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Rice University, Ph.D., 1978
RICE UNIVERSITY

Localization of Na\textsuperscript{+}, K\textsuperscript{+}-Activated Adenosine Triphosphatase Activity in Teleost Chloride Cells: Cytochemical and Cell Isolation Studies on the Branchial Epithelium of the Pinfish, *Lagodon rhomboides*

by

Seth R. Hootman

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

THESIS DIRECTOR'S SIGNATURE

[Signature]

Houston, Texas

May, 1978
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TEXT ABBREVIATIONS

ACTH  adenocorticotropic hormone
ATP   adenosine 5'-triphosphate
BSS   balanced salt solution
BTO   1-p-bromotetramisole oxalate
DNA   deoxyribonucleic acid
EDTA  ethylenediamine tetraacetic acid
K⁺-NPPase potassium-dependent nitrophenylphosphatase
Mg²⁺-ATPase magnesium-dependent adenosine triphosphatase
Na⁺,K⁺-ATPase sodium, potassium-activated adenosine triphosphatase
NPP   p-nitrophenylphosphate
Tris  tris (hydroxymethyl) aminoethane
UMP   uridine 5'-monophosphate
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INTRODUCTION

Background and Rationale for the Study

The question of how vertebrates maintain a relatively constant internal ionic environment in the face of variable external conditions is one of the most fundamental in biology and one that has accordingly received a vast amount of attention. The overall strategies responsible for ionic and osmotic homeostasis have now been elucidated in most classes and the interplay of the various organ systems principally involved has been outlined. The finer details of the biochemical and structural features of the specialized cell types that comprise these organs are also being analyzed and in the process are revealing fundamental aspects of cellular phenomena in general.

This is particularly well illustrated with respect to the state of knowledge concerning osmoregulation among teleost fish. Teleosts are remarkably well suited for studies of ionic and osmotic regulation, since they can be maintained in chemically defined media for long periods and many species can withstand significant alterations, both qualitatively and quantitatively, in the medium constituents. For example, many fish can be transferred directly from freshwater to seawater or vice versa with no ill effects. Since freshwater and seawater are, respectively, 1% and 400% as saline as the interstitial fluids of the fish, it is remarkable that the ionic constituents of the interstitial fluids remain relatively constant following transfers. Maintenance of controlled internal ion concentrations when faced with abrupt changes in the external salinity argues for the existence of
very efficient control mechanisms in these euryhaline fish, a fact that was recognized many years ago by Smith ('30, '32) and Krogh ('39), who formulated the basic principles underlying osmoregulation in teleosts. They suggested, on the basis of both anatomical and physiological data, that four organ systems played principal roles in the control of ion and water movements in teleosts; the intestine, kidneys, integument, and the gill. Until this time the gill had been associated primarily with respiratory functions. However, Smith pointed out that the considerable amplification of the branchial epithelium necessary for adequate gaseous exchange provided a surface area that exceeded by an order of magnitude the remaining area of body surface exposed to the environment and was thus the major site of interface between the external medium and the internal body fluids. As such it could be expected to play a dominant role in water and solute control.

Keys ('31a, '31b) was the first to show that transport mechanisms in the gill were indeed central to the maintenance of ionic and osmotic equilibrium in teleosts. With his elegant isolated and perfused heart-gill preparation from the eel, Anguilla anguilla, he demonstrated that Cl\(^-\) was secreted against a concentration gradient when the gill was bathed with seawater, thus confirming the branchial epithelium as a major osmoregulatory surface. Seeking the cellular basis for active Cl\(^-\) secretion, Keys and Willmer ('32) followed these studies with a histological investigation of the gills of several species of teleosts and one species of elasmobranch. In all species of teleosts, they observed large granular cells clustered about the
bases of the respiratory lamellae. These cells were easily distinguishable by their large size, marked affinity for eosin, and the fact that they spanned the width of the otherwise stratified branchial epithelium. No cells of this type were seen in the one elasmobranch species studied. Keys and Willmer were able to readily distinguish between these large cells and the also numerous mucous goblet cells and made two other observations of note: that the tentatively termed "chloride-secreting cells" were concentrated around the afferent branchial blood vessel, and that the cells were less abundant in freshwater-adapted than in seawater-adapted fish. While this combination of observations did indeed suggest a salt secretory role for the chloride cells, the evidence was far from definitive and it was in many respects a bold move for Keys and Willmer to make this claim. It was not surprising, therefore, that their conclusions were quickly challenged. Bevelander ('35, '36) was the first to question their assertions, maintaining on the basis of his histological studies that the Keys-Willmer cells were a type of mucous cell and were probably not directly concerned with salt secretion. Yet in several of the next investigations to appear in the literature a strong link was forged in the chain of evidence indicating that the chloride cell did in fact play a central role in electrolyte regulation. This was the discovery of the adaptive response. Liu ('42) was the first to demonstrate this response, showing that variations in the external salinity to which a teleost is exposed have significant effects on the population of chloride cells in
the branchial epithelium. By adapting paradise fish, *Macropodus opercularis*, to freshwater containing table salt to a salinity of 2.73% (930 mOsm), Liu was able to induce proliferation of chloride cells. Not only were the cells in the salt-stressed fish more numerous than those of control fish maintained in freshwater, the individual cells were much larger. This combined proliferation and hypertrophy of chloride cells following transfer of euryhaline teleosts to hyperosmotic environments has been observed repeatedly since then (Copeland, '48, '50; Jozuka, '66; Olivereau, '70; Shirai and Utida, '70; Thomson and Sargent, '77). In certain euryhaline species, however, no proliferation of chloride cells occurs, although in these fish the hypertrophy of pre-existing cells is usually quite evident. Perhaps the most striking example of this response was reported by Karnaky et al. ('76a) following their studies on salinity adaptation in the pupfish, *Cyprinodon variegatus*. The pupfish will adapt to extremely saline conditions and Karnaky was able to obtain excellent survival of these fish after transfer to 200% seawater. Histological examination of gill filaments from these fish and from others maintained in 50% and 100% seawater revealed that the chloride cells from the 200% seawater-adapted specimens were roughly twice the diameter of those from fish adapted to either of the two lower salinities.

The other major response to salt stress visible at the light microscopic level is the remodeling of the apical pole of the cell. The apical surfaces of chloride cells from fish adapted to freshwater support short, irregular projections. By contrast, in fish adapted to seawater the cell surface characteristically displays a large, crypt-like invagination. This crypt was first seen by Bevelander ('35, '36), who
mistakenly classified the crypt-bearing cells as "vacuolated mucous cells." Copeland ('48, '50), however, was the first to recognize that the apical crypt is a diagnostic feature of chloride cells in fish maintained in hyperosmotic environments. Both Copeland ('50) and Getman ('50) observed the appearance of apical crypts in chloride cells within six hours after transfer of euryhaline species from freshwater to seawater. It thus appeared that those chloride cells present in the gills of teleosts adapted to low salinities could rapidly respond to a change in the salinity of the external environment in a way that was expressed in a radical alteration of the chloride cell's cytoarchitecture.

Analysis of chloride cell morphology was carried beyond the light microscopic level in the late 1950's and early '60's as several groups of researchers began to use the electron microscope as an investigative tool. Papers by Parry et al. ('59), Kessel and Beams ('60, '62), Doyle and Gorecki ('61), Straus and Doyle ('61), Threadgold and Houston ('61, '64), Holliday and Parry ('62), and Philpott and Copeland ('63) appeared in rapid succession, and although the various authors continued to disagree with respect to the possible roles of the chloride cell in salt secretion, the basic fine structural characteristics of freshwater and seawater-adapted chloride cells were elucidated. Two features of fine structure were uniformly observed in chloride cells at both high and low environmental salinities. The first of these was the large population of mitochondria, a characteristic that immediately distinguished chloride cells from the other cell types of the branchial epithelium. These mitochondria were uniformly distributed throughout most of the cell and were enveloped in a labyrinth of anastomosing tubules, the second characteristic feature of chloride cell morphology. These tubules filled most of
the cellular volume and closely enmeshed all of the other organelles. Although several early references refer to this network as smooth endoplasmic reticulum, frequent observation of direct continuity between tubules of the labyrinth and basal and lateral cell surfaces suggested that the labyrinth was actually a functional extension of the chloride cell plasma membrane, a relationship later definitively shown using tracers of the extracellular space such as horseradish peroxidase and lanthanum (Philpott, '66, '67, '68; Ritch and Philpott, '69). This combination of features in chloride cells was highly significant, since studies on many other tissues were at this time establishing that an amplified cell surface together with abundant mitochondria were the two universal hallmarks of cells specialized for electrolyte transport (Bulger, '63; Kornick, '63a, '63b; Tormey, '63, '64; Abel and Ellis, '66; Berridge and Gupta, '67; Copeland and Fitzjarrell, '68). Thus although the aggregate information supplied by electron microscopic studies of chloride cells did not definitively establish these cells as the primary sites of branchial ion regulation, they did show that the chloride cell typically possessed the two fine structural features characteristic of electrolyte transporting cells in general.

Concurrent with these electron microscopic studies which were rapidly identifying the fine structural hallmarks common to transporting cells from diverse sources were studies aimed at elucidation of the biochemical events associated with active transport phenomena. One of the most rewarding discoveries to come from these investigations was the discovery of sodium, potassium-activated adenosine triphosphatase (Na⁺,K⁺-ATPase). This enzyme was described by Skou ('57) in a microsomal fraction derived from crab nerve and has since been the subject
of several hundred papers and numerous reviews (see Skou, '65, '75; Albers, '67; Bonting, '70; Baker, '72; Schwartz et al., '72; Dahl and Hokin, '74; Glynn and Karlish, '75; Jørgensen, '75). From this massive and rapidly expanding literature has emerged a reasonably clear outline of the molecular characteristics and physiological significance of the enzyme.

The Na⁺,K⁺-ATPase appears to be an oligomeric enzyme consisting in the purified state of two subunits, a large "catalytic" protein (mol wt 90-100,000) and a smaller (mol wt 45-60,000) glycoprotein. Several studies (Kyte, '75; Perrone et al., '75; Giotta, '76) have suggested that in the native membrane the functional enzyme consists of at least an α₂β₂ complex and possibly larger aggregates. The enzyme is a characteristic marker of plasma membranes in eukaryotic cells and to date has not been definitively localized in any of the internal cellular membrane systems. Both subunits are integral proteins, the catalytic subunit spanning the plasma membrane and the glycoprotein subunit lying embedded in the outer lipid leaflet.

The overall reaction catalyzed by Na⁺,K⁺-ATPase involves a sequence of steps. Basically, the enzyme catalyzes the hydrolysis of the γ-phosphate of ATP in a two step process that includes a Na⁺-dependent phosphorylation of the catalytic subunit followed by hydrolysis of the phospho-enzyme in the presence of K⁺. This sequence of events involves conformational changes in the state of the enzyme and results, in the native membrane, in the translocation of the two activating cations across the bilayer. Stoichiometrically, for every molecule of ATP hydrolyzed, two K⁺ are transferred from the external to the cytoplasmic membrane surface while three Na⁺ are pumped out. The Na⁺,K⁺-ATPase thus appears to be
the enzymatic expression of the "sodium pump" which, by maintaining specific intracellular cation concentrations, underlies virtually all cellular metabolic processes. This permissive control is in many specialized cells also remodeled to support specific activities such as excitability in nerve and fluid transport across epithelia. This latter specialization has received much attention, since precise control of fluid and solute segregation within and among the various compartments that comprise multicellular organisms is fundamental to survival. The comparative studies that followed discovery of Na⁺,K⁺-ATPase revealed that the enzyme was a ubiquitous component of animal cell membranes and that the activity of the enzyme was highest in tissue specialized for electrolyte and fluid transport. This finding is illustrated in table I. The tissues included display a wide range of activities with the highest activities recorded in those organs (dogfish rectal gland, duck salt gland) that are primarily concerned with elaboration and secretion of highly saline exudates. Excellent correlation of Na⁺,K⁺-ATPase activity with previously established transport capabilities in virtually all tissues examined convincingly supported a direct role for the enzyme in ion translocation across epithelial surfaces.

This emerging correlation induced Epstein et al. (’67) to examine the enzyme in the gills of the killifish, Fundulus heteroclitus, a common euryhaline teleost. Their initial studies revealed a five-fold difference in Na⁺,K⁺-ATPase activity in gill homogenates from fish adapted to seawater when compared to the freshwater control fish. The much higher activities recorded in the salt-stressed animals strongly suggested that Na⁺,K⁺-ATPase played an active role in cation efflux across the branchial epithelium and that tissue levels of the enzyme were con-
TABLE I. Specific activities of Na⁺,K⁺-ATPase in selected vertebrate tissues. Values are expressed as μmoles Pi/mg protein/hour measured in crude tissue homogenates at 37°C.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Na⁺,K⁺-ATPase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat pancreatic islets</td>
<td>0.3</td>
<td>Lernmark <em>et al.</em> ('76)</td>
</tr>
<tr>
<td>Bovine heart</td>
<td>3.0</td>
<td>Pitts and Schwartz ('75)</td>
</tr>
<tr>
<td>Bovine brain cortex</td>
<td>6.4</td>
<td>Uesugi <em>et al.</em> ('71)</td>
</tr>
<tr>
<td>Pinfish pseudobranch</td>
<td>13.7</td>
<td>Dendy <em>et al.</em> ('73a)</td>
</tr>
<tr>
<td>Rat duodenum</td>
<td>14.7</td>
<td>Silva <em>et al.</em> (75)</td>
</tr>
<tr>
<td>Rat kidney medulla</td>
<td>22.7</td>
<td>Fisher <em>et al.</em> ('75)</td>
</tr>
<tr>
<td>Duck salt gland</td>
<td>31.2</td>
<td>Stewart <em>et al.</em> ('76)</td>
</tr>
<tr>
<td>Dogfish rectal gland</td>
<td>38.0</td>
<td>Hokin <em>et al.</em> ('73)</td>
</tr>
</tbody>
</table>
trolled to some extent by extrinsic factors such as environmental salinity. These initial findings have since been verified repeatedly (Jampol and Epstein, ’68, ’70; Kamiya and Utida, ’69; Motais, ’70; Milne et al., ’71; Bornancin and De Renzis, ’72; Sargent and Thomson, ’74), and the level of gill Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in seawater-adapted euryhaline teleosts was usually two to four-fold greater than the freshwater level. Thus a part of the characteristic response of euryhaline teleosts, when abruptly challenged with a hyperosmotic environment, is the augmentation of transport ATPase activity in the branchial epithelium.

