Summer 1972

An electrophoretic comparison of the gill plasma membrane proteins of freshwater and saltwater-adapted killifish, Fundulus heteroclitus

Earl Dean Hammit

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A Thesis

Presented to the Faculty of the Graduate School

of the University of Richmond

in Partial Fulfillment of the Requirements of the

Degree of Master of Arts

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Texas A&M University

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ACKNOWLEDGEMENTS

I sincerely thank the following members of the faculty of the Biology Department of the University of Richmond: Dr. David W. Towle. my major professor, for suggesting the problem and for his advice through the course of investigation; Dr. William S. Woolcott, Jr., Dr. Francis B. Leftwich, and Dr. John W. Bishop for their critical reading of the manuscript.

Appreciation is also extended to Dr. Keith Shelton, Department of Biochemistry, Medical College of Virginia Health Science Division of Virginia Commonwealth University, for his assistance in the densitometric analysis of thesis materials and Dr. Robert M. Terry, Department of Modern Foreign Languages, University of Richmond for his assistance in helping me fulfill the language requirement.

Finally, I would like to especially thank my wife, Patty, whose patience, understanding and encouragement were indispensable during the course of this study.

ABSTRACT

A study was undertaken to compare proteins present in the gill membranes of freshwater and saltwater adapted killifish, Fundulus heteroclitus. For this purpose, a method for the preparation of gill plasma membranes was developed.

Polyacrylamide gel electrophoresis indicated that the two types of gills possessed many membrane proteins in common. However, the freshwater adapted group possessed a single protein not found in the saltwater adapted group and the latter contained a protein not found in the freshwater adapted group.

INTRODUCTION

The killifish, Fundulus heteroclitus is a euryhaline teleost that tolerates environments which range from freshwater to salinities in excess of 35 ppt (De Sylvia et al., 1962). To better understand the adaptive ability of heteroclitus, as well as other euryhaline fish, in vitro and in vivo investigations have been conducted by many researchers.

The gut, gill and kidney interact to maintain the proper water and mineral balance in fish (Maetz, 1971). In freshwater, water enters through the gills, and the kidneys remove the excess water by excreting abundant amounts of dilute urine. Renal sodium and chloride loss are compensated by an active uptake of ions by the gills (Conte, 1969 b). In a marine environment, water lost across the gills is replaced by active absorption in the gut. Excess monovalent ions absorbed in the gut are excreted by the gills (Conte, 1969 b). Although the role of the gill in freshwater is limited mainly to respiration and secondarily to ion uptake (Conte and Lin, 1967), one of the most important roles of the saltwater gill is the active excretion of monovalent ions (Kamiya and Utida, 1968). Tosteson, et al., (1962) found by measuring the electric potentials that chloride transport

in the gills is an active process. Maetz et al. (1964) used radioactive tracer techniques to determine that sodium transport across gill membranes is also active and independent of the chloride mechanisms. The turnover rate of sodium ions across the gills is much higher in marine fish than in freshwater fish (Motais & Maetz, 1964, Motais, 1967).

The molecular mechanism of chloride transport has not been elucidated (Motais & Garcia-Romeu, 1972). It is not known, for example, whether the activity of carbonic anhydrase, the chloride transport enzyme, changes during adaptation of fish to altered salinity. More is known, however, about the mechanism of sodium transport. Most animal cells transport sodium outward and potassium inward, usually against concentration gradients, in an energy requiring process. Much evidence suggests that the $Na^+ + K^+$-activated ATPase in the plasma membrane participates in this active transport process (Skou, 1965). When a euryhaline fish is subjected to a marine environment, the Na^+ absorbed through the gut must be eliminated by the gill against a high chemical gradient. The increased requirements placed upon the cation transport mechanism appear to be reflected by the increased activity of $Na^+ + K^+$-activated ATPase in the saltwater gills (Epstein, et al., 1967; Kamiya & Utida, 1968; Conte, 1969). This increase in

