; 28°C, gels 5,6.

(See Appendix B, graph VII).

(b) **20 days acclimation**

8°C, gels 1,2; 21°C-24°C, gels 3,4; 28°C, gels 5,6.

Split gel 8°C/28°C, gel 7. (See Appendix, graph VIII).
Fig. 8. (a) **31 days acclimation**

8°C, duplicate gels 1,2 and 3,4; controls, 19°C-24°C, gel 5, and duplicate gels 6,7. (See Appendix, graph IX).

(b) **38 days acclimation**

8°C, duplicate gels 1,2 and 3,4; controls, 16°C-21°C, duplicate gels 5,6 and 7,8; 28°C, duplicate gels 9,10 and 11,12. (Appendix B, graph X).
Fig. 9. 45 days acclimation

$8^\circ C$, duplicate gels 1,2; controls ambient sea water $16^\circ -21^\circ C$, duplicate gels 3,4; $28^\circ C$, duplicate gels 5,6. Amido black stained gels, repeats of the same sequence. (Appendix B, graph XI).
Fig. 10  **52 days acclimation**

Each gel is from separate fish: 28°C, gels 1,2,3; 8°C, gels 4,5,6; controls, gels 7,8. Split gels 28°C/8°C.
Fig. 11. (a) **Low speed v.s. high speed post mitochondrial supernatants.**

Basic gels: Low speed, 28°C, gels 1,2; controls, gels 3,4. Acid gels: Low speed, 28°C, gels 5,6; controls, gels 7,8. High speed, 28°C, gels 9,10; controls, gels 11,12.

(b) **Mitochondria triton-x-100 soluble proteins.**

Acid gels.
Fig. 12  Acid gels of liver tissue sub-cellular fractions.
Fig. 13. Sodium dodecyl sulfate acrylamide gels of mitochondria SDS, 2-mercaptoethanol soluble proteins:

(a) Triplicate gels: 8°C; ambient sea water. Duplicate gels: 28°C. Split gels, 8°C/28°C. (Appendix B, graph XII for gels 3, 5, 8).

(b) Duplicate gels of each temperatures stained with Coumassie Brilliant Blue and Amido Black dyes. Gel 1, bovin serum albumin.
Fig. 14. Fresh $24^\circ C$ trout tissues: left to right - brain, liver, muscle. Stained with Coumassie Brilliant Blue, gels 1-3, and with Amido Black, gels 4-6.
Fig. 15. Frozen trout tissues:

(a) Liver. Each gel from separate fish. Two gels each for 4°C, 12°C, and 17°C acclimations. Stained with CBB, gels 1-6, and with AS, gels 7-12.

(b) Muscle: Same as for liver for acclimation temperatures. Stained with CBB, gels 1-4, and with AS, gels 5-8.
3. Polyribosome and monoribosome profile

Results from the sucrose density gradients (10-35%) did not indicate clearly any shift in the polyribosome and monoribosome profile for studies of 2 weeks acclimation in *Gillichthyes mirabilis*. 
V. DISCUSSION

1. Electrophoretic results and tissue comparisons

Polyacrylamide gel electrophoresis separates proteins based upon their charge and size differences. A discussion of principles involved in polyacrylamide gel electrophoresis and the conditions that contribute to optimal results is given in appendix A.

The results from the present studies indicate remarkable reproducibility of protein distribution patterns between fish from all acclimation temperatures. The minor variations in band separation in the regions of bands 3, 4, and 5 were quantitative rather than qualitative and may reflect phenomena intrinsic to the gel near the origin, a phenomena also observed by other investigators and called a "haze zone" by Matson (1965). However, since the slight variations were reproducible, and where most apparent, they may reflect changes induced during warm acclimation.