Epstein et al. (’67) immediately recognized a possible relationship between the fine structural changes in chloride cells observed by previous investigators and their observed increase in gill Na\textsuperscript{+},K\textsuperscript{+}-ATPase following adaptation of fish to seawater. However, the first serious attempt to correlate the two lines of evidence was the cytochemical attempt to localize ATPase activity in the gills of the Japanese eel, Anguilla japonica, by Mizuhira et al. (’70). These authors demonstrated reaction products lining the external surface of the tubular labyrinth membranes in chloride cells, but since deposition was not affected by inclusion of ouabain (the specific inhibitor of Na\textsuperscript{+},K\textsuperscript{+}-ATPase) in the reaction medium, the localized reaction products could not be definitively attributed to the activity of the transport enzyme. Other barriers to the unambiguous localization of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, including inactivation of the enzyme by Pb\textsuperscript{++} and glutaraldehyde and substantial non-enzymatic hydrolysis of ATP during the cytochemical incubations, further suggested that the observed reaction products in this study did not localize sites of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. Other studies by this group of researchers were more profitable, however. In two subsequent papers (Shirai and Utida,
'70; Utida et al., '71), they presented results that demonstrated a statistically significant linear relationship between the activity of Na\(^+\),K\(^+\)-ATPase and the number of chloride cells in the branchial epithelium of the eel during seawater adaptation. It appeared from these studies that the bulk of the transport enzyme measured in the gill could be attributed to Na\(^+\),K\(^+\)-ATPase molecules located within chloride cells. This conclusion has since been verified by two separate lines of investigation.

In 1972, Kamiya developed a procedure for dissociation of the branchial epithelium and separation of the resultant cellular suspension into fractions enriched in particular cell types. One of the fractions obtained from the gills of seawater-adapted eels was greatly enriched in chloride cells, and the specific activity of Na\(^+\),K\(^+\)-ATPase in this fraction was at least twice as high as that measured in any of the other fractions obtained. Isolated chloride cells from eels adapted to freshwater were smaller and less numerous and no enrichment of the transport enzyme was noted. Using similar cell isolation techniques, Sargent et al. ('75) observed that Na\(^+\),K\(^+\)-ATPase and succinic dehydrogenase were enriched in a cellular fraction containing a high percentage of chloride cells, again demonstrating a positive correlation between chloride cells and Na\(^+\),K\(^+\)-ATPase.

While the results of cell isolation experiments presented strong evidence for localization of transport ATPase in chloride cells, the data was not definitive. Definitive localization was established in an elegant series of experiments (Karnaky et al., '76b) in which \(^{3}\)H-ouabain was injected into the vascular system of the killifish and
then localized in the gills by light microscopic autoradiography. Parallel experiments showed that the injected radioactive ouabain was specifically bound to Na\(^+\),K\(^+\)-ATPase and that this binding was tight enough to resist repeated washing. The autoradiographs revealed that the majority of the ouabain bound in the gills was associated with chloride cells. At higher magnifications, it was quite evident that the heaviest depositions of exposed silver grains were localized over the labyrinth areas of the cells, while binding at the apical surfaces was minimal. Unfortunately, the limitations in resolution inherent in this type of study did not permit definitive localization at the subcellular level.

Suggestive evidence for localization of Na\(^+\),K\(^+\)-ATPase in membranes of the chloride cell tubular labyrinth has, however, come from another line of investigation, one that offers a very much higher level of resolution. Addition of soluble lanthanum salts to fixative solutions during preparation of tissues for electron microscopic examination results in deposition of electron-opaque amorphous precipitates in the extracellular space of the tissue. This fact was first exploited by Revel and Karnovsky ('67) in an investigation of intercellular junctions in the heart and liver and shortly thereafter by Philpott (Philpott, '67, '68; Ritch and Philpott, '69) in studies of the teleost gill and pseudobranch. In these latter studies, the elaborate tubular networks that characterize both the branchial chloride cell and the parenchymal cell of the pseudobranch were readily penetrated by the electron dense tracer, thereby demonstrating continuity of the tubular lumens with the extracellular space. A more intriguing observation, however, was that the external surfaces of the tubule membranes displayed a definite substructure. At high magnification, arrays of particles delineated by the surrounding
amorphous lanthanum deposits could be seen projecting into the tubules. These particles displayed a center-to-center spacing of 60A in longitudinal and transverse sections of individual tubules and a suggestion of shallow helical arrays of particles on the membrane surface could be seen when tubules were sectioned tangentially. Ritch and Philpott ('69) suggested, on the basis of the hypothesized role of the chloride cell in electrolyte transport, that these particles represented portions of the enzyme complexes responsible for active ion transport (e.g., Na⁺,K⁺-ATPase). Further investigations on pseudobranch membranes (Dendy et al., '73a, '73b) have reinforced this suggestion. Negative staining of Na⁺,K⁺-ATPase-rich microsomal fractions from the pseudobranch of the pinfish, Lagodon rhomboides, revealed similar particulate surface structures on membranes derived from the tubular plasma membrane systems of the intact pseudobranch cells. Nakao ('74, '77) has since also demonstrated helical arrays of particles on the luminal surfaces of labyrinthine tubules in chloride cells from the Japanese lamprey, Lampetra japonica. The particulate substructure of these membranes was surprisingly visible without the use of electron dense stains, and showed a center-to-center spacing of 160A. These particulate arrays have also been observed in the chloride cells of the European lamprey, Lampetra fluviatilis (Morris and Pickering, '76; Pickering and Morris, '76), although they are not seen in conventionally stained chloride cells from a North American lamprey species, Petromyzon marinus (Youson and Freeman, '76). Since recent freeze-etch studies on purified preparations of Na⁺,K⁺-ATPase (Van Winkle et al., '76; Deguchi et al., '77; Vogel et al., '77) have shown that the enzyme complex protrudes from the membrane surface, it is possible that the particles seen on the membranes of the chloride cell
labyrinth and on the membranes of related cell types are in fact a portion of Na\(^+\), K\(^+\)-ATPase. This possibility as yet remains to be critically tested, however.

**Objectives of the Study**

It is now generally accepted that the chloride cell plays an integral role in teleost regulation. Yet the details of the way in which this role is mediated are far from clear, primarily because the molecular components of the transport systems involved have not been fully characterized nor have their positions within the cytoarchitecture of the chloride cell been ascertained. The Na\(^+\), K\(^+\)-ATPase, for example, appears to be a crucial component of the transport machinery, yet its subcellular localization has not been definitively established. This information is essential if one is to determine the part this enzyme plays in the actual transepithelial flux of electrolytes. The principal objective of this study, therefore, was localization of Na\(^+\), K\(^+\)-ATPase at the fine structural level in the chloride cells of a typical euryhaline teleost, the pinfish, *Lagodon rhomboides*. This was accomplished by application of the ultracytochemical procedure developed by Ernst ("72a, "72b, "75) which is based on the K\(^+\)-dependent phosphatase activity manifested by Na\(^+\), K\(^+\)-ATPase. This technique was developed through a kinetic analysis of enzyme activity under conditions of cytochemical incubation and is generally recognized as the single valid cytochemical procedure for Na\(^+\), K\(^+\)-ATPase localization at the fine structural level.

Several other experiments were also carried out in order to provide information necessary for interpretation of the cytochemical data. These are as follows: 1) the fine structural morphology of the pinfish branchial epithelium was characterized for pinfish adapted to isoosmotic and
hyperosmotic environments; 2) the activities of Na\(^+\),K\(^+\)-ATPase and several other putative plasma membrane markers were determined in gill homogenates from fish adapted to isoosmotic and hyperosmotic media; and 3) the temporal patterns of increase in activity during adaptation to the hyperosmotic medium were determined for those enzymatic markers that exhibited significantly elevated activities in the higher salinity.

In addition, a number of experiments were performed in an attempt to develop a procedure for the rapid isolation of chloride cells from the branchial epithelium. Such isolation is a prerequisite for further studies of the molecular topography of the labyrinth membranes using large molecular weight probes of membrane structure and binding sites such as lectins and antibodies, since these compounds do not readily pass the endothelial and connective tissue barriers that guard the basal surface of the branchial epithelium \textit{in situ}. Although both Kamiya ('72a) and Sargent \textit{et al.} ('75) had previously been successful in isolation of chloride cells from the gills of eels, their limited morphological observations suggested that the isolated cells had sustained substantial fine structural damage during the isolation procedure. For this reason and with further experiments of the above nature in mind, a procedure was developed that yielded an enriched fraction of morphologically well preserved chloride cells from the branchial epithelium of seawater-adapted pinfish.
MATERIALS AND METHODS

Collection of Fish and Adaptation Protocol

Pinfish, *Lagodon rhomboides*, ranging between 10 and 20 cm in length were handlined from the brackish waters (400-800 mOsm) in Offat's Bayou, Galveston, Texas and transferred on the day following capture to aerated 15 gallon aquaria containing either 1/3 strength (350-400 mOsm) or full strength (11-1200 mOsm) artificial seawater (Utility Marine Mix, Paterson, NJ). The aquaria were maintained at 20 ± 2°C and exposed to a natural photoperiod. Fish were removed and sacrificed for experiments on selected days for up to three weeks after transfer to the aquaria.

Sources of Chemicals

Most chemicals were obtained from Fisher Scientific Co., Mallinckrodt Chemical Works, or Sigma Chemical Co. The alkaline phosphatase inhibitor, 1-p-bromotetramisole oxalate, was obtained from Aldrich Chemical Co. Fixatives, plastics, and other reagents for processing of tissue for electron microscopic examination were purchased from Tousimis Research Corp. and Electron Microscopy Sciences, Inc.

Biochemical Assays on Gill Homogenates

Preparation of homogenates

For particular experiments, four fish were usually chosen at random from the captive population and quickly killed by spinal and caudal transsection. The eight gill arches from each fish were excised, blotted on filter paper, and their combined weight was determined to a tolerance of ± 0.2 mg on a Mettler balance. The combined arches from each fish were then homogenized with 10 strokes of a teflon homogenizer at a con-
centration of approximately 50 mg of tissue/ml in ice-cold medium containing 250 mM D-mannitol, 2 mM Na$_2$EDTA, and 20 mM Tris-HCl (pH 7.5). Homogenates were filtered through 250 μm mesh bolting silk and frozen immediately at -70°C. Homogenates to be assayed for sialic acid content were homogenized in ice-cold distilled water before being frozen. All frozen samples were thawed and assayed within 10 days of collection. None of the enzymes assayed showed any significant loss of activity over this time period.

Assays

All assays were standardized and appropriate blanks were included to assure that the values obtained as optical densities were directly proportional to the concentration of the solute being measured. The assays were also checked to assure that the hydrolysis of substrates was linear for the reaction times and conditions indicated. Duplicate determinations were performed on all samples in each of the assays.

1) Adenosine triphosphatases

Activities of 100 μl samples of homogenates were determined in 1.5 ml reaction mixtures containing the following concentrations of reagents: 5 mM Na$_2$ATP, 10 mM MgCl$_2$, 80 mM NaCl, 30 mM KCl, 0.25 mM Na$_2$EDTA, and 92 mM Tris-HCl (pH 7.5). Na$^+$,K$^+$-ATPase activities were determined from the decrease in total ATPase activity on addition of 0.5 mM ouabain to the reaction medium. The residual activity was referred to as Mg$^{++}$-ATPase. The above concentrations of reagents were chosen in order to optimize reaction conditions as suggested by Dendy et al. ('73a) in their kinetic characterization of Na$^+$,K$^+$-ATPase activity in pinfish pseudobranchs. The reaction was initiated by addition of homo-
enate, run for 10 minutes at 37 ± 1°C with continuous shaking, and stopped with 2.5 ml of ice-cold 10% trichloroacetic acid. Inorganic phosphate liberated during the reaction was measured spectrophotometrically in a Bausch and Lomb Spectronic 20 following the method of Fiske and Subbarow ('25).

2) K⁺-nitrophenylphosphatase

Nitrophenylphosphatase activities of 100 μl samples of homogenates were assayed in 1.0 ml reaction mixtures containing 5 mM Na₂NPP, 10 mM MgCl₂, 0.25 mM Na₂EDTA, and 100 mM Tris-HCl (pH 7.5). K⁺-NPPase activity was determined as the difference in activity resulting from the addition of 20 mM KCl to the reaction medium. The reaction was allowed to proceed for 10 minutes at 37 ± 1°C and was stopped with 0.5 ml of ice-cold trichloroacetic acid; 4.5 ml of 0.35 M Trizma base was added to restore alkaline pH and p-nitrophenol was measured spectrophotometrically at 400 nm.

3) 5'-nucleotidase

Activities of 100 μl samples of homogenates were assayed in 1.5 ml reaction mixtures containing 5 mM Na₂UMP, 10 mM MgCl₂, and 50 mM Tris-HCl (pH 7.5). Reactions were run for 20 minutes at 37 ± 1°C, stopped with 2.5 ml of ice-cold 10% trichloroacetic acid and assayed for inorganic phosphate by the method of Fiske and Subbarow ('25).