$Na^+ + K^+$-activated ATPase activity may be the result of (1) synthesis of new enzyme molecules when an increase in relative quantity of ATPase protein would be expected or (2) activation of preexisting enzyme molecules in which the same amount of $Na^+ + K^+$-activated ATPase protein would be found in either freshwater or saltwater adapted fish. Previous investigations of this question have been inconclusive (Cvervanka & Conte, 1970; Conte, personal communication). The rapid turnover of gill epithelial cells observed upon transfer to saltwater (Conte & Tripp, 1970) suggests that new membrane components may be synthesized. In addition, immunochemical studies by Conte and Morita (1968) showed that adaptation to seawater causes an increase in several cellular proteins. There was a 4-8 fold increase in antigenicity of the cellular components, plasma membranes being much higher than microsomal preparations of the saltwater adapted gills. However, no direct analyses of individual gill membrane proteins have been recorded.

The purpose of the present study was to electrophoretically compare membrane proteins from gills of freshwater and saltwater adapted Fundulus heteroclitus. Another objective was to develop a simple method for the preparation of gill plasma membranes.

MATERIALS AND METHODS

Killifish, Fundulus heteroclitus, were collected from Mobjack Bay, Severn, Virginia by sein during the months January through April, 1972. After transport to the laboratory, the fish were maintained in filtered water collected at the site of capture. Water temperature was maintained at 19-21 C and its salinity was determined using the method of Strickland and Parsons (1960). Male heteroclitus were placed in aquaria containing fresh water (0.02 ppt) or saltwater (34.7 ppt) and allowed to acclimate for a minimum of fourteen days before tissue was removed for assay. A total of twelve electrophoretic analyses were performed. In each test, the gills from 10-15 fishes were pooled.

Gill plasma membranes were prepared according to Fitzpatrick et al. (1969) with modifications in volumes and centrifugal forces employed. All steps were carried out at 4 C. Gill filaments were removed from excised gill tissues and kept in ice-cold isotonic sucrose medium (0.25 M sucrose-1 mM EDTA). The gill filaments were pooled until approximately 500 mg of tissue were accumulated. The tissue was then homogenized in three volumes of isotonic sucrose medium in a Potter-Elvehjem apparatus with a teflon pestle at 1725 rpm until all tissue was broken up. The homogenate

was filtered through two layers of gauze. A portion of this homogenate was examined for enzyme activity with the remainder being centrifuged at 1475 g for 10 min at 0 C. The supernatant was decanted and the sedimented material was resuspended in one volume 2M sucrose, mixed thoroughly with a glass rod, and centrifuged 15 minutes at 18,000 g. Seven volumes cold distilled water were added to the supernatant which was then centrifuged at 35,000 g for 15 min. The resulting pellet consisted of two layers. The upper layer containing the plasma membrane fragments, was removed by gently swirling 1 ml isotonic sucrose above the pellet. This suspension was thoroughly mixed with a glass rod and centrifuged for 15 min at 35,000 g. The supernatant was decanted and the pellet was resuspended in 0.1 ml distilled water, and a portion was removed for enzyme assay. To this plasma membrane preparation, urea was added to an 8 M concentration and mixing was accomplished by vigorous vortexing. Phenol-acetic acid 2:1 (w/v) was added to make a total volume of 0.4 ml and the mixture again vortexed vigorously. The preparation was used immediately for electrophoresis or frozen at -17 C for later use. Frozen preparations were used within 24 hours after freezing.