Factors that interfere with good resolution and cause variations can be due to mechanical irregularities produced at the stacking gel and running gel interphase, or due to protein aggregation and plugging of the gel interphase by larger insoluble proteins. Mechanical factors in these experiments were minimal. There was a problem of protein aggregation and plugging of the gel, which affected the relative migration rates and reduced resolution of minor proteins, as observed in the 31 days acclimation study. Plugging of gel pores at the upper gel interphase can interfere with even electrophoretic field and cause joule heating and protein streaking artifacts. To overcome this problem, high speed centrifugation and SDS and mercaptoethanol treatments of homogenates and gels were used. High speed centrifugation...
reduced the number of proteins and the intensities of the protein bands obtained. Triton-x-100 treatment greatly improved post mitochondrial supernatant results, but was not sufficient for solubilization of membrane proteins. Triton-x treatment of mitochondrial and microsomal fractions in rat liver studies (Dehlinger and Schimke, 1971) indicated that 50% of rat liver membrane proteins were solubilized and that these had molecular weights greater than 50,000; triton-x insoluble proteins were heterogeneous in size. SDS and mercaptoethanol treatment and electrophoresis with SDS gels produced good protein band resolutions with no observable protein plugging of gel surface.

Two points should be kept in mind when considering the protein bands distributions: (1) Before a band can be considered a true single unit protein, correlative studies must be performed with known proteins. Single chains of serum albumin have been shown to aggregate and give multiple zones in acid gels (Poulik, 1966). (2) Since the amount of dye that binds to a protein varies with proteins, densitometry graphs provide only relative quantitative comparisons unless reference quantities of the proteins have been electrophoresed at the same time. Comparisons of relative quantities of proteins from the same experiment should therefore be more reliable than between graphs from separate runs.

The present studies would seem to indicate that thermal acclimation induces no apparent major changes in the proteins of *Gillichthyes mirabilis* for the tissues investigated as described. Longer term acclimation may affect minor protein species found in the regions of slower migration larger sized proteins. Longer term acclimation may also have overall effects on protein band concentrations by either influencing structural properties of the proteins, similar to those produced by freeze storage (which likewise produced loss of
resolution), or by decreasing the pool of tissue proteins. The protein patterns that were obtained from warm stressed starved fish indicated definite distribution changes and therefore greater restructuring of proteins. These effects were in the faster migration protein species. The observed decrease in proteins from liver tissue of fish subjected to prolonged warm acclimation without starvation may therefore be reflective of depletion of protein pools. This would suggest that warm acclimation at 28°C imposed greater stresses for Gillichthyes m. than did cold acclimation. This was also apparent by the gross observations of weight loss and liver atrophy over prolonged holding at the elevated temperature. Questions arise from these for considering how the capacity to adapt metabolically may correlate with the eurythermic nature of this species. What are some of the limits set by the thermal environments encountered in the ecology of this species, and what might be some of the mechanisms that enable it to become functionally adapted to eurythermic conditions?

2. **Acclimation adaptations**

Although no overall major restructuring of proteins were observed by gel electrophoresis, the gross metabolic and physiological adjustments observed (changes in lipid content, physiological activities, and in the different adaptive capacity to cold and warm stress) would indicate that induction of metabolic processes do occur in response to temperature. Early experiments by Summer and Doudoroff (1935) showed that Gillichthyes exposed to warm and cold for as little as 30 minutes became more resistant to subsequent lethal temperatures. The resistance increased at diminishing rates over 30 minutes to ten days exposures. It was found that previous warm acclimation provided better resistance to warm than did previous cold acclimation to cold stress. It would
seem then that whatever the adaptive processes evolved by this eurythermic species, the capacity for heat adaptation could be a more important phenomena, a point that may be especially relevant to the ecology of this species.

Seasonal variations of intertidal pool temperatures have been shown to range from 12°C to 34°C, while surface sea water ranged from 14°C to 22°C for the year monitored (Norris, 1963). (See fig. 1; surface water temperatures for this study.) The burrowing behavior of *Gillichthyes* and their ability for buccal respiration would also indicate that this species has evolved the capacity to adjust to conditions of heating and low oxygen tensions of intertidal ecology. The prolonged stress at 28°C in the present studies would be towards the upper limits of tolerance for *Gillichthyes*, and therefore might account for the observed variations that were accentuated with prolonged holding at this temperature. In nature, *Gillichthyes* experience short term diurnal heating, and these fluctuations would induce prolonged resistance adaptations. It has been reported that fish can respond to as little as 0.5°C temperature changes (in Fry & Hochachka, 1971). In *Gillichthyes*, behavioral selection may lessen the degree of temperature changes encountered in diurnal temperature fluctuations. However, the intertidal ecology would still subject the fish to thermal ranges far greater and quicker than those encountered by seasonal adaptors. Temperature, then, has to be translated into more immediate physiological parameters.