4) Alkaline phosphatase

Alkaline phosphatase activity was assayed by a modification of the procedure of Cvancara and Conte ('70). Activities of 100 μl samples of homogenates were determined in 1.0 ml reaction mixtures con-
taining 5 mM \(\text{Na}_2\text{NPP}\), 10 mM \(\text{MgCl}_2\), and 20 mM glycine (pH 10.5). The re-
action was initiated by addition of homogenate and allowed to proceed
for 10 minutes at 23 ± 1°C, at which time it was stopped with 0.5 ml of
ice-cold 10% trichloroacetic acid. Alkaline pH was restored by addition
of 4.5 ml of 0.35 M Trizma base and p-nitrophenol was measured at 400 nm.

5) N-Acetylneuraminic acid (sialic acid)

The assay was modified from that of Jourdain et al. ('71).
A 0.5 ml sample of homogenate was mixed with 0.1 ml of 40 mM periodic
acid and held on ice for 20 minutes, after which 1.25 ml of resorcinol
reagent (0.6% resorcinol, 0.25 mM \(\text{CuSO}_4\), and 18.5% HCl) was added. The
solution was then vortexed and returned to the ice bath for 5 minutes.
The samples were then heated to 100°C for 15 minutes, cooled to room
temperature, and 1.25 ml of 95% t-butyl alcohol was added. The solu-
tions were vigorously mixed, heated to 37°C for 5 minutes to stabilize
the color, and centrifuged for 5 minutes. Optical density of the solu-
tion was determined at 630 nm within 15 minutes.

6) Protein

Protein in samples was measured by a modification of the
method of Lowry et al. ('51) using crystalline bovine serum albumin as
a standard. Samples of homogenates were diluted as required with dis-
tilled water, usually to a 1:10 dilution. Aliquots of 0.25 ml were
withdrawn and mixed with 0.25 ml of 1.0 N NaOH and 5.0 ml of Lowry
reagent A. The combined solutions were then vortexed briefly and
allowed to stand at room temperature for 60 minutes at which time 0.5
ml of Lowry reagent B was added. The solutions were vigorously vortexed
and the optical density was read at 650 nm after 20 minutes.
Cytochemical Localization of Na\(^+\),K\(^+\)-ATPase

Determination of the effects of fixation on Na\(^+\),K\(^+\)-ATPase

The activity of Na\(^+\),K\(^+\)-ATPase is markedly sensitive to aldehyde fixation although not all aldehydes are equally inhibitory. Ernst and Philpott ('70) demonstrated that formaldehyde was much less inhibitory than was glutaraldehyde, even when used in much higher concentrations. However, fixation with formaldehyde alone often does not adequately preserve tissue fine structure. Recently Ernst ('75) showed that a combination fixative consisting of 1% formaldehyde and 0.25% glutaraldehyde would allow acceptable preservation of tissue while preserving enzyme activity adequate for cytochemical localization, provided that the fixation interval was kept short. The effects of these two fixatives, formaldehyde or the formaldehyde-glutaraldehyde combination, on the activity of Na\(^+\),K\(^+\)-ATPase in the pinfish gill was therefore examined before beginning the cytochemical localizations.

Pinfish were sacrificed and the four paired gill arches were excised. The arches were then blotted and weighed individually and the four arches from the left buccal cavity were held separately in homogenizing medium at 4\(\degree\)C. The four arches from the right side were then immersed in either of the following fixatives at 4\(\degree\)C: 1) 2% formaldehyde or 2) 1% formaldehyde-0.25% glutaraldehyde. Both of the fixatives were buffered to pH 7.4 with 0.15 M cacodylate buffer. At regular intervals arches were removed from the fixative, rinsed in several changes of ice-cold buffer and homogenizing medium, and homogenized as previously described. The matching control gill arch was likewise homogenized and both were frozen at -70\(\degree\)C. Activity of Na\(^+\),K\(^+\)-ATPase in these homogenates was determined as previously described and the relative inhibi-
tory effects of the two fixatives were calculated from the difference in activity between the fixed arches and their paired controls. Each experiment included at least two fixation intervals and each was repeated twice.

Cytochemical procedures

For cytochemical determinations, 12 to 14 day seawater-adapted fish were killed by spinal severance and the pericardial cavity was exposed. Fixation was initiated by rapid perfusion through the bulbous arteriosus of 2 to 3 ml of ice-cold 1% formaldehyde-0.25% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4). Gill arches that had blanched, indicating successful perfusion, were then quickly excised and immersed in fresh fixative at 4°C for 5 minutes. Following fixation, arches were sliced freehand with a scalpel such that individual gill filaments were transsected into 1 mm pieces. These pieces were then rinsed three times in ice-cold 0.1 M cacodylate buffer (pH 7.4) with 0.2 M sucrose and three times in 0.1 M Tris-HCl (pH 7.5) with 0.2 M sucrose. After a final rinse in 0.1 M Tris-HCl (pH 9.0) with 0.2 M sucrose at room temperature, the tissue was divided into approximately equal portions and incubated in 10 ml of the K⁺-NPPase reaction medium of Ernst ('75). Incubations were allowed to proceed for either 30 or 60 minutes at 25 ± 1°C with continuous agitation. The standard incubation medium contained the following: 10 mM Na₂NPP, 20 mM MgCl₂, 30 mM KCl, 10 mM SrCl₂, and 200 mM Tris-HCl (pH 9.0). In addition, control media were prepared and the following variations were carried out.

1) Ouabain was added to the complete medium to a concentration of 10 mM.

2) Since K⁺ is an obligatory cofactor for the phosphatase activity of
the Na\(^+\),K\(^+\)-ATPase, KCl was omitted from some reaction media.

3) Since interpretation of the cytochemical reaction is complicated by the presence of a highly active alkaline phosphatase in teleost gills (Cvancara and Conte, '70; this study), steps were taken to specifically inhibit its activity. Firth ('74) pointed out that this nonspecific phosphatase readily hydrolyzes NPP at the alkaline pH of the incubation medium in the Ernst K\(^+\)-NPPase localization technique and recommended inclusion of suitable alkaline phosphatase inhibitors in the medium. A potent inhibitor of the enzyme, 1-p-bromotetramisole oxalate, was therefore added at a concentration of 0.5 mM to sets of both the complete and control media in order to clearly differentiate between the precipitates arising from the activity of alkaline phosphatase and the depositions produced by the activity of Na\(^+\),K\(^+\)-ATPase. B-glycerophosphate, a substrate hydrolyzed by alkaline phosphatase but not by Na\(^+\),K\(^+\)-ATPase, was also substituted for NPP in some of the media.

4) In order to differentiate between artifactual precipitates and those that were enzymatically-produced, two further variations were carried out. Tissue pieces were incubated for 60 minutes in a medium from which substrate had been deleted. Pieces were also incubated in substrate-deficient medium for 30 minutes, after which KH\(_2\)PO\(_4\) was added to a concentration of 10 mM and the pieces were further incubated for 30 minutes.

Following incubation in the various media, the tissue was rinsed in Tris-HCl (pH 9.0) as before and treated with 2\% Pb(NO\(_3\))\(_2\) in 0.2 M suc-
rose for 10 minutes at room temperature. This treatment was necessary in order to convert the deposits of Sr$^{++}$-precipitated inorganic phosphate to the more electron opaque lead salts. After this conversion, the pieces of gill tissue were rinsed briefly in 0.25 M sucrose and 0.2 M cacodylate buffer (pH 7.4), postfixed for 60 minutes at 4°C in 1% OsO$_4$ prepared in the same buffer, and dehydrated in ethanol. Embedding was carried out in Epon by the method of Luft ('61).

Cell Isolation Procedures

Fish were removed from aquaria, paralyzed by spinal transsection, and perfused through the bulbous arteriosus with 5 to 10 ml of chilled Ca$^{++}$,Mg$^{++}$-free balanced salt solution (BSS) consisting of 185 mM NaCl, 10 mM KCl, 5 mM NaHCO$_3$, 0.5 mM KH$_2$PO$_4$, 1.5 mM Na$_2$HPO$_4$, 5 mM glucose, and 2 mM Na$_2$EDTA (pH 7.2-7.4; 380-390 mOsm). Gill arches were then excised and the second left and right arches were removed for enzyme assay. The remaining 6 arches were incubated at 4°C in Ca$^{++}$,Mg$^{++}$-free BSS for 30 minutes with frequent gentle agitation. Following incubation, the branchial epithelium was carefully scraped from the supporting cartilage rods of the gill filaments and pooled in 20 ml of Ca$^{++}$, Mg$^{++}$-free BSS. This suspension was filtered through 250 μm mesh bolting silk, passed four times through a 10 ml pipette (1 mm bore diameter) with minimal pressure, filtered through 40 μm mesh bolting silk, and repipetted as before. The resulting suspension consisting primarily of single cells and subcellular debris was centrifuged at 150 x g for 5 minutes at 4°C in a Sorvall HB-4 rotor operated in a Sorvall RC-3 refrigerated centrifuge. The cell pellet was gently resuspended in 5 ml of Ca$^{++}$,Mg$^{++}$-free BSS and layered over a discontinuous gradient consisting of four steps (10 ml each) of 5, 10, 15, and 20% Ficoll
(mol wt 400,000) in Ca\textsuperscript{++},Mg\textsuperscript{++}-free BSS. Gradients were centrifuged at 2000 x g for 45 minutes at 4°C in the HB-4 rotor. Three bands of cells routinely formed and were collected by displacement with an ISCO density gradient fractionator. Band A was usually collected from 10 to 18 ml, band B from 20 to 28 ml, and band C from 30 to 38 ml. The fractions were diluted to 50 ml with Ca\textsuperscript{++},Mg\textsuperscript{++}-free BSS and centrifuged at 200 x g for 5 minutes. Pellets from the three bands were either fixed and embedded as follows or resuspended in up to 5 ml of Ca\textsuperscript{++},Mg\textsuperscript{++}-free BSS or homogenizing medium. Resuspended pellets that were to be assayed for ATPase activities were homogenized and frozen at -70°C. Assays were carried out as described for tissue homogenates. Rates of oxygen consumption on 2 ml samples from band C pellets resuspended in Ca\textsuperscript{++},Mg\textsuperscript{++}-free BSS were determined with a YSI-Clark 2510 oxidase probe.

**Fixation and Examination**

Gill filaments were fixed by perfusion with 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer (pH 7.4) which also contained 1 mM CaCl\textsubscript{2}. Samples were fixed both at 4°C and at room temperature and for times ranging from 60 minutes to 24 hours. After the initial fixation, filaments were rinsed three times in ice-cold 0.1 M cacodylate buffer (pH 7.4) containing 0.2 M sucrose and 1 mM CaCl\textsubscript{2} and postfixixed for 90 minutes at 4°C in 1% OsO\textsubscript{4} prepared in the same buffer. The samples were then dehydrated in ethanol and embedded in Epon 812.

Embedded material was observed with both the light and electron microscopes. For light microscopic examination, thick sections (0.5-1.0 μm) were cut with glass knives on a Porter-Blum MT-2 ultramicrotome, mounted on glass slides, and stained with 1% methylene blue. These thick sections were then viewed and photographed with a Zeiss Ultraphot
light microscope.

For electron microscopic examination, thin sections were obtained with diamond knives on the ultramicrotome and mounted on uncoated copper grids. Sections were observed either unstained or stained with aqueous uranyl acetate and alkaline lead citrate in a Philips 200 electron microscope operated at 60 kV.
RESULTS

Anatomy and Histology of the Gills

The anatomy of the pinfish gill is similar to that described for many other species of teleosts (see representative papers by Bajtel, '49; Newstead, '67; Morgan and Tovell, '73). Four pairs of gill arches occupy the buccal cavity with a single set of four lying on each side of the head. Along each gill arch and projecting distally from its outer circumference are positioned the gill filaments. These are organized into two rows, with filaments from the rows alternating in a manner identical to that described in the trout, *Salmo gairdneri*, by Morgan and Tovell ('73). Histologically, individual filaments are supported by a cartilaginous gill ray, which is a continuous extension of the cartilaginous portion of the gill arch proper. Lacunae of chondrocytes are evident in the transsected shaft of the gill ray (figs. 1 and 2). At either side of this supportive rod are the principal blood vessels of the filament. The afferent branchial artery is situated immediately posterior to the shaft and the larger efferent artery is located at the anterior surface, although separated from it by an oblong lymph sinus and a knot of loose connective tissue containing a small bundle of unmyelinated nerves. Encompassing the gill ray, connective tissue, and the blood and lymph vessels is the thick basal lamina which supports the branchial epithelium proper, a combined simple columnar/stratified epithelium consisting of several cell types. The stratified portion is comprised of basal and supportive cells, pigment and mucous cells, rodlet cells, mast cells, and an external layer of squamous pavement cells. The simple columnar aspect of the epithelium is
contributed by chloride cells which extend from the basal lamina to the free surface. Chloride cells, mucous cells, and roddlet cells all interrupt the pavement layer, but only chloride cells span the entire width of the epithelium.

The paired secondary lamellae extend from the central filament at regular intervals and form the major respiratory surfaces of the gill. These leaflets arise at an oblique angle much as the rays of a feather and several are usually seen in parallel array when filaments are observed in transverse section (fig. 1). The lamellae, which provide a greatly amplified surface for gaseous exchange, are composed of a network of shallow anastomosing blood spaces defined by the spool-shaped pillar cells, a highly specialized endothelial cell type. These spaces communicate directly with both the afferent and efferent vessels. In order to facilitate gaseous exchange, the epithelium covering the lamellae is very thin and consists only of a layer of squamous basal cells and the usual pavement cells. Since the pavement cells are directly exposed to the external environment, surface amplification is once again emphasized in that the apical surfaces of these cells are disposed as a series of more or less concentric microplicae that appear like short microvilli in sectioned material.