A modified Janicki and Kinter (1971) ATPase

assay was run in duplicate with one incubation blank. Reagents were added to the incubation tubes so that the final concentrations were 20 mM imidazole-HCl (pH 7.8), plus either 100 mM NaCl and 20 mM KCl, or 120 mM NaCl and 1mM ouabain in a final volume of 2 ml. All tubes received 0.1 ml of the sample material with the exception of the incubation blank which received the same amount of the suspension medium. After incubation at 25 C for 30 min, 0.2 ml 50 mM ATP in 50 mM $MgCl_2$ was added to each sample and allowed to react for 30 min. The reaction was halted by the addition of 2 ml 10% trichloroacetic acid. The test mixtures were placed in ice for 10 min and then centrifuged at 10,000 g for 10 min at 0 C. One ml of the supernatant was analyzed for phosphate according to Fiske and SubbaRow (1925). Na^++K^+-activated ATPase activity was determined as the difference between the total activity in the presence of Mg^{++}, Na^+, and K^+, and the activity (Mg^{++}-dependent) measured in the presence of Mg^{++}, Na^+, and ouabain. It is expressed as micromoles of phosphate released per mg protein per min. A protein assay was performed on both the homogenate and final membrane preparation according to Lowry, et al. (1951).

In preliminary work, an electrophoretic system employing sodium dodecylsulfate (SDS) as outlined by Fairbanks et al. (1971) was attempted

without reliable results. This method was abandoned and the electrophoresis technique of Takyama et al. (1964) was used throughout the subsequent test period. Polyacrylamide gels were prepared by the method of Takyama et al. (1964) as modified by Chignell and Titus (1969). Glass tubes (5 mm ID x 80 mm long) were lightly fire polished and scored at 50 mm and 75 mm lengths. They were then silanized by immersion in 1% dichlorodimethyl silane in benzene for four hours to facilitate removal of the gels. After rinsing in distilled water several times and thorough drying, the tubes were stoppered at their bases and positioned upright in test tube racks. Seven and one-half percent acrylamide gels were prepared to the following specifications: Stock solution A contained 6 g acrylamide, 0.16 g N,N'-methylenebisacrylamide (BIS), 12 g urea, 28 ml glacial acetic acid and distilled water to a total volume of 60 ml. Stock solution B, prepared fresh daily, consisted of 0.3 g ammonium persulfate, 12 g urea, and distilled water to a volume of 20 ml. Stock solutions A and B were mixed with N,N, N', N'-tetramethyl ethylenediamide (TEMED) (3:1:0.02, v/v/v) to give the final gel solution. The gel solution was carefully pipetted into electrophoresis tubes to a height of 50 mm and overlayed with 75% acetic acid to 75 mm. The tubes were incubated at 50 C for 15 min to polymerize the gel. The 75% acetic acid was shaken

from the tube and the gel was rinsed with 10% acetic acid before being immersed 4-7 days in a solution of phenol-acetic acid-water, 2:1:1 (w/v/v) containing 2 M urea.

The soaked gels were inserted vertically into holders of the electrophoresis apparatus and a 10% acetic acid overlay was applied to the gel. One hundred microliters of the gill plasma membrane preparation, suspended in phenol-acetic acid-water, 2:1:1 (w/v/v) containing 2 M urea, was layered on the gels underneath the 10% acetic acid (pH 2.3). Electrophoresis was conducted toward the cathode (-) at 3 C for four and one-half hours at 2.5 mA per tube. Both freshwater and saltwater gill membrane preparations were run simultaneously to provide an accurate comparison. Upon completion of electrophoresis, the gels were carefully removed from the tubes by injecting 10% acetic acid between the gel and glass tube with a 10 cc glass syringe equiped with a 1.5 in 20 gauge needle. After removal from the glass tubes, the gels were placed in a marked staining chamber filled with 10% acetic acid. All gels were then drained and simultaneously stained with 1% amido-schwartz in 7% acetic acid for 1 hr. After staining, the gels were cleared with several changes of 7% acetic acid at 35 C. The cleared gels were scanned in a Gilford Model 2410

Linear Transport with Adapter model 2411 mounted to a Beckman DU monochromater to determine the density and distribution of the protein bands. Electrophoretographs were obtained at 500 mu at a scanning rate of 2 cm/min and a chart speed of 1 in/min.

The $Na^+ + K^+$-activated ATPase protein was labeled with ^{32}P in the presence of ouabain (Chignell and Titus, 1969) and separated by electrophoresis. The gels were sliced longitudinally; one-half was stained and the other half transversely sectioned. Measurements of radioactivity per mm of gel were obtained by a liquid scintillation counting.