3. **Protein and enzymic functional adaptations**

The possibility that eurythermic capacity was derived from thermally induced changes in protein synthesis rates, or changes in enzymic species during acclimation was shown to be negative, from the
present studies of polysomes and monosomes, and from the extensive list of enzymes investigated (table I). The question of thermally induced changes in proteins during acclimation has also been studied in Gillichthyes from the point of protein degradation (Somero & Doyle, 1973). It was found that protein degradation rates were different in different tissues of warm and cold acclimated fish. All these findings lead to the question: What are the intrinsic qualitative structural properties of enzymes from eurythermal fish that give the animal its biochemical flexibility under varying thermal conditions. Also, how might temperature affect enzyme function by influencing local cellular environments, and therefore enzymic activities.

On the one hand, simple models in nature have often been held to be the rule, and so in some cases of poikilotherms, adaptive compensation to thermal changes may be by restructuring of certain regulatory enzymes (Hochachka, 1968, Hochachka & Lewis, 1970). On the other hand, cellular metabolism consists of complex integrated processes. These processes have to be orchestrated to all the finely changing nuances that enable the organism to achieve delicate balances, the summation of which encompasses physiological viability. Temperature as only one modulatory force might therefore affect metabolism by influencing molecular processes on overall physiological levels.

Various ideas have been suggested that focus upon possible intrinsic qualitative differences in the nature of proteins that may account for their biochemical adaptability. One view suggests that gene duplication has provided means for slight variations in functional proteins and that these slight differences were preferentially exploited during ontogeny (Ohno et al., 1967). Widespread natural variation of
many proteins have been amply reported (Bailey et al., 1969; Dando, 1974; Hattingh, 1973; Johnson, 1971). The greater degree of protein pattern variations observed in the fish from batch III in the present investigation might be reflective of increased polymorphic expressions induced by ecological parameters experienced by that batch of fish in their developmental history.

Other studies comparing eurythermic and stenothermic species have indicated differences in enzymes, reflective of their biological thermal niche. In one study, eurythermic rainbow and brook trouts, which experience seasonal temperature changes, exhibited heterogeneity in the multiple forms of soluble isocitrate dehydrogenases. Stenothermic lake trout, which experiences very stable temperature, exhibited only one subunit form of the enzyme (Moon & Hochachka, 1971). Comparisons of the capacity for acclimation to eurythermic or stenothermic conditions in two closely related species have indicated that the glycolytic enzymes, lactate dehydrogenase and pyruvate kinase, exhibited different abilities to acclimate, reflective of their species natural habitat (Wilson et al., 1974). Tissue specific enzymic activity differences for an enzyme from homeothermic and heterothermic tissues in the same animal have also been shown (Behrisch & Percy, 1974). The theory which these and other experiments have suggested is that, in evolutionary terms, stable environments induce little enzymatic heterogeneity, whereas eurythermic conditions do, and this enables greater physiological flexibility. Gene duplication, in this way, provides a means for conservative modification of the basic model for protein function.

What might be some of the properties contributing to greater functional capacity? Comparisons of pig heart and rainbow trout liver
soluble NADP-linked isocitrate dehydrogenases have shown that stable characteristics for the enzyme are molecular weights, cationic and cofactor requirements, inhibitor specificity, and activation energy. Properties which accounted for enzymic differences were electrophoretic mobility, and Km parameters with respect to temperature, cosubstrate, and a specific inhibitor, ADP (Moon, 1972).