These common elements of morphology were observed whenever gill filaments from pinfish adapted to either the lower or higher experimental salinity were examined. There were, however, differences that were immediately apparent when filaments from fish adapted to seawater were compared with filaments from fish adapted to 1/3 seawater. Most notably these were alterations in both the total numbers and specific cytological characteristics of the chloride cells.
While a detailed morphometric analysis of chloride cell numbers was not attempted during the present series of experiments, a simple experiment confirmed that the initial visual impression, that chloride cells were more numerous in the gills of seawater-adapted pinfish, was correct. Preliminary observations showed that chloride cells were common along the entire length of the gill filament in both salinities. One micrometer thick transverse sections were therefore taken midway between the proximal and distal ends of four filaments from each of four fish adapted to either 1/3 seawater or to seawater. The numbers of chloride cells with visible nuclei were counted in representative sections and the means ± S.E.M. were calculated. In the fish adapted to 1/3 seawater, the average was 29 ± 4 cells per section while in those sections from seawater-adapted pinfish gill filaments the average was 51 ± 5. A marked proliferation of chloride cells must, therefore, occur in the branchial epithelium of pinfish during adaptation to seawater.

In filaments from pinfish fixed immediately after capture or after two weeks in 1/3 seawater (fig. 1), chloride cells were located primarily along the lateral surfaces of the gill ray. In addition, a few chloride cells were scattered in the epithelium surrounding the afferent branchial artery. These cells ranged from 5 to 10 μm in diameter and were both cuboidal and columnar, the latter configuration predominating in the region of the afferent vessel. The apical surfaces of the cells usually appeared flat at low magnification, but higher magnification revealed irregular projections. Cells were also occasionally observed which displayed crypt-like invaginations of the apical cell surface, but they accounted for only a small percentage of the total
cell population.

After 12 to 14 days in seawater (fig. 2), the more numerous chloride cells occupied prominent positions along virtually the entire width of the filament and were rare only in the region of the epithelium overlying the efferent artery. In addition to being more numerous, the seawater chloride cells were in general slightly larger with diameters averaging around 10 μm. Where fortuitous planes of section revealed chloride cells in direct contact with the external environment, the apical cell surfaces were invaginated to form the characteristic crypts so diagnostic of seawater-adapted fish. Large vesicular-appearing structures, presumably crypts slightly out of the normal plane of section, were also observed in the apices of those cells that appeared to be beneath the layer of squamous pavement cells. Thus while only a few cells in the lower salinity displayed invaginations of the cell surface, apical crypts seem to be a universal feature of seawater-adapted chloride cells in the pinfish, a finding in accord with those of previous studies on other teleost species.

Adaptation of teleosts to different conditions of salinity has also been reported to affect the population of mucous cells in the gills. Jozuka ('66) described a decline in the numbers of branchial mucous cells in the Japanese eel following transfer of these fish to seawater. This decline did not begin until several days after the transfer and was complete, with only a few mucous cells remaining, at the end of a month. Mucous cells in the pinfish gill are less abundant than in the eel, but are evident in fish adapted to both salinities. They are most numerous in the region of the epithelium that overlies the efferent branchial artery but are also scattered along the lateral surfaces of
the filament and in the epithelium that surrounds the afferent vessel. There are usually somewhat fewer mucous cells in this latter region in fish adapted to seawater, although mucous cells were still abundant near the anterior pole of the filament. Since this decline was not strongly evident, a careful morphometric study such as that carried out by W.S. Marshall ('76) would appear to be necessary in order to precisely determine what effect salinity changes have on the population of branchial mucous cells in the pinfish.

**Fine Structure of the Branchial Epithelium**

**Miscellaneous cell types**

The heterogenous nature of the various cell types represented in the branchial epithelium is most evident where it is thickest, in the regions of the two major blood vessels. The area shown in figure 3 encompasses a slice of the epithelium that surrounds the afferent branchial artery. The epithelium is separated from the artery by the squamous endothelium and two layers of smooth muscle, the inner circularly and the outer longitudinally arranged. Fibroblasts and an occasional unmyelinated nerve fiber are also seen between the endothelium and the epithelium. Underlying the epithelial layer is the basal lamina, which averages around 0.7 μm in thickness in this region although it decreases to less than 0.05 μm along the lateral surfaces of the gill ray.

The basal surface of the branchial epithelium proper is lined by cells of a single type which form a confluent layer superimposed on the surface of the basal lamina (fig. 3). The confluency of these basal cells is interrupted only by chloride cells. The basal cells are often elongate, sending narrow processes between the other cells of the epithelium and distally toward the free surface. Basal cells could be
identified by their electron dense cytoplasm within which were seen bundles of microfilaments and numerous clear vesicles. Other cell types of indeterminate function were also present in the basal and central regions of the epithelium. Most contained an assortment of filaments and vesicles and some occasionally contained large inclusions resembling lysosomes, lipid droplets, and pigment granules. For lack of a more definitive functional term, these cells were collectively referred to as supportive cells. Four other cell types were also common in the branchial epithelium; the squamous pavement cells that form the surface layer, mucous cells, mast cells, and rodlet cells. This last cell type is enigmatic and its functional roles are unknown, although its fine structure has been described by Leino ('74) and Desser and Lester ('75) in other teleosts. Rodlet cells are observed in many other organs besides the gill and are not thought to play a role in osmoregulation.

Fine structure of branchial chloride cells

The fine structure of chloride cells in the pinfish branchial epithelium is similar to that described in many other species of teleosts. The majority of the cellular volume in chloride cells from both salinities was occupied by the labyrinth of anastomosing tubules (figs. 6-8). This extensive network of plasma membrane invaginations ramified throughout virtually the entire cell, being excluded only from the basally located nucleus and a narrow rim of cytoplasm immediately subjacent to the apical crypt membrane. The reticular networks thus formed enveloped all of the major cellular organelles, most prominent of which were the numerous mitochondria. Apposition of mitochondrial and tubular membranes was of the most intimate nature, with inter-membrane distances of less than 200A commonly observed. Chloride cell
mitochondria were uniformly distributed within the labyrinth and were usually oval or bean-shaped, although some were noticeably elongate. Cristae were oriented both transversely and longitudinally in the moderately electron dense matrix. Also present in the inner mitochondrial matrix were small (~300Å) granules, usually one or two per mitochondrial profile. Also identifiable in the cytoplasmic ground substance of the chloride cell were cisternae of endoplasmic reticulum and free polyribosomes, microtubules and microfilaments, and many small vesicles. These last were especially numerous in the cytoplasm surrounding the Golgi complexes, which were usually observed in a supra-nuclear position. Two or more Golgi complexes were commonly observed in a single section, so a given chloride cell may contain several of these organelles. The morphology of Golgi complexes differs little in chloride cells from fish adapted to either salinity. In each they consist of four to five parallel flattened cisternae, the complex as a whole being slightly curved. Surrounding the stack of cisternae were abundant vesicles and droplets of differing sizes and degrees of electron opacity. No specific orientation of these organelles with respect to the baso-apical axis of the cell was noted.

The primary morphological difference between chloride cells from pinfish adapted to 1/3 seawater or to seawater, aside from the increased size of the seawater-adapted cells, was in the structure of the apical cell surface. In chloride cells from pinfish adapted to the lower salinity, the apical surface was extended into irregular projections (figs. 4 and 5). In planes of section parallel to the surface of the epithelium it was apparent that these projections formed bifurcating microridges rather than microvillous extensions. The outer surface of
the membrane was covered with a filamentous glycoalyx. Seawater-
adapted chloride cells, on the other hand, were characterized by a crypt
formed by invagination of the apical plasma membrane (fig. 6). The
lumen of the crypt was usually filled with a mass of fine filaments
which appeared to be continuous with the glycoalyx of the crypt mem-
brane. Two or more chloride cells often impinged on a common apical
crypt, and where this was the case, their respective lateral surfaces
interdigitated and overlapped one another.

Immediately beneath the apical plasma membrane and separating it
from the tubules of the labyrinth is a narrow zone of cytoplasm usually
less than 1 μm in thickness. Smaller organelles such as polyribosomes,
microtubules and microfilaments, an occasional centriole, and small
vesicles are common in this zone (figs. 5 and 6). Both electron dense
and electron lucent vesicles were observed, often in intimate associa-
tion with the plasma membrane. Instances of what may represent fusion
of these vesicles with the crypt membrane were also observed (fig. 6,
insets), suggesting that at least some of these vesicles may be involved
in turnover of apical plasma membrane constituents, although whether
they represent endocytic or exocytic mechanisms remains to be demonstra-
ted.

In addition to the population of typical chloride cells in the
branchial epithelium, a cell type was observed that shared many of the
same characteristics, yet was morphologically distinct. These cells
were smaller than chloride cells and could be readily distinguished by
their smaller and more electron dense mitochondria and by a slight dis-
tension of the tubules of the labyrinth (fig. 8). These cells usually
contained one or more large autophagic lysosomes and a single supra-
nuclear Golgi complex. Cells of this type have previously been observed in the teleost pseudobranch by Dunel and Laurent ('73), who termed them "accessory cells," although they have not been heretofore described in the branchial epithelium of the gill proper. While several groups of workers have described differences in the density of chloride cells in the same filament (Doyle and Gorecki, '61; Straus, '63; Threadgold and Houston, '64; Coleman et al., '77), a review of the data suggests that these differences are the result of fixation artifacts and/or natural variation in cell morphology in the chloride cell populations and that the electron dense chloride cells previously described are not identical to accessory cells. In the pinfish branchial epithelium, accessory cells were frequently observed in either salinity and invariably occupied positions adjacent to chloride cells. In both salinities, these cells contributed to the free surface of the epithelium but they were never observed in the lower strata of the epithelium or in contact with the basal lamina. In fish adapted to seawater, they often shared a common apical crypt with one or more chloride cells (fig. 6). The absence of recognizable intergrades between accessory cells and chloride cells mediates against the possibility that the former are replacement cells for the chloride cell population. The common observation of autophagic lysosomes in accessory cells, however, suggests that these cells may represent chloride cells that have undergone regression, although the changes such as mitochondrial degeneration and labyrinth fragmentation previously described by Shirai and Utida ('70) and Doyle and Epstein ('72) in regressing chloride cells were not observed in pinfish accessory cells.

The only definitive evidence for cellular renewal in the branchial
epithelium gained during the present study was a single observation of mitosis in the gill of a pinfish fixed on the eighth day of adaptation to seawater. A cell in late prophase was observed in the lowest stratum of the epithelium. Since this cell did not display fine structural features diagnostic for any of the recognizably differentiated cell types found in the branchial epithelium, it was impossible to characterize the cell with respect to its lineage. It is possible that cell division is more common in the pinfish branchial epithelium than this single observation would seem to suggest and that a more thorough study would reveal further evidences of cellular turnover.

Survey of Plasma Membrane Markers in the Gill

The activities of several putative plasma membrane marker enzymes and the levels of sialic acid, a common carbohydrate constituent of membrane glycoproteins and glycolipids, in gills from pinfish adapted to the two experimental salinities are presented in table II. Since extensive differential fractionation of homogenates was not attempted in the present study, it cannot be stated with certainty that each of the enzymes is exclusively localized to the plasma membrane in cells of the pinfish branchial epithelium. In fact, three of the enzymes listed (e.g., 5'-nucleotidase, alkaline phosphatase, and Mg$^{++}$-ATPase) have been reliably demonstrated in other subcellular locations in other tissues (Leonard and Provenza, '72; Widnell, '72; Borgers, '73; Farquhar et al., '74; Sikstrom et al., '76; Saccomani et al., '77) and thus may have at least dual localizations. However, if these membrane constituents are raised to significantly higher levels during the chloride cell hypertrophy that follows transfer of pinfish to seawater, elevated tissue activities should be noted unless compensatory decreases in those
TABLE II. Levels of putative plasma membrane marker enzymes and sialic acid in pinfish gill homogenates. Fish were adapted to the two salinities for a minimum of 14 days and activities were measured as described in the Materials and Methods. Each value represents the mean ± S.E.M. from determinations on homogenates from four fish. Enzyme activities are expressed as μmoles substrate hydrolyzed/mg protein/hour. Sialic acid levels are expressed as μmoles sialic acid/mg protein.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>1/3 Seawater</th>
<th>Seawater</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-nucleotidase</td>
<td>7.3 ± 1.3</td>
<td>7.2 ± 0.7</td>
<td>0.500</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>1.37 ± 0.01</td>
<td>1.24 ± 0.23</td>
<td>0.100</td>
</tr>
<tr>
<td>Mg^{++}-ATPase</td>
<td>15.4 ± 0.6</td>
<td>19.8 ± 0.7</td>
<td>0.005</td>
</tr>
<tr>
<td>Na^+,K^+-ATPase</td>
<td>9.6 ± 0.9</td>
<td>25.8 ± 1.9</td>
<td>0.001</td>
</tr>
<tr>
<td>K^+-NPPase</td>
<td>0.60 ± 0.04</td>
<td>1.90 ± 0.17</td>
<td>0.001</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>17.1 ± 0.7</td>
<td>15.6 ± 0.2</td>
<td>0.200</td>
</tr>
</tbody>
</table>
activities in other cellular locations occur. Due to this latter possibility, interpretation of these results must therefore be made with discretion. Nonetheless, it is plain that the activities of Mg$^{++}$-ATPase, Na$^+$,K$^+$-ATPase, and K$^+$-NPPase are significantly higher in gills of fish adapted to seawater than in the gills of 1/3 seawater-adapted fish. It is also noteworthy that the increase in Mg$^{++}$-ATPase is only around 22% while those of Na$^+$,K$^+$-ATPase and K$^+$-NPPase are much greater, 270% and 310%, respectively. As previously suggested, the Mg$^{++}$-ATPase activity measured may result from the contributions of at least two enzymes. In the chloride cells of the killifish, the major subcellular localization of Mg$^{++}$-ATPase activity demonstrable by the Wachstein-Meisel cytochemical technique was shown to be the inner mitochondrial membrane (Ernst and Philpott, '70). Activity was also localized to the outer surfaces of the chloride cell labyrinth membranes in this study, but the reaction here was much less intense. Likewise, in cell fractionation studies of the pinfish pseudobranch, Dendy et al. ('73a) demonstrated that most of the Mg$^{++}$-ATPase activity derived from mitochondrial membranes while a minor fraction was associated with plasma membrane fragments. Based on these studies, it may be suggested that the higher level of Mg$^{++}$-ATPase measured in the gills of pinfish adapted to seawater results mainly from an increase in mitochondria that accompanies the observed chloride cell proliferation and hypertrophy. On the other hand, the high Na$^+$,K$^+$-ATPase activity in seawater-adapted pinfish gills most probably results from amplification of the membrane labyrinth. As discussed in the introduction, Na$^+$,K$^+$-ATPase is exclusively localized in plasma membranes and its activity has been closely correlated with chloride cell size and number in the teleost branchial epithelium. This
observed increase is therefore consistent with previously published observations and with the cytological evidence presented in the present study. The activity of K⁺-NPPase is also increased and to nearly the same extent as Na⁺,K⁺-ATPase in the higher salinity, a finding consistent with the current interpretation that this non-specific phosphatase activity is a manifestation of the K⁺-dependent dephosphorylation step in the Na⁺,K⁺-ATPase reaction sequence. Turnover of NPP is between 5 and 10% of the rate of ATP hydrolysis, which is also consistent with previously published data (see Dahl and Hokin, '74).