RESULTS AND DISCUSSION

One of the objectives was to develop a simplified method for obtaining a subcellular fraction of gill that is rich in plasma membranes. It was desirable to obtain a method to provide a membrane fraction that sedimented rather quickly at lower centrifugal speeds, as an ultracentrifuge, which is required for most membrane preparations, was not readily available. One widely used method for plasma membrane preparation utilizes homogenization and sonication and eventually ultracentrifugation (Fisher

et al., 1970). The older, classical Neville (1960) technique for isolating membrane fractions, used density gradient centrifugation at lower speed, but eventually ultracentrifugation was employed. The procedure adopted for the present study was derived from a modification of a technique Fitzpatrick et al. (1969) used to isolate membranes from mammalian kidney homogenates at lower centrifugal forces. A modified flow sheet of the gill plasma membrane preparation is shown in Figure 1.

The ATPase assay conducted to ascertain the quality of the membrane preparation is shown in Table 1. Since sodium-potassium-activated ATPase is a structural part of the plasma membrane (Skou, 1965), its specific activity in the final preparation relative to the homogenate is an indication of the degree of membrane purification. In the gill plasma membrane preparation, the specific activity of the $Na^+ + K^+$-activated ATPase increased as the purification of the membrane proceeded. The plasma membrane fraction, when compared with the original homogenate showed an 11.3 fold increase in $Na^+ + K^+$-activated ATPase specific activity (Table 2). The specific activity of Mg^{++}-dependent ATPase showed only a 1.2 fold increase in specific activity indicating that the procedure employed was able to separate these two enzymes. These results may be compared with those of Epstein, et al., (1967) who found a five fold increase

in $Na^+ + K^+$-dependent ATPase activity using microsomal preparations and with Fitzpatrick et al., (1969) who obtained five fold increases in $Na^+ + K^+$-activated ATPase and Mg^{++}-dependent ATPase activities using kidney plasma membrane preparations.

The electrophoretographs of gill membrane proteins from saltwater adapted fish indicated twelve major bands, as did the electrophoretographs from the freshwater adapted fish (Figures 2 and 3). Although most of the bands are homologous, the saltwater membranes contained one protein not present in the freshwater membranes (at 17 mm) and the freshwater membranes contained one protein not found in the saltwater (at 36 mm). Quantitative differences between proteins of the two groups were not analyzed; however the different magnitude of similar peaks suggest some differences between similar proteins. These studies represent the first direct evidence for protein differences related to salinity adaptation. The protein differences may reflect the altered transport requirements faced by saltwater and freshwater adapted heteroclitus. Whether these proteins are components of $Na^+ + K^+$-activated ATPase or carbonic anhydrase enzyme systems remains to be investigated. The possibility that these differences could be due to other factors, (i.e. sexual maturity, nutrition) has not been entirely eliminated.

Seigel et al. (1969) reported that in the presence of K^+, ouabain stimulates inorganic phosphate, in the form of ^{32}P, to be incorporated into protein in $Na^+ + K^+$-activated ATPase. This labeled enzyme complex may be identified electrophoretically (Towle, 1970). An attempt was made, using the technique of Chignell and Titus (1969), to determine whether the $Na^+ + K^+$-activated ATPase was one of the proteins undergoing change. When gill plasma membrane preparations were incubated in the presence of ^{32}P and ouabain and electrophoresed, radioactivity was found to be dispersed at random throughout the gel. The reason for the disparity between these results and those of Chignell and Titus is unknown. Although inconclusive results were obtained from the attempt to label the ATPase enzyme complex, identification of specific proteins in gills of freshwater and saltwater adapted fish is an area worthy of further investigation.