In *Gillichthyes*, tissue specific enzymic-substrate-inhibitions of pyruvate kinases and lactate dehydrogenases indicated one means for selective channeling towards glycolysis (under cold, high $O_2$ conditions), or towards lactate formation (under high temperatures, low $O_2$ conditions) (Somero, 1973). Specificity of enzymic action necessitates ordered chemical processes, which have been described by the concept of a one to one enzyme substrate interaction. However, the tissue specific differences and catalytic flexibility might suggest a more flexible structural model for eurythermic enzymes. Increased structural flexibility might have been derived from modifications of minor molecular groups in the proteins which are significant in protein structural stabilities. Changes in weak bond interactions of the proteins would then render the enzymes more sensitive to modulators. Negative and positive cooperative effects by modulators have been found to be very significant influences of enzymic activities (Behrisch & Johnson, 1974a; Moon, 1972). The consequence of these would be to extend the capacity for metabolic integration over wider ranges of change. This seems to be a reasonable tentative explanation for eurythermic adaptability that fits in with current theories for enzyme function. Thus, instead of changes in the complexity of enzymic forms or in protein quantities, capacity for adaptation is extended by increased sensitivity to a complexity of biochemical modulatory forces.
APPENDIX A

1. Polyacrylamide gel electrophoresis

The process of polymerization of acrylamide and cross-link bisacrylamide is initiated by TEMED. and ammonium persulfate or riboflavin in the presence of light (Chrambach, 1971):

\[
\begin{align*}
\text{Acrylamide} & \quad \text{bisacrylamide} & \quad \text{polyacrylamide} \\
\text{CH}_2=\text{CH} & \quad \text{CH}_2=\text{CH} & \quad \text{initiators} \\
\text{C}=0 & \quad \text{C}=0 & \quad \text{C}=0 \\
\text{NH}_2 & \quad \text{NH} & \quad \text{NH}_2 \\
\text{NH}_2 & \quad \text{NH} & \quad \text{NH}_2 \\
\text{NH} & \quad \text{C}=0 & \quad \text{NH}_2 \\
\text{CH}_2=\text{CH} & \quad \text{CH}_2=\text{CH} & \quad \text{CH}_2=\text{CH} \\
\end{align*}
\]

Pore size (Davies, 1964) of the formed gel is a function of both monomer and co-monomer concentrations. As the concentration of monomer is increased, the concentration of co-monomer should be decreased in order to maintain gel flexibility. The equal increase or reduction of both components to extremes give gels that are too brittle or too soft. In low concentration gels, sucrose (40%) is included to provide osmotic strength, as is the case for stacking gels given in the methods. Riboflavin catalyst produces larger pore size than does ammonium persulfate, but these have a tendency to shrink with time. Stacking gels should therefore be made shortly before use, whereas separation gels can be stored overnight if necessary. A 7.5% gel will have an average pore size
of 50 Å and a hydrated chain diameter of about 10 Å. This provides some frictional resistance for most cellular proteins. Proteins between 200 Å to 400 Å are sharply separated, while larger and globular proteins are excluded (Ornstein, 1964). Most proteins exist in their charged forms at the pH selected.

Migration of proteins is based upon ionic charge mobility. Electrical mobility is directly proportional to the net charge and inversely proportional to the size of the molecule and the solution viscosity (i.e. frictional resistance provided by pore size) (Ornstein, 1964). A system of ions and pH gradients was developed to facilitate the phenomenon of stacking and to facilitate efficient protein migration in the running gel. It has been argued by Richard and Lecanidou (1974) that the stacking phenomenon depends upon sample mass and buffer ionic strength alone and that stacking gel is unnecessary to bring about reduction of sample volume into a thin band at the running gel interphase in their studies of RNA, and discussion for protein. According to these authors the optimum load depends upon the ionic strength of buffers used and on the mobility of the anion, with increased loads possible at higher buffer concentrations. Nevertheless, stacking gels have been in wide use and the phenomenon will be considered in greater detail in order to understand the summary of necessary conditions for polyacrylamide gel electrophoresis given further down. According to Williams and Reisfeld (1964), the process of protein migration and resolution into bands during acrylamide gel electrophoresis in a discontinuous buffer system is explained as follows:

By selecting a leading ion with highest mobility and a trailing ion with mobility lesser than any protein, and a buffer pH discontinuity at the stacking running gel interphase, a voltage pH gradient is set up
during electrophoresis which produces protein stacking. When current is turned on, the leading ions in the buffer migrate ahead, creating an increased voltage gradient in respect to the slower migration species. This increased voltage gradient accelerates the slower migration species and a steady state migration band is established whereby proteins become stacked in a millimicron thick zone. When proteins enter the separation gel, migration is influenced by sieving effects and the higher pH gradient. The leading and trailing ions will therefore migrate ahead of the proteins. The proteins will become separated based upon their size and charge.

The summary of the necessary conditions for anionic and cationic (in bracket) systems below is from Williams and Reisfeld (1964):

1) pH of running gel should be such that proteins are biologically stable and have the same sign of charge at that pH.
2) Chloride (potassium) ion is used as leading ion.
3) Trailing ion is a weak acid (weak base) or amino acid with a \( pK_a \) less than (greater than) the pH unit of the running gel.
4) pH of sample and stacking gel is 2 to 3 pH units less than (greater than) the \( pK_a \) of the trailing ion so that the trailing ion is weakly charged and therefore migrates slowly.
5) Buffer systems should have a \( pK_a \) of equal or less (equal or greater) than one pH unit from the running gel to provide for good buffering capacity.
6) 1N HCl (KOH) is used in stock solution for making gels.

It has been found by Poulik (1966) that the quality of protein separation in acrylamide gel electrophoresis depends upon ionic concentrations for any system of ions used. Low ionicity of the buffer
medium allowed for high potential gradients without adverse joule heating effects. According to Poulak better resolution will also result with low ionic strength since ion binding to proteins will be reduced and intrinsic differences in protein charges at a given pH will not be masked.

2. Sodium dodecyl sulfate acrylamide gel electrophoresis

For SDS gel electrophoresis, proteins are first reduced in mercaptoethanol and bond to sodium dodecyl sulfate \( \text{C}_{12}^\text{OSO}_3^- \). This causes extensive disruption of hydrogen bonds, and hydrophobic and disulfide linkages, which results in solubilization of many relatively insoluble proteins. Identical amounts of SDS are bound to the protein on a gram to gram basis by predominately hydrophobic binding (Shapiro et al., 1967; Fish et al., 1970; Reynolds & Tanford, 1970). Reduction of disulfide bonds is required to optimize availability of hydrophobic residues and to obtain maximal binding. In the process, the native structure of the protein is changed to an extended rod-like conformation with a moderately high content of \( \alpha \)-helix in which hydrophobic residues are externally exposed for SDS binding (Nozaki et al., 1974). The forces of attraction between the denatured protein and detergent are greater than between SDS, thus preventing the formation of detergent micelles (Tanford, 1968). Binding equilibrium is established at less than 0.1% SDS concentrations (Nozaki et al., ibid). The resultant SDS-protein complex is highly ordered in structure and has been found to have identical hydrodynamic shapes differing only in dimensions (Stokes radius), which are dependent upon the protein molecular weight (Fish et al., ibid).
The SDS treated proteins are therefore separated in electrophoresis based upon their size differences alone. Plots of $\log R_s$ (Stokes radius) versus $\log$ molecular weights were found to be linear for proteins in the ranges 15,000-75,000 m wt (Fish et al., ibid). At the lower molecular weight limits, protein-SDS complexes assume approximately spherical shapes instead of the rod like particles.