Although neither 5'-nucleotidase nor alkaline phosphatase showed elevated activities in the seawater gill homogenates, minor differences cannot be definitively ruled out because of the ambiguities mentioned earlier. However, relative increases of the magnitude shown by Na⁺,K⁺-ATPase clearly do not occur. Likewise, the levels of sialic acid are similar in the gills of pinfish adapted to both experimental salinities. This last finding was to some extent unexpected, since cell surface-associated polyanionic substances, presumably including sialic acids, have been demonstrated in both the apical crypt and plasma membrane labyrinth of chloride cells (Karnaky, '67, '70; Philpott, '68; Karnaky and Philpott, '69). Furthermore, the glycoprotein subunit of Na⁺,K⁺-ATPase contains sialic acid residues (Kyte, '72; Perrone et al., '75; M.O. Marshall, '76). One might therefore expect the increased Na⁺,K⁺-ATPase activity in seawater pinfish gills to be reflected in increased sialic acid levels. Although no significant difference was noted, an increase in sialic acid levels may in fact have accompanied the chloride cell response and yet been insignificant when compared to background tissue levels. The squamous pavement cells of the branchial epithelium
display an elaborate glycocalyx and the mucous that coats the teleost
gill contains significant amounts of sialic acid (Bolognani and Bolognani-
Fantin, '63; Olivereau and Lemoine, '71; Harris and Hunt, '73; Lemoine,
'74), so it remains possible that residues associated with chloride cell
membranes may comprise only a small percentage of the total tissue con-
tent of this carbohydrate. Additionally, a decrease in the number of
branchial mucous cells following transfer of pinfish to seawater could
counteract increases in sialic acid levels associated with chloride cell
proliferation and hypertrophy.

Temporal Patterns of Enzyme Activity during Seawater Adaptation

Although the above experiments demonstrated high Na\(^+\),K\(^+\)-ATPase acti-

vity in the gills of pinfish held in seawater for 14 days, they did not

reveal whether or not a steady state level indicative of full adaptation

had been reached. For this reason, the pattern of Na\(^+\),K\(^+\)-ATPase activity

variation over the two-week period following transfer was determined

(fig. 9). In addition, the patterns of increase of both K\(^+\)-NPPase and

Mg\(^++\)-ATPase were investigated in order to determine whether or not they

coincided with that of Na\(^+\),K\(^+\)-ATPase.

A slight but nonsignificant decline in Na\(^+\),K\(^+\)-ATPase activity was

noted over the first two days of adaptation and was followed by a period

of rapid increase in activity that reached a plateau around the 10th day

after transfer. This pattern of change, resulting in a two- to three-fold

increase in activity, is virtually identical to that described by Utida

et al. ('71), Forrest et al. ('73a), and Thomson and Sargent ('77) for

eel species and by Evans and Mallery ('75) for the fat sleeper,

Dormitator maculatus, when these fishes were transferred from freshwater
to seawater. The dip in activity noted around the eighth day was also
observed by Forrest et al. ('73a) and verified in a separate experiment during the present investigations. The significance of this transient decrease remains to be elucidated.

$\text{Na}^+,\text{K}^+$-ATPase activity measured in gill homogenates from pinfish held in 1/3 seawater did not change significantly during the 14 day adaptation period, indicating that the increase in activity noted in the gills of fish adapting to seawater resulted totally from their transfer to this hyperosmotic environment.

Activities of $\text{K}^+$-NPPase from aliquots of the same homogenates are shown in figure 10. The curve differs from that in figure 9 only in minor respects and the two show a highly significant linear correlation ($P < 0.001$) when the means are compared by regression analysis (fig. 11). These observations supported the hypothesis that $\text{K}^+$-NPPase activity measured in the gill homogenates represents the phosphatase component of $\text{Na}^+,\text{K}^+$-ATPase, a critical piece of evidence with reference to the cytochemical localization of the transport enzyme. They also assured that the 12 to 14 day seawater-adapted pinfish had a high steady state level of $\text{Na}^+,\text{K}^+$-ATPase in their gills.

Activity of $\text{Mg}^{++}$-ATPase from aliquots of the same homogenates is illustrated in figure 12. Although the activity measured in seawater gills is increased significantly at the end of the 14-day adaptation period, it is apparent that the increase does not coincide with that of $\text{Na}^+,\text{K}^+$-ATPase. Augmentation of $\text{Mg}^{++}$-ATPase activity appears to be more gradual and is delayed at least two days behind $\text{Na}^+,\text{K}^+$-ATPase. The point of half maximal increase for $\text{Na}^+,\text{K}^+$-ATPase is reached by the sixth day of adaptation, while that point in the $\text{Mg}^{++}$-ATPase increase curve is not reached until about the ninth day. These results suggest that the
increase in Mg\(^{++}\)-ATPase activity reflects a sequence of subcellular events that is quite separate and distinct from the plasma membrane hypertrophy reflected by changes in Na\(^{+}\),K\(^{+}\)-ATPase activity. Since the major site of Mg\(^{++}\)-ATPase activity in the teleost branchial epithelium appears to be mitochondrial, the observed change may reflect the proliferation of mitochondria that is one of the salient morphological responses of the chloride cell when euryhaline fish are transferred to hyperosmotic environments.

**Cytochemical Localization of Na\(^{+}\),K\(^{+}\)-ATPase Activity in the Gill**

**Effects of fixation on Na\(^{+}\),K\(^{+}\)-ATPase**

The effects of 2% formaldehyde and 1% formaldehyde-0.25% glutaraldehyde on the activity of Na\(^{+}\),K\(^{+}\)-ATPase in pinfish gills are shown in figure 13. Inactivation of the enzyme by formaldehyde alone is gradual, with over 50% of the control activity remaining after 60 minutes of fixation at 4°C. In contrast, addition of 0.25% glutaraldehyde to the fixative results in a rapid decrease in activity to less than 20% of control values after only 30 minutes. For this reason, formaldehyde alone was used as the fixative of choice in the initial cytochemical experiment. Gill tissue from seawater-adapted pinfish was fixed for 60 minutes at 4°C in cacodylate-buffered 2% formaldehyde and processed for K\(^{+}\)-NPPase localization. Electron microscopic examination unfortunately revealed that preservation of tissue fixed in this manner was very poor, with severe morphological disruption noted in all samples. Accordingly, the combination fixative was employed in the second cytochemical experiment that was performed, and the fixation interval was shortened to less than 10 minutes at 4°C. Preservation of morphology was still less than ideal in these samples, although it was in general
much improved over the previous experiment. Furthermore, there appeared to be abundant reaction precipitates in most blocks of the processed tissue. This latter fixation protocol was therefore used in all of the subsequent cytochemical experiments.

Electron microscopic localization of Na\(^+\),K\(^+\)-ATPase

In gill filaments incubated in the complete cytochemical medium containing 30 mM KCl, distinctly granular precipitates were deposited along the cytoplasmic surfaces of the plasma membranes of most of the cell types which comprise the branchial epithelium. This reaction product was sparse, however, in most cells except for chloride cells, even after 60 minutes of incubation. In chloride cells after 30 minutes of incubation, discrete foci of precipitate were evident in the crypt region and along the tubules of the labyrinth (fig. 15). Consistent with the known polarity of Na\(^+\),K\(^+\)-ATPase activity, these deposits were exclusively localized to the cytoplasmic surfaces of the membranes and did not intrude into the luminal spaces of the labyrinth. Likewise, although mitochondria were closely surrounded by tubular meshworks, staining of mitochondrial membranes was rarely observed. The amount of reaction product varied widely from cell to cell, with chloride cells adjacent to each other often exhibiting quite different levels of activity. The distribution of precipitates within a given cell was also variable, with heavier deposition frequently observed in the basal regions of the labyrinth. At the apical cell surface, a few focal deposits were associated with the cytoplasmic surfaces of the crypt membrane and the vesicles in the underlying rim of apical cytoplasm.

After 60 minutes of incubation, deposition on the labyrinth mem-
branes was much more pronounced and no appreciable gradient in the amount of precipitation along the baso-apical cell axis was seen (fig. 14), although in many cells deposition was lighter in the area of the labyrinth immediately surrounding the nucleus. Precipitates often surrounded individual tubules and filled the cytoplasmic ground substance between the tubules and the closely apposed mitochondria (figs. 14, 16-23). Minor accretion of granules into the lumens of tubules was frequently observed following this longer incubation, although mitochondria continued to remain free of deposits except for occasional peppering of very fine precipitates (fig. 16).

Deposition was also more pronounced at the apical cell surfaces after 60 minutes of incubation (figs. 14, 17, 19, 20, 21). Precipitation was more apparent at the crypt membrane and the vesicles of the apical zone stained more heavily. Deposition was as before on the cytoplasmic surfaces of the membranes. In addition, small deposits of reaction product were associated with Golgi lamellae and with the membranes of vesicles in the Golgi regions. Deposition here was much lighter than in the surrounding labyrinth, but as in the case of the tubules, staining was observed only on the cytoplasmic aspects of the respective membranes.

Other deposits besides those granular precipitates associated with the cytoplasmic surfaces of membranes were observed in sections from tissue incubated in both complete and control media. Granular precipitates were scattered over nuclei in many cells after 60 minute incubations (figs. 14, 20, 23) and large focal deposits were observed within the basal lamina of the epithelium and associated with collagen bundles in the underlying connective tissue (fig. 32). In addition, precipitates of a
more amorphous nature were widely distributed along the plasma membranes of most cell types, but were localized to the extracellular surfaces of the membranes (figs. 14, 18, 27). Lateral and basal surfaces were frequently more heavily stained than the apical surfaces, an effect particularly noticeable in the squamous pavement cells of the epithelium (figs. 18 and 27). Chloride cells exhibited these extracellular precipitates primarily along the crypt membrane (figs. 14, 19, 20, 26), where deposition was often heavy.

Because of the variety of the observed precipitates, control incubations were carried out in order to delineate bona fide sites of K⁺-NPPase activity. In order that the membrane-associated depositions could be definitively ascribed to the phosphatase component of Na⁺,K⁺-ATPase, two essential controls were independently satisfied. One control incubation included ouabain and in the other control, K⁺ was deleted from the reaction medium, since K⁺ is a cofactor necessary for the hydrolysis of NPP by the transport enzyme. As shown in figures 24-29, deposition of precipitates on the cytoplasmic surfaces of chloride cell labyrinth membranes was much reduced in both of these controls. The inclusion of 10 mM ouabain virtually abolished the reaction depositions in tissue incubated for 30 minutes (figs. 27 and 28) while sections from tissue incubated in a medium lacking KCl showed only light and scattered deposits (fig. 24). Although the morphological preservation of both controls following 30 minute incubation was comparable to that of tissue samples incubated in the complete medium, control samples incubated for 60 minutes showed substantial morphological deterioration (figs. 25 and 29), the ouabain controls more so than the controls in which KCl was deleted. This deterioration was regularly seen and was usually manifes-
ted as a decreased definition of membrane profiles and an overall disruption of tissue integrity. In these controls, a uniform peppering of fine precipitates covered mitochondria and labyrinth membranes alike, while the characteristic membrane-associated precipitates of chloride cells from tissue blocks incubated in the complete medium were notably absent (figs. 25 and 29). Precipitation within chloride cell nuclei was also usually reduced in these controls, although deletion of KCl or inclusion of ouabain had no consistent effects on the intensity of precipitation on the basal lamina or on the deposition of the amorphous precipitates occasionally associated with the extracellular surfaces of cell plasma membranes. Reduction in the intensity of nuclear precipitation is enigmatic, since Na⁺,K⁺-ATPase has never been reliably localized in nuclei. A likely explanation for the occurrence of nuclear precipitates, therefore, is that they result from diffusion of inorganic phosphate into the nuclei from cytoplasmic sites of high enzymatic activity. If such is indeed the case, inhibition of K⁺-NPPase should result in significant reduction of the intranuclear precipitates in chloride cells. This is in fact what was observed, suggesting that the nuclear precipitates observed following long incubation times are artifactual in nature.

Deletion of NPP from the incubation medium further revealed which depositions were the result of enzymatic hydrolysis and which resulted from non-enzymatic catalysis of substrate. In tissue blocks incubated in the substrate-deficient medium, fine punctate deposits were randomly distributed over mitochondria and other cytoplasmic organelles and along cell membranes (fig. 30). Precipitation within nuclei was usually sparse although the cisternae of nuclear envelopes were usually filled with precipitates. In addition, amorphous deposits associated with the extra-
cellular surfaces of plasma membranes were only slightly reduced, although staining of the basal lamina was abolished. Tissue blocks incubated in substrate-deficient medium to which 10 mM KH$_2$PO$_4$ was added after 30 minutes exhibited an identical pattern of staining except for the presence of large, granular precipitates at the free surface of the epithelium. The occurrence of these various deposits in blocks incubated in the absence of substrate suggests that they arise from nonspecific precipitation of lead and/or strontium and that they do not reveal sites of enzymatic hydrolysis.