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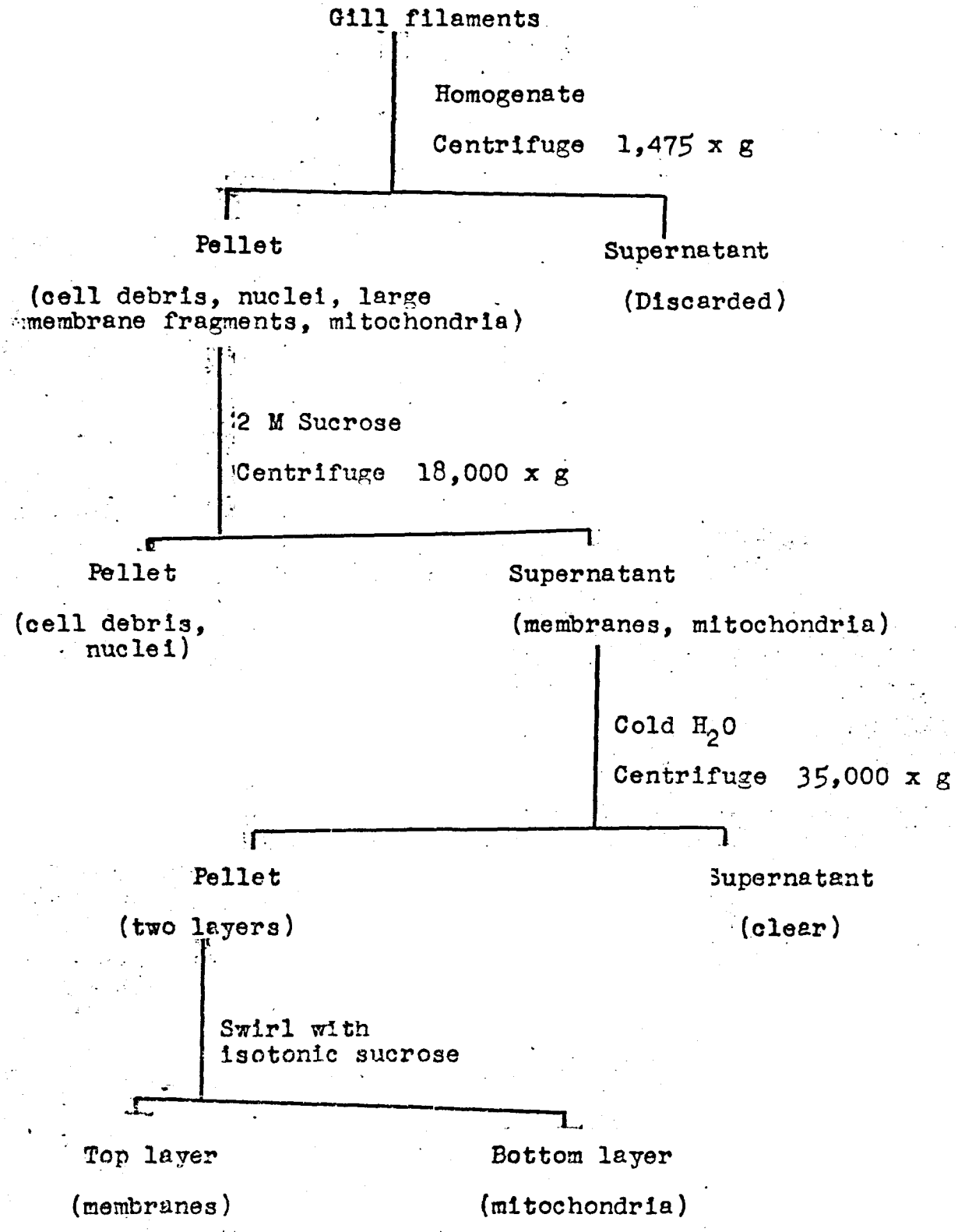
Table 1. ATPase assay of homogenate and plasma membrane preparations of gill tissue from Fundulus heteroclitus.