The slight variabilities observed in the upper portions of the SDS gels ('a' in the graphs) may be due to these lower limits of resolution, or other factors influencing separation at the region near the origin as discussed by Richter-Lansberg et al. (1974). Factors discussed include reconstitution of disulfide bridges by dissociation of dodecyl sulfate-protein complexes through sieving in their system of discontinuous gel electrophoresis and protein mobility changes due to alteration in ionic environment of the sample during electrophoresis. According to these authors some of these problems can be overcome by the addition of mercaptoethanol and sodium dodecyl sulfate to the upper buffer chamber to prevent reoxidation and dissociation. This was done in the present studies.
APPENDIX B

Densitometry graphs from polyacrylamide gel electrophoresis shown in the results given in section IV (pages 27-39), for Gillichthyes mirabilis. Numbers on graphs refer to gross peak areas and not to specific numbers of protein bands nor do they indicate unique separated units of protein. For a band to be labeled as a unit protein, correlative studies of known proteins electrophoresed at the same time are necessary. Likewise, only relative quantitative comparisons should be made of peak areas, keeping in mind that due to density differences, minor bands will appear as 'humps' off major peaks, or they can be obscured. b = bromophenol blue tracking dye. // = end of gel.
Graph 1. *Gillichythes mirabilis* muscle

13 days acclimation. From fig. 2, page 27.
GRAPH I: GILLICHYSES MUSCLE.
Graph II. Liver, non-frozen tissue.

13 days acclimation. Compare with graph III, quick frozen one week stored tissue from the same protein sample. From fig. 4 a, page 29.
Graph III  Liver, one week frozen stored tissue.

From fig. 4 b, page 29.
GRAPH III: LIVER, ONE WEEK STORAGE FROZEN

8°C ACCLIMATION (fig. 4b gel 1)

15°C-24°C CONTROL (fig. 4b gel 3)

28°C ACCLIMATION (fig. 4b gel 5)
28°C STARVED STRESSED
(fig. 6 gel 1 - LIVER)

(fig. 6 gel 2 - LIVER)
Graph IV. 28°C starved stressed - liver.

From fig. 6, page 31.
Graph V. \textsuperscript{28^\textdegree}C starved stressed - muscle.

From fig. 6, page 31.
28° C STARVED STRESSED
(fig. 6 gel 3 - MUSCLE)
(fig. 6 gel 4 - MUSCLE)
Graph VI. $28^\circ C$ starved stressed - brain.

From fig. 6, page 31.
28° STARVED STRESSED; (fig. 6 gel 6 = BRAIN)
Fig. VII. 13 days acclimation

From the same tissue as in graph II, but stored frozen over night in homogenate form. Graphs of gels from fig. 7 a, page 32.
8°C ACCLIMATION
(fig. 7a gel 1)

19°C-24°C Ambient sea water CONTROLS
(fig. 7a gel 3)

28°C ACCLIMATION
(fig. 7a gel 5)
Graph VIII. 20 days acclimation

From fig. 7 b, page 32.
Graph IX. 31 days acclimation

From fig. 8 a, page 33.
C ACCLIMATION
(fig. 8a gel 1)

CONTROL 19°-24° C ambient sea water.
(fig. 8a gel 7)
Graph X. 38 days acclimation

From fig. 8 b, page 33.
8°C ACCLIMINATION
(fig. 8b, gel 1)

(fig. 8b, gel 4)

CONTROL ambient sea water 16°C-21°C
(fig. 8b, gel 5)

(fig. 8b, gel 7)

28°C ACCLIMATION
(fig. 8b, gel 9)

(fig. 8b, gel 12)
Graph XI. 45 days acclimation

From fig. 9, page 34. Duplicate gels for each set of temperatures.
80°C ACCLIMATION
(fig. 9, gel 1)

(fig. 9, gel 2)

CONTROL ambient sea water 19°C-24°C
(fig. 9, gel 3)

(fig. 9, gel 4)

28°C ACCLIMATION
(fig. 9, gel 5)

(fig. 9, gel 6)
Graph XII. Sodium dodecyl sulfate acrylamide gels

From fig. 13 a, page 38. 'a' = region in the gels where minor bands are visible. In the other numbered peaks areas the minor bands are less obvious in these densitometry graphs due to the great difference in the density of protein stains. These minor bands are more obvious in the photograph.
8°C ACCLIMATION
(fig. 13, gel 3)

CONTROL ambient sea water 16°-21°C
(fig. 13, gel 5)

28°C ACCLIMATION
(fig. 13, gel 8)
BIBLIOGRAPHY