A major complication in the localization of sites of Na$^+$,K$^+$-ATPase activity is the presence of alkaline phosphatase activity in the pinfish gill (see table II). Incubation of tissue for the cytochemical demonstration of K$^+$-NPPase activity must be carried out at pH 9.0, since the efficiency of phosphate precipitation by Sr$^{++}$ is significantly reduced at lower pH's. This incubation pH is not optimal for pinfish gill Na$^+$,K$^+$-ATPase, which has a pH optimum of approximately 7.5 (Dendy et al., '73a). The pinfish gill alkaline phosphatase, on the other hand, displays a rather broad pH-activity curve, with an optimum between pH 10.5 and 11.0 (Natalia Raphael, unpublished observations). Although the pH of the cytochemical medium is thus suboptimal for both enzymes, both Na$^+$,K$^+$-ATPase and alkaline phosphatase will hydrolyze NPP at pH 9.0. In order that deposits resulting from the activity of one enzyme not be ascribed to the activity of the other, suitable means for differentiation of the respective activities had to be incorporated in the cytochemical procedures. Both enzymes are fortunately subject to inhibition by different compounds, a fact that was exploited in the present study. The Na$^+$,K$^+$-ATPase was inhibited as previously described
by the addition of 10 mM ouabain to the incubation medium. Alkaline phosphatase activity is not affected by ouabain. It is, however, markedly reduced in the presence of either cysteine (Ernst, '75) or 1-tetramisole, a recently developed broad spectrum antihelminthic (Van Belle, '72; Borgers, '73; Borgers and Thoné, '75; Jalanka and Lindberg, '75). Addition of one or the other of these two compounds to the K⁺-NPPase reaction medium thus seemed an appropriate way by which to delineate sites of alkaline phosphatase activity in the pinfish branchial epithelium. In order to determine which compound was the more inhibitory, their respective effects on NPPase activity at pH 9.0 in pinfish gill homogenates was analyzed. Cysteine, at a concentration of 10 mM, reduced K⁺-independent NPPase (alkaline phosphatase?) activity to an average of 42 ± 7% of control values (four determinations), but it also inhibited K⁺-dependent NPPase activity by 39 ± 10%. A particularly stable analog of 1-tetramisole, 1-p-bromotetramisole oxalate (BTO) proved to be a more potent and specific inhibitor. At a concentration of 0.5 mM, BTO inhibited K⁺-independent NPPase activity by an average of 79 ± 1% while causing only a only a 14 ± 2% decrease in K⁺-dependent NPPase activity. Inhibition of the former was not increased by raising the concentration of BTO to 1.0 mM, suggesting that approximately 10% of the measured activity was insensitive to this inhibitor. These results agreed with those of Firth and Marland ('75) and Reynolds and Dew ('77), who also demonstrated alkaline phosphatase activities that were resistant to 1-tetramisole and its analogs even at inhibitor concentrations greater than 10⁻³ M. Thus although the competitive effects of alkaline phosphatase activity were greatly reduced, they were not eliminated by addition of BTO to the cytochemical medium. The
significant reduction of alkaline phosphatase activity by the inhibitor, however, allowed identification of sites of alkaline phosphatase activity. In those tissue samples incubated in the presence of 0.5 mM BTO, the only difference in the pattern of precipitation uniformly noted was a reduction in the quantity of deposits that covered the basal lamina and connective tissue. The amorphous deposits associated with the extracellular surfaces of plasma membranes were also reduced in some tissue blocks, although this was not a consistent observation. Alkaline phosphatase may contribute to these membrane-associated deposits, but their persistence in the absence of substrate suggests that they are primarily artifactual in nature. Staining of chloride cell labyrinth membranes appeared to be slightly heavier in the presence of BTO (compare figures 14-19 with 20-23), although this effect was noticeable only when reviewing micrographs of many chloride cells incubated both with and without the inhibitor.

When B-glycerophosphate was substituted for NPP in the incubation medium, scattered precipitates were observed along the extracellular surfaces of most cell membranes and heavier focal deposits covered the basal lamina and dense layers of connective tissue collagen (fig. 31). Chloride cell membranes were devoid of precipitates, however, except for a few small granular deposits at the cell periphery. In most of the previous studies in which alkaline phosphatase has been localized at the fine structural level (Hugon and Borgers, '66; Mayahara et al., '67; Seligman et al., '70; Borgers, '73; Lin et al., '76), precipitates have been confined to the extracellular membrane surfaces and to the interior of cytoplasmic vesicles, although Leonard and Provenza ('72) demonstrated abundant reaction products among the collagen fibrils of
developing bone following incubation of that tissue in the medium of Hugon and Borgers ('66). In addition, Gravis ('78) has recently demonstrated alkaline phosphatase activity in the basal lamina underlying the seminiferous epithelium.

For the purposes of the present study, the most important conclusion to be derived from the alkaline phosphatase controls is that the enzyme is not abundant in chloride cells, at least in the pinfish. Since reaction of plasma membranes for alkaline phosphatase activity reveals reaction product on the external surfaces while precipitates resulting from activity of the $K^+$-NPPase are invariably localized on the cytoplasmic sides, the respective activities can be differentiated without the use of inhibitors. It seems prudent, however, to include BTO or another suitable inhibitor in the medium when incubating tissue samples for $K^+$-NPPase localization in order to inhibit the alkaline phosphatase which otherwise competes for and thereby reduces the effective concentration of NPP available for hydrolysis by $Na^+,K^+$-ATPase.

**Isolation of Chloride Cells from the Branchial Epithelium**

General comments on the isolation

The general appearance and position of the three cell bands formed during centrifugation of suspensions from seawater-adapted gills are illustrated in figure 33. Light microscopic observation of sections from the pelleted cells revealed that band C (fig. 35) contained a high percentage (50 to 70%) of large, granular cells tentatively identified as chloride cells. These cells were usually round or oval with a mean diameter of approximately 13 μm. Preservation of morphology of the pelleted chloride cells was variable, with a mixture of well preserved
and obviously damaged cells routinely observed. Damaged cells could be identified at this level by their extensively vacuolated appearance. The proportion of cells in band C showing such damage varied considerably, but was usually around 30% of the total number of chloride cells in the sample. Since the goal of these experiments was to design a technique for the rapid isolation of chloride cells with good fine structural integrity, yields were not optimized. Haemocytometer counts, however, routinely showed around 3 x 10⁶ chloride cells per fish in band C from seawater gills. Aggregates of smaller cells were the chief contaminants in this band. Occasionally, when perfusion was incomplete, substantial numbers of erythrocytes were also observed in band C. Band A contained small cells ( <5 μm diameter) resembling lymphocytes, free cell nuclei, and unidentified fragments of cells. Band B was comprised of cells of intermediate size (fig. 34). These included many of an indeterminate nature, although mucous cells, mast cells, and an occasional rodlet cell could be identified. Chloride cells were also observed in band B, although they never accounted for more than 10% of the total cell population.

In gradients derived from the gills of fish adapted to 1/3 seawater, the only conspicuous difference in the appearance of the gradients was a substantial decrease in the amount of material in band C. Only a few chloride cells were observed in pellets from this band, usually less than 20% of the total. These cells averaged 7 μm in diameter, roughly half the diameter of isolated seawater-adapted chloride cells. Band B likewise contained some chloride cells along with many cells of other types, but as in band C they represented a minor percentage of the total. The particular density gradient system developed that was used success-
fully to isolate an enriched fraction of chloride cells from seawater-adapted pinfish appeared to be, therefore, less favorable when applied to cell suspensions from 1/3 seawater-adapted fish.

Rates of oxygen consumption of samples from band C of the seawater cell gradients varied between 1 and 3 μg-atoms oxygen/mg protein/min. This rate was slightly higher than that reported by Sargent et al. ('75) but was still quite low when compared to reported values from other isolated cell types. Obviously, some of the chloride cells experienced metabolic as well as morphological damage.

Adenosine triphosphatase activities

The specific activity of Na\(^+\),K\(^+\)-ATPase in gill homogenates from seawater-adapted pinfish was two to three times the activity measured in homogenates from fish adapted to 1/3 seawater in these experiments (table III), a finding in accord with results presented in table II. The difference in the absolute levels of Na\(^+\),K\(^+\)-ATPase activity noted between the two experiments most probably resulted from variation in the time of year of collection. Na\(^+\),K\(^+\)-ATPase levels in pinfish gill homogenates were generally lower in the summer months, when the cell isolation experiments were performed, than in the winter. Nonetheless, the relative difference in activities between fish adapted to either 1/3 seawater or to seawater was invariably the same.

The data presented in table III demonstrate that the bulk of Na\(^+\),K\(^+\)-ATPase activity in seawater gill gradients is associated with the cells in gradient band C. It is significant that bands A and B showed no enrichment of the enzyme relative to the homogenate, while the activity in the chloride cell-containing fraction was three to four-fold higher. In contrast, the lower activity of gradients derived from the gills of
TABLE III. ATPase activities in homogenates and cell fractions isolated by density gradient centrifugation from disrupted pinfish gill suspensions. Fish were adapted to the two salinities for a minimum of 12 days and activities were measured as described in the Materials and Methods. The values represent the means ± S.E.M. from four experiments in each salinity and are expressed as μmoles inorganic phosphate/mg protein/hour. Gradient band values that differ significantly (P < 0.01) from corresponding homogenate values are indicated (*).

<table>
<thead>
<tr>
<th>Adaptation Conditions</th>
<th>Fraction</th>
<th>Specific Activity Mg²⁺-ATPase</th>
<th>Specific Activity Na⁺,K⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3 Seawater</td>
<td>Homogenate</td>
<td>13.8 ± 1.3</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Band A</td>
<td>13.5 ± 1.1</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Band B</td>
<td>23.9 ± 1.7*</td>
<td>8.6 ± 1.0*</td>
</tr>
<tr>
<td></td>
<td>Band C</td>
<td>24.4 ± 1.1*</td>
<td>10.3 ± 0.2*</td>
</tr>
<tr>
<td>Seawater</td>
<td>Homogenate</td>
<td>16.1 ± 1.0</td>
<td>10.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Band A</td>
<td>17.9 ± 2.9</td>
<td>5.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Band B</td>
<td>24.5 ± 4.3*</td>
<td>9.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Band C</td>
<td>32.2 ± 2.1*</td>
<td>37.2 ± 4.4*</td>
</tr>
</tbody>
</table>
1/3 seawater-adapted pinfish was distributed between bands B and C. The decreased density of chloride cells in fish adapted to the lower salinity obviously contributed to the difficulty in obtaining a clean separation with this type of gradient system.

The specific activity of Mg\(^{++}\)-ATPase was highest in band C, although the activity in band B was also significantly higher than that measured in homogenates. These results support those previously discussed and suggest that chloride cells display high Mg\(^{++}\)-ATPase activities, most probably as a result of their numerous mitochondria.

**Fine structure of the isolated cells**

The majority of those cells recovered from gradient bands B and C exhibited excellent preservation of fine structural features. Cells of five representative types are illustrated in figures 36 through 42. In general the smaller cells such as mast cells and pavement cells withstood the rigors of the isolation procedure better than did the larger cell types. The large mucous cells seemed especially fragile, with obviously damaged cells often being observed in band B, although those recovered from band C were usually intact. Rodlet cells were only occasionally observed, but when seen were also usually in a satisfactory state of preservation.

The integrity of fine structure of cytoplasmic organelles was also well maintained in the majority of the isolated chloride cells from both salinities (figs. 40-44), although the isolation procedure caused loss of the structural polarization of organelles and the subjacent cytoplasm of the free surface that is so evident in situ. Only in rare instances were the specializations of the apical surface preserved. In-
stead, the cells usually displayed a rounded profile and a thin but
well defined cortical zone around their periphery (figs. 40-42). This
zone of cytoplasm most often separated the tubules of the labyrinth
from the enveloping plasma membrane, a situation quite different from
that observed in the intact tissue where the labyrinth membranes are
contiguous extensions of the plasma membrane. In those isolated chlor-
ide cells that exhibited good overall preservation of fine structural
features, the entire periphery of the cell could usually be scanned
without encountering a single instance of labyrinth-plasma membrane
continuity (fig. 41). A few cells showing satisfactory preservation of
morphology were observed that also exhibited many instances of contact
between tubules of the labyrinth and the plasma membrane (fig. 42, in-
set). As a general rule, however, cells showing these connections
usually also displayed swollen tubular elements, disrupted mitochondria,
and other conspicuous signs of morphological damage. This correlation
suggests that the sealing off of the labyrinth from the external medium
is possible only in those cells that escape serious damage during the
isolation procedure. Thus the decrease in profiles of plasma membrane-
labyrinth continuity in the isolated cells may be an event based on the
maintenance of metabolic integrity in the isolated cells. Whether this
sealing off of the labyrinth is total or only partial is an important
question in planning further experiments which utilize large molecular
weight probes of membrane topography.

The only discernible morphological difference between chloride
cells isolated from seawater-adapted and 1/3 seawater-adapted pinfish
gills was the increased size of the seawater-adapted cells. The diame-
ters of ten well preserved cells from each salinity were measured and
the standard deviations of the means were calculated. The mean diameter of the seawater cells, 13.2 ± 1.4 μm, was nearly twice that, 7.0 ± 0.6 μm, of the cells from fish adapted to 1/3 seawater. This corresponds to volume estimates of 1200 μm³ and 180 μm³, respectively, for the seawater and 1/3 seawater chloride cells.

The fine structure of the isolated chloride cells did not vary significantly from that observed in situ, except for the previously mentioned alterations in cell polarity and the formation of the cortical zone. Mitochondria and other organelles were in all respects identical to those seen in chloride cells in the intact tissue (figs. 43 and 44). Golgi complexes were not as frequently observed in the isolated cells, although when seen they displayed their typical in situ morphology.
DISCUSSION

The results discussed in the previous section support the current view that the chloride cell is responsible for active solute regulation in euryhaline teleosts and that Na\(^+\),K\(^+\)-ATPase is essential to these processes. Moreover, these studies have indicated that the chloride cell presents an advantageous model system for the study of such key problems in cellular biology as elucidation of the controls over cell proliferation \textit{in situ} and delineation of the mechanics of plasma membrane hypertrophy and mitochondrial proliferation.