	Micromoles Pi Per Milliter Per Minute					
	Total ATPase			Mg^{++}ATPase		
	Tube 1	Tube 2	Ave.	Tube 3	Tube 4	Ave.
Homogenate	0.96	0.86	0.91	0.80	0.78	0.79
Plasma membranes	0.237	0.230	0.234	0.090	0.095	0.093

Table 2. Specific activity of $Na^+ + K^+$-activated and Mg^{++}-activated ATPase in homogenate and plasma membranes of Fundulus heteroclitus

		$Na^+ + K^+$ ATPase		Mg^{++} ATPase	
	Total protein mg/ml	umole P/ ml/min	umole P/ mg/min	umole P/ ml/min	umole P/ mg/min
Homogenate	2.60	0.208	0.080	1.264	0.486
Plasma membranes	0.25	0.226	0.904	0.149	0.596

Figure 1. Flow sheet for plasma membrane preparation from gills of Fundulus heteroclitus.



Suspend plasma membrane fraction

in H_2O, 8 M urea, phenol/HAc (2:1)

Figure 2. Gel photograph and electrophoretograph of saltwater-adapted killifish, Fundulus heteroclitus.

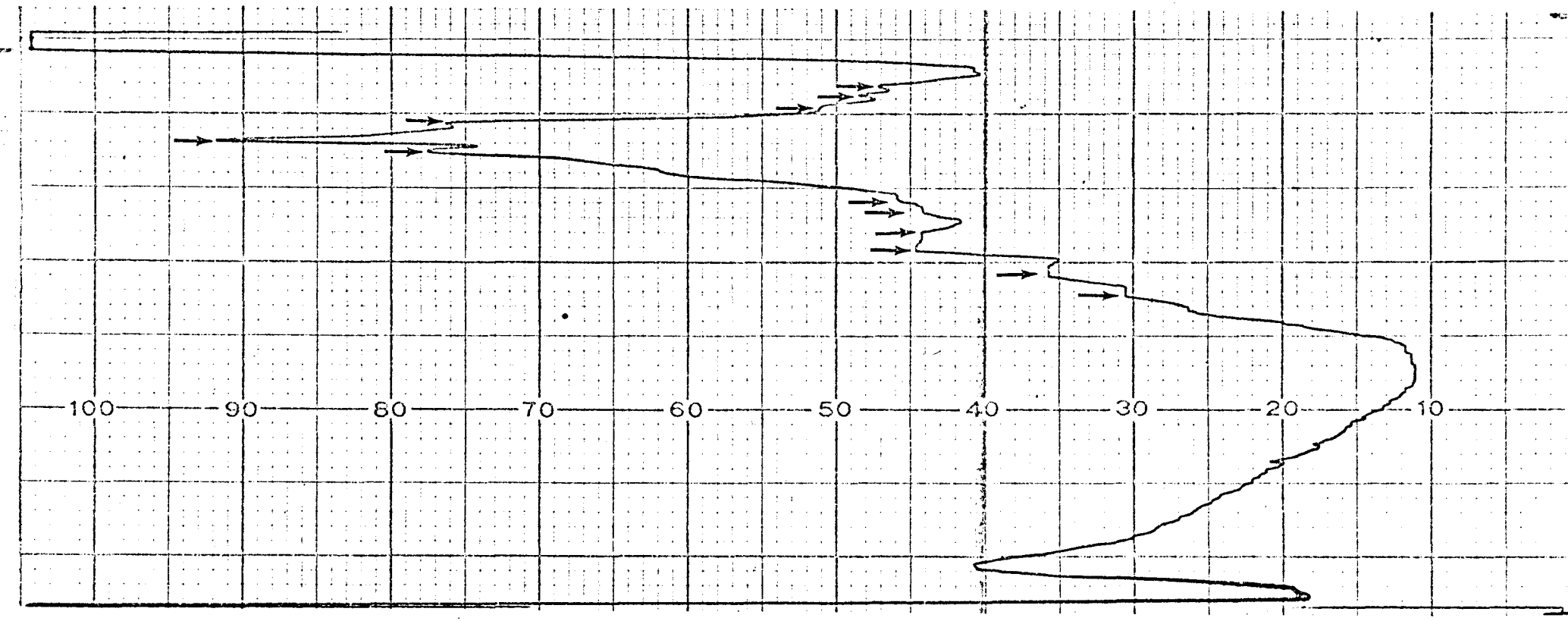

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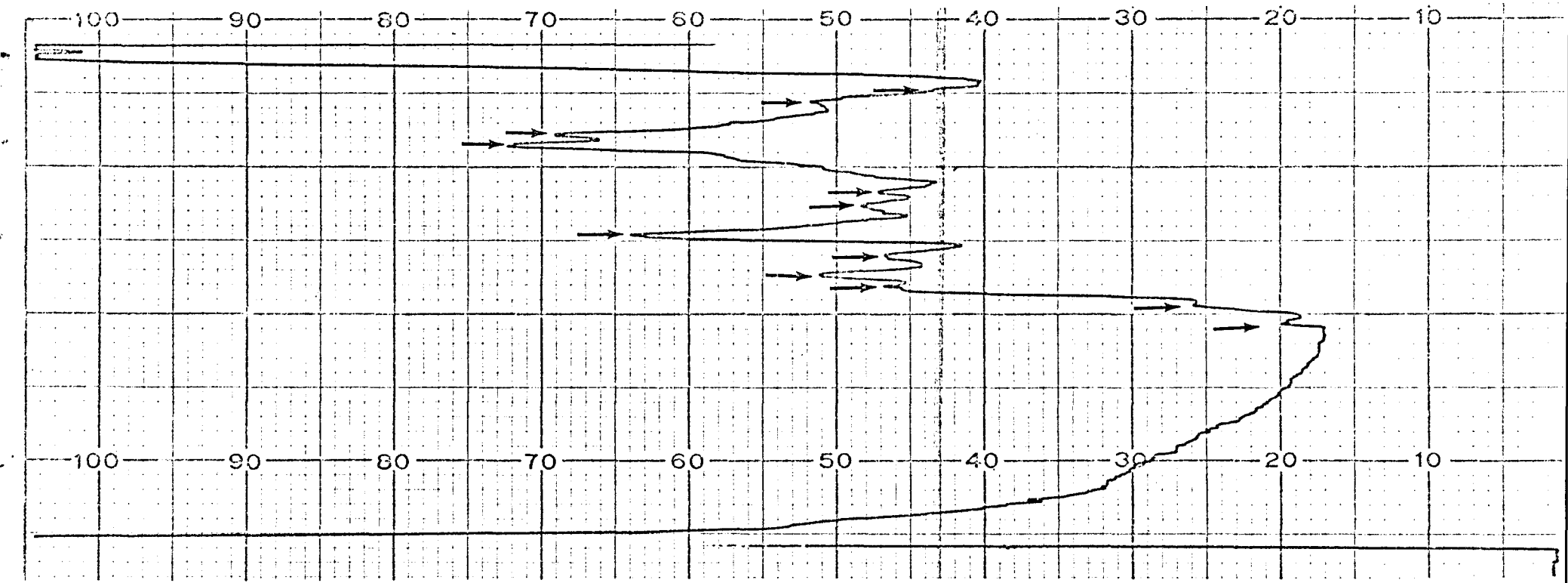
Figure 3. Gel photograph and electrophoretograph of freshwater-adapted killifish, Fundulus heteroclitus.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VITA

Earl Dean Hammic was born May 17, 1945 in Rotan, Texas. He attended Rotan High School graduating in May, 1963. He entered Texas A & M University, College Station, Texas in September, 1963. He was graduated January, 1968 with a Bachelor of Science degree in Animal Science. After two and one-half years service in the United States Army as a commissioned officer, he began graduate studies in Biology at the University of Richmond in September, 1970. While at the University of Richmond, he was initiated into Beta Beta Beta Honorary Biological Society and received an A.D. Williams Memorial Scholarship. He is a candidate for the Master of Arts degree in June 1973. He is married to the former Jane Patrick Sandifer of Franklin, Texas. He will enter the Medical College of Virginia, Health Science Division of Virginia Commonwealth University in September, 1972 where he plans to work toward the degree of Doctor of Philosophy in Pharmacology.