The Adaptive Chloride Cell Response in Euryhaline Teleosts

In the pinfish as in the eel and other euryhaline species, transfer of fish from hypoosmotic or isoosmotic environments to a suitably hyperosmotic environment results in proliferation of chloride cells in the branchial epithelium. This response is predictable and easily induced, yet little is known regarding either the causal factors involved or the manner in which new chloride cells arise during this adaptive response. Studies utilizing both irradiation (Conte, '65; Monie, '68) and antibiotics (Maetz \textit{et al.}, '69) have, however, demonstrated that osmotic homeostasis in teleosts is dependent on cellular renewal in the branchial epithelium and that this renewal is more critical under hyperosmotic conditions. In the former studies, selective irradiation of the gill-buccal area in coho salmon, \textit{Oncorhynchus kisutch}, with a dose of X-rays sufficient to completely inhibit further cell division resulted in rapid salt-loading and death in those fish adapted to seawater. In siblings exposed identically but adapted to hypoosmotic media, survival
was not seriously impaired. These results suggested that turnover of the cellular elements involved in branchial electrolyte regulation was more rapid in the seawater-adapted fish, a fact later demonstrated by experiments utilizing $^3$H-thymidine to label the DNA of cells in the branchial epithelium (Conte and Lin, '67). In these experiments, cellular renewal rates in gill samples (expressed as the time for turnover of 50% of the labelled DNA) were determined to be 15.8 ± 1.0 days and 5.8 ± 1.0 days for freshwater-adapted and seawater-adapted salmon, respectively. The labelled replacement cells were localized autoradiographically and found primarily along the basal lamina of the epithelium in the interlamellar regions. The authors suggested that these cells, which were cytologically unspecialized at the time of labelling, later differentiated into chloride cells. Olivereau ('70) came to a similar conclusion on the basis of her histological studies of the branchial epithelium in European eels. She observed an increase in chloride cell numbers within a few hours after transfer of freshwater-adapted fish to seawater and a wave of cell division in the basal layer of the epithelium beginning at about two days and lasting until the seventh day after transfer. She interpreted the initial response as the differentiation of chloride cells from an unspecialized stem cell line and the secondary response as a replacement of the stem cell stock. Shirai and Utida ('70) confirmed this increase in chloride cell numbers in the gills of Japanese eels during adaptation to seawater, but they did not report cells undergoing mitosis although they made histological observations of the branchial epithelium on the first, third, fifth, seventh, and fourteenth days after transfer.

The observation that chloride cells are approximately twice as
abundant in the branchial epithelium of seawater-adapted pinfish as in those fish adapted to 1/3 seawater again suggests that proliferation from an unidentified stem cell line occurs during the adaptive response. Yet although fish were sacrificed on the fourth, fifth, and seventh days after transfer to seawater, no cells that could be definitively classified as partially differentiated chloride cells on the basis of morphological criteria were observed. Nor were mitotic figures frequently seen in these samples or in the gills of pinfish fully adapted to either experimental salinity. Thus while the single observation of a cell in mitosis in the branchial epithelium of a day-seven seawater pinfish verified Olivereau's observation of cell division in the teleost gill, a more rigorous study, perhaps utilizing pharmacological agents such as colchicine or vinblastine to arrest dividing cells in metaphase, is necessary in order to establish rates of cell division in the branchial epithelium and to identify the line of germinal cells that are the ultimate effectors responding to changes in the environmental salinity.

As previously mentioned, little is definitively known about the finer points of control of chloride cell proliferation and turnover, although mediation of salinity-related changes via the pituitary-interrenal axis has been strongly suggested (see reviews by Johnson, '73; Chester Jones, '74). Both steroid and polypeptide hormones have been implicated in the control of electrolyte transport. For example, injection of cortisol, which is normally produced by the interrenal bodies in teleosts and is controlled via ACTH secretion by the anterior pituitary, has been reported to increase Na⁺ efflux and Na⁺,K⁺-ATPase activity (Mayer et al., '67; Cyamon et al., '69; Pickford et al., '70; Epstein et al., '71; Butler and Carmichael, '72; Kamiya, '72b; Forrest et al., '73a, '73b;
Scheer and Langford, '76) and to simultaneously stimulate proliferation and hypertrophy of chloride cells (Doyle and Epstein, '72). Prolactin, on the other hand, decreases branchial Na\(^+\) efflux and Na\(^+\),K\(^+\)-ATPase activity in most teleosts (see reviews by Ensor and Ball, '72; Johnson, '73; Bern, '75), yet in some species prolactin may positively affect chloride cell development. Mattheij and Stroband ('71) reported that chloride cells were numerous in the branchial epithelium of the cichlid, *Cichlasoma bicellatum*, adapted to freshwater, but that they were sparse in these fish when they were adapted to 25% seawater, a nearly iso-osmotic medium. In 25% seawater-adapted fish treated with three injections of prolactin on alternate days and sacrificed after one week, however, chloride cells were as numerous as in the freshwater-adapted fish.

Clearly both cortisol and prolactin, and most probably other hormones, interact in euryhaline teleosts to control the active state of chloride cells in the branchial epithelium. Yet a direct effect of these endocrine agents on the chloride cell has not been demonstrated. It is not known, for example, whether cortisol acts directly on the genome of presumptive chloride cells in the germinal line or whether a more indirect interaction gives rise to the observed proliferative response. The former possibility is exciting, since if it is indeed the case it presents a model system for the study of steroid hormone induction of membrane hypertrophy in teleosts.

A portion of the results in the present study is quite significant with respect to the induction of specific membrane-associated proteins. The activity of Na\(^+\),K\(^+\)-ATPase in gill homogenates increases in concert with chloride cell hypertrophy, a fact previously established by
several studies and verified during the course of the present investigations. Other enzymes that might be involved in the adaptive response, however, have received only a modest amount of attention, and much of the information to be found in the literature is contradictory. For example, a two-fold increase in succinic dehydrogenase activity in chloride cells following transfer of eels to seawater has recently been reported (Sargent et al., '75), a not unexpected finding considering the mitochondrial proliferation commonly observed in the chloride cells of seawater-adapted fish. This result is, however, at variance with those of Conte and Tripp ('70) and Epstein et al. ('67), who reported no change and a decrease, respectively, in succinic dehydrogenase in the gills of other species. Questions also exist with regard to possible salinity-related changes in the levels of alkaline phosphatase (Cvancara and Conte, '70; this study), carbonic anhydrase (Maetz and Bornancin, '75; Mashiter and Morgan, '75), anion-stimulated ATPase (Maetz and Bornancin, '75; DeRenzis and Bornancin, '77; Van Amelsvoort et al., '77), and Mg\(^{++}\)-ATPase (Epstein et al., '67; Kamiya and Utida, '69; Lasserre, '71; Karnaky et al., '76a). Many other enzymes that might be expected to vary during chloride cell proliferation and hypertrophy have not been examined. A list of those that might be profitably investigated includes acyl transferase, adenylate cyclase, cytochrome oxidase, glycosyl transferases, and monoamine oxidase, to name only a few.

The activities of both alkaline phosphatase and 5'-nucleotidase were measured in pinfish gill homogenates during the present studies to determine whether other plasma membrane markers besides Na\(^+\),K\(^+\)-ATPase were involved in the chloride cell membrane hypertrophy that is a sali-
ent aspect of the adaptive response. The fact that neither phosphatase increased in activity following transfer of pinfish to seawater suggested two possibilities: that chloride cells contain low levels of these en-
zymes in both salinities, or that the new membrane synthesized during adaptation contains primarily Na\(^+\),K\(^+\)-ATPase and the associated lipids that comprise its microenvironment. The cytochemical localization of alkaline phosphatase favors the first possibility, although it does not rule out the second. No cytochemistry of 5\'-nucleotidase was carried out during the present studies, so little can be said about localization of this enzyme. Independent support for the second possibility can be gained, however, from the work of Sargent and Thomson ('74). They demonstrated by polyacrylamide gel electrophoresis of microsomal fractions derived from the gills of eels adapted to freshwater and to seawater that only two membrane proteins differed quantitatively when prepara-
tions from the two salinities were compared. These two proteins were identified as the subunits of Na\(^+\),K\(^+\)-ATPase, and they were increased from two to three-fold in seawater microsomes. Essentially identical results were obtained by Stewart et al. ('76) in their studies of the avian salt gland. The results of these investigations and the lack of a discernible increase in the levels of plasma membrane marker enzymes other than Na\(^+\),K\(^+\)-ATPase in the pinfish gill indicate that the hyper-
trophy associated with salt stress is accomplished through quantal addi-
tion of new membrane which contains only one protein component, trans-
port ATPase. If future investigations substantiate this suggestion, the chloride cell should prove to be an excellent model system for the study of specific protein induction. One can envision a series of ex-
periments probing the molecular events of Na\(^+\),K\(^+\)-ATPase and membrane
biogenesis in chloride cells, beginning with the initial interaction of a hormonal inducer with the germinal cell and culminating in insertion of the integral glycoprotein transport enzyme into the plasma membrane of the differentiating chloride cell.

Significance of Cell Isolation Studies to Elucidation of Chloride Cell Physiology

One factor that has hampered biochemical analysis of chloride cells is the inherent complexity of the gill. Chloride cells account for less than 20% of the total cell population that comprises the branchial epithelium, a fact that introduces an element of ambiguity into interpretation of biochemical results. One way to circumvent this problem is the development of cell isolation procedures that yield enriched populations of the transport cells. This approach has been successfully implemented in studies on other transporting epithelia, including urinary bladder (Scott et al., '74; Sapirstein and Scott, '75; Pisam and Ripoche, '76), gastric mucosa (Munro et al., '75; Romrell et al., '75), mammary gland (Kraehenbuhl, '77), and exocrine pancreas (Amsterdam and Jamieson, '72, '74a, '74b; Metz et al., '77; Williams, '77). The investigations of Kamiya ('72a) and Sargent et al. ('75) demonstrated that tissue disruption and cell isolation procedures could also be used to obtain enriched fractions of chloride cells from the branchial epithelium of teleosts. The isolated chloride cells recovered in both of these studies, however, appeared severely damaged. In the present study, an attempt was made to isolate an enriched fraction of chloride cells with acceptable fine structural integrity that would be suitable for further biochemical and cytochemical investigations. This goal was in many respects achieved, although certain reservations should be expressed concerning the prepara-
tion. This study has demonstrated that chloride cells with excellent preservation of fine structural features can readily isolated from the gills of seawater-adapted teleosts without enzymatic digestion of the tissue and that high Na⁺,K⁺-ATPase activity is associated with the isolated chloride cells. However, the low rate of oxygen consumption by the isolated cells and the frequent observation of severely disrupted chloride cells in the final preparations suggest that the isolation procedure should be substantially modified before metabolic studies on the isolated cells are attempted. Addition of amino acid and antibiotic supplements, brief enzymatic digestion, and less vigorous mechanical treatments may yield a greater percentage of viable cells. These experiments remain to be carried out. Despite these reservations, the chloride cell isolation procedure developed in the present study should facilitate further investigations of the molecular topography of the chloride cell labyrinth membranes using large molecular weight membrane probes. Although compounds such as horseradish peroxidase (mol wt 40,000), when injected into the circulatory system of teleosts, readily pass the endothelial and connective tissue barriers and rapidly penetrate the basolateral openings of the chloride cell labyrinth (Philpott, '66), larger molecules such as lectins and antibodies are ordinarily excluded at the endothelium or the basal lamina. Isolation of chloride cells should expedite diffusion of these larger membrane probes into the luminal spaces of the network of anastomosing tubules, provided that at least some of the opening from the labyrinth to the enveloping cell membrane remain patent following isolation.

The isolated chloride cell preparation should also lend itself well to combined cytochemical and biochemical studies because of the
ease with which known numbers of cells can be exposed to defined media. Moreover, use of the isolated chloride cells holds an advantage over the intact tissue in cytochemical experiments in that artifacts introduced by diffusion gradients are minimized, since components of buffers, fixatives, substrates, capture ions, inhibitors and so on are interfaced directly with the isolated cell surfaces.

Significance of the Cytochemical Localization of Na⁺,K⁺-ATPase Activity to Mechanisms of Electrolyte Transport by the Chloride Cell

Cytochemical investigations have played a major role in elucidation of the mechanisms responsible for electrolyte transport in salt secreting epithelia, as have morphological, biochemical, and physiological studies. Collation of evidence garnered from each of these avenues of approach has allowed the synthesis of a plausible model for electrolyte secretion that appears to be applicable to many transporting epithelia. This model closely fits the data derived from studies of four tissues in particular, the avian salt gland (Ernst and Mills, '77), the elasmo-branch rectal gland (Silva et al., '77b), and the teleost gill (Silva et al., '77a) and operculum (Degnan et al., '77). The two principal components of the tentative model are Na⁺,K⁺-ATPase and a carrier for the co-transport of Na⁺ and Cl⁻. In the model, Na⁺,K⁺-ATPase serves two functions; it maintains low concentrations of intracellular Na⁺ and simultaneously concentrates Na⁺ in the intercellular spaces of the epithelium. The transport enzyme would thereby create a favorable gradient for the passive influx of Na⁺ across the basolateral membranes. Coupling of this influx with the uphill movement of Cl⁻ via a neutral carrier would transport Cl⁻ into the cytoplasm of the secretory cell. The net extrusion of Cl⁻ from the epithelium could then be accomplished
by diffusion, as long as the interior of the secretory cell was electronegative with respect to the external environment. This would require no carrier-mediated transport at the apical cell surfaces. The net transport of Na\(^+\) would be accomplished via a parallel "leak" pathway; Na\(^+\) concentrated in the lumens of the basolateral invaginations would diffuse to the cell periphery and across the junctional complexes under the influence of the favorable electrical gradient afforded by net Cl\(^-\) transport.

Several operational requirements for the validity of this model have now been tested and satisfied. For example, the model predicts that the luminal or external surfaces of these epithelia are electronegative with respect to their basal surfaces. This prediction has been verified for the seawater-adapted gill (Potts and Eddy, '73; House and Maetz, '74) and operculum (Degnan et al., '77; Karnaky et al., '77). In both, the transepithelial potential difference is approximately 20 mV, with the apical surface negative. A second requirement of the model is the absolute dependence of Cl\(^-\) secretion on the presence of Na\(^+\) in the fluids surrounding the basal surfaces of the secretory cells. Substitution of choline for Na\(^+\) in the media bathing either the isolated operculum (Degnan et al., '77) or the isolated, perfused rectal gland (Silva et al., '77b) virtually abolished Cl\(^-\) efflux from either of these two epithelia, thus satisfying this requirement. The model further requires that the junctional complexes between adjacent secretory cells are readily permeable to Na\(^+\). The primary barriers to free diffusion of solutes across epithelia are the zonulae occludens, which in physiologically "tight" epithelia consist of several interwoven strands of protein that form confluent bands between adjacent cells (Friend and Gilula,
'72; Claude and Goodenough, '73; Staehelein, '73, '74; Hull and Staehelein, '76). Epithelia that allow relatively free passage of water and small solutes, on the other hand, characteristically display zonulae oculudens consisting of only one or two strands. Freeze-etch studies of the avian salt gland (Ellis et al., '77; Riddle and Ernst, '77) have shown that the zonulae oculudens in this tissue are of the latter type. The junctional complexes that unite the secretory cells in the rectal gland Bulger, '63; Komnick and Wohlfarth-Bottermann, '66) and those that link chloride cells to adjacent cells in the seawater-adapted teleost gill (Philpott and Copeland, '63; Bierther, '70; this study) also appear to be "leaky" on the basis of fine structural observations.

The best evidence for intracellular transport of Cl\textsuperscript{−}, another feature of the proposed model, is derived from a study by Philpott ('65) wherein gills from killifish adapted to seawater were fixed in unbuffered silver acetate-osmium tetroxide. The chloride cells in gills fixed in this manner displayed dense, granular precipitates along both surfaces of the apical crypt membranes. Comparison of these deposits with pure silver chloride by selected area electron diffraction revealed strikingly similar patterns, suggesting that the anions precipitated at the crypt membrane by silver ions in the fixative were halides, the bulk of which were most probably Cl\textsuperscript{−}. Petrik ('68) has since carried out similar experiments on the branchial epithelium of seawater-adapted eels with the same results.

Since Na\textsuperscript{+},K\textsuperscript{+}-ATPase figures prominently in the proposed model, evidence establishing its localization and the obligatory nature of its activity is essential. Micromolar concentrations of ouabain rapidly abolish the net efflux of both Na\textsuperscript{+} and Cl\textsuperscript{−} in the teleost gill and oper-
culum (Degnan et al., '77; Karnaky et al., '77; Silva et al., '77a) and in the elasmobranch rectal gland (Silva et al., '77b), and the inhibitor is most effective when applied to the basal surfaces of the epithelia. These experiments indicate that the enzyme is in fact localized on the basolateral membranes of the secretory cells, a suggestion substantiated by autoradiographic localization of $^3$H-ouabain binding sites (Karnaky et al., '76b, '76c; Thompson and Cowan, '76; Ernst and Mills, '77). In each of these studies, exposed silver grains were copiously located over regions of basolateral membrane amplification, while few grains were observed over the apical membranes of the secretory cells. Direct visualization of Na$^+$,K$^+$-ATPase activity via K$^+$-NPPase cytochemistry (Ernst, '72a, '72b; Ellis and Goertemiller, '74, '76; Goertemiller and Ellis, '76) has verified this localization.

In the present study, the tubules of the chloride cell labyrinth, which are simply elaborate extensions of the basolateral cell surface, exhibit copious ouabain-sensitive, K$^+$-dependent depositions. Localization of precipitates attributable to the activity of Na$^+$,K$^+$-ATPase on the cytoplasmic surfaces of labyrinth membranes further shows that the enzyme maintains its conventional orientation in the chloride cell, pumping Na$^+$ from the cell cytoplasm into the intratubular spaces. This localization definitively establishes the link between the increase in activity of Na$^+$,K$^+$-ATPase and the observed membrane hypertrophy in chloride cells during adaptation to seawater. It also supports the hypothesis for electrolyte transport previously discussed.

In addition to the reaction deposits on the cytoplasmic surfaces of labyrinth membranes, precipitates were observed on the cytoplasmic surfaces of vesicles in the crypt region and on the cytoplasmic sur-
face of the apical crypt membrane. This localization appears to be unique to the chloride cell, since staining of the luminal cell surfaces was not observed in the cytochemical studies previously alluded to on the avian and reptilian salt glands and the elasmobranch rectal gland. There seem to be, therefore, two possibilities with regard to cytochemical depositions associated with the apical membranes in pinfish chloride cells. The first is that the deposits observed in the crypt region are artifactual in nature and result from diffusion of inorganic phosphate in a manner identical to that which produces nuclear staining. While this possibility cannot be definitively ruled out, it seems unlikely, since the observed deposits are discretely localized to particular sites and do not resemble the fine deposits seen in controls.

The second possibility, of course, is that these precipitates do in fact localize sites of Na\(^+\),K\(^+\)-ATPase activity. An alternate hypothesis for electrolyte transport by the chloride cell that might explain Na\(^+\),K\(^+\)-ATPase activity on the membranes of vesicles in the apical zone has recently been advanced by Maetz and Pic ('77). In this model, vesicles in the apical region would function as shuttling devices between the tubules of the labyrinth and the crypt membrane by budding from the tubular system and transporting Na\(^+\) concentrated in the tubules of the labyrinth by Na\(^+\),K\(^+\)-ATPase to the apical surface of the epithelium. This mode of quantal transport is supported by the observations of Masoni and Garcia-Romeau ('72) and Masoni and Payan ('74) that several organic molecules including inulin and methylene blue are concentrated and excreted by chloride cells in the gills of seawater-adapted eels. Since these compounds do not readily pass cell membranes, bulk transport of solutes is clearly indicated. The frequent observation
of what appear to be vesicular fusion profiles on the crypt membranes of seawater-adapted pinfish chloride cells also supports this hypothesis as does the frequent observation of microfilaments and microtubules in this region. The latter would satisfy criteria for mechanisms of movement and direction for migrating vesicles. At least a part of the observed vesicles are, however, undoubtedly involved in secretion of the crypt mucous. Philpott ('68) has convincingly demonstrated that these vesicles in the killifish chloride cell contain a polyanionic material that stains in a manner identical to the crypt mucous when exposed to colloidal thorium at low pH. Additionally, Karnaky ('72) has demonstrated that \(^3\)H-galactose is localized in the crypt mucous of chloride cells in the pupfish within 30 minutes after intraperitoneal injection of the labelled sugar, suggesting that the mucins in the crypt are rapidly renewed. Since the renewal of plasma membrane glycoproteins has been shown in several other cell types to involve vesicular transport from the Golgi complex to the cell surface (Flickinger, '75; Michaels and Leblond, '76; Haddad et al., '77), it would not be surprising if many of the vesicles in the cytoplasm subjacent to the apical crypt membrane were engaged in this function. Since at least two types of vesicles can be delineated in the apical cytoplasm of pinfish chloride cells on the basis of morphological criteria, multiple functions for these enigmatic organelles are indicated. While the cytochemical reaction products associated with vesicles in this area in the present study provide suggestive evidence for involvement of vesicles in transport across the epithelium, it must be emphasized that this localization does not definitively establish bulk transport as the mechanism of \(\text{Na}^+\) transport. There is no reason \textit{a priori}, however, why such a transport
system could not be integrated with a paracellular shunt pathway in chloride cells. Investigations specifically designed to differentiate vesicular from paracellular pathways using metabolic tracers such as myoglobin and horseradish peroxidase should be of value in this respect.

Thus although the localization of Na\(^+\),K\(^+\)-ATPase in chloride cells, through its K\(^+\)-NPPase manifestation, has been definitively established in the present studies, further correlative investigations appear necessary in order to delineate between the possibilities suggested by this localization. It is clear, however, that the tubular extensions of the plasma membrane which comprise the labyrinth represent the dominant sites for transport activity and that the cytochemical findings presented here are consistent with the previous correlation of chloride cell hypertrophy with increased gill Na\(^+\),K\(^+\)-ATPase activity during adaptation of euryhaline teleosts to hyperosmotic environments.
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Figure 1  (1/3 seawater-adapted)

Figure 2  (seawater-adapted)

Representative 1 μm transverse sections through gill filaments after adaptation to the two experimental salinities. The cartilagenous gill ray (GR) supports the afferent (A) and efferent (E) branchial arteries and the branchial epithelium proper. Respiratory lamellae (RL) flank the filaments. Chloride cells, a few of which are indicated by arrows, are identifiable by their relatively large size and by their pale staining cytoplasm. Note that the number of chloride cells is much increased in figure 2.

Figure 1, 310X

Figure 2, 280X
Figure 3 (seawater-adapted)

A low magnification electron micrograph of the branchial epithelium. Several cell types are represented, including mucous cells (M), squamous pavement cells (P), basal cells (B), and supportive cells (S). Portions of several chloride cells (1-5) are also visible. A section of the afferent branchial artery is seen at lower left, separated from the epithelium by layers of connective tissue, smooth muscle, and a thick basal lamina.

4000X
The apical regions of two chloride cells. Figure 4 illustrates the typical appearance of the apical cell surface in chloride cells from fish adapted to the lower experimental salinity. The apical plasma membrane is folded into a complex pattern of irregular, branching microridges. In figure 5, a less common configuration is illustrated. Although the conspicuous folding of the plasma membrane is still apparent, the cell surface as a whole is invaginated to form a crypt-like pocket. The outer surface of the membranes in both cells supports a fine, filamentous glycocalyx and numerous vesicles of differing sizes and degrees of electron density occupy the cytoplasm immediately subjacent to the apical plasma membrane. Three multivesicular bodies (arrows) are apparent in figure 4, and a few microtubules (arrowheads) are seen in figure 5.

Figure 4 - 20,000X
Figure 5 - 28,000X
The apical crypt region of a typical chloride cell. Portions of two accessory cells (AC) are seen flanking the chloride cell and sending processes into the crypt that is filled with a mass of filamentous material. Insets: a) An area of the crypt membrane shows several vesicles, including one at upper left that contains a crystalline inclusion. A possible instance of vesicular fusion with the crypt membrane is also visible (arrow). b) A higher magnification view of a fusion profile on the crypt membrane. Note the microfilament which extends from the tip of a microtubule (arrow) to the membrane. c) A centriole observed in the cytoplasm just beneath the crypt membrane.

Figure 6 - 19,000X
Inset A - 33,000X
Inset B - 75,000X
Inset C - 69,000X
Figure 7 (seawater-adapted)

A field of chloride cell cytoplasm illustrating the intimate association between numerous mitochondria and tubules of the plasma membrane labyrinth. Polyribosomes and cisternae of granular endoplasmic reticulum are common in the cytoplasmic ground substance. Inset: In fortuitous tangential sections, the anastomosing pattern of the labyrinth tubules is readily apparent.

Figure 7 - 27,000X
Inset - 52,000X
Figure 8 (seawater-adapted)

An accessory cell (AC) near the free surface of the branchial epithelium. Note the decreased size and increased electron density of mitochondria in this cell as compared to those in the portion of a chloride cell (CC) that fills the lower third of the figure. The ground substance of the accessory cell is also slightly more electron dense than that of the chloride cell.

20,000X
Changes in the activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and K\textsuperscript{+}-NPPase in the gills of pinfish following transfer from brackish water (625 mOsm) to artificial seawater (●) or to 1/3 seawater (○). Points indicate the means ± S.E.M. of determinations from four fish. Assay conditions were as described in the text.
Correlation between pinfish gill Na⁺,K⁺-ATPase and K⁺-NPPase activities during the adaptation sequences illustrated in figure 9 and 10. Points represent correlation of enzyme activities from fish in seawater (●), 1/3 seawater (○), and brackish water (▲). There is a highly significant (P < 0.001) linear correlation in both experimental salinities. $r_{SW} = 0.996; \ r_{1/3\ SW} = 0.939.$
Figure 12

The change in activity of the Mg\textsuperscript{++}-ATPase in the gills of pinfish following transfer from brackish water (625 mOsm) to artificial seawater (●) or to 1/3 seawater (○). Points indicate the means ± S.E.M. of determinations from four fish. Assay conditions were as described in the text. It is apparent from a comparison of this figure with figure 9 that the increases in the activities of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and Mg\textsuperscript{++}-ATPase noted in those fish adapting to seawater do not coincide temporally.
The effects of fixation on the activity of Na⁺,K⁺-ATPase in the gills of pinfish adapted to seawater. Gills were fixed at 4°C for the periods indicated in either 2% formaldehyde (○) or 1% formaldehyde-0.25% glutaraldehyde (○) as described in the text. Activity remaining at the end of the various fixation intervals is plotted as a percentage of the activity in unfixed paired controls. Each point represents the average of two experiments. Note that the addition of glutaraldehyde to the fixative significantly increases the rate of inactivation of the enzyme.
Figure 14  (seawater-adapted)

Electron microscopic localization of $K^+$-NPPase activity in the branchial epithelium. Tissue was incubated for 60 minutes in the complete cytochemical medium with 0.5 mM BTO. Unstained section. Reaction products are localized primarily to the membranes of the chloride cell labyrinth. The membranes of the accessory cell in the upper right corner of the figure show only minor indications of activity. Precipitates are also seen on the external surface of the chloride cell crypt membrane and in nuclei.

16,500X
Cytoplasmic details from chloride cells in tissue blocks incubated in the complete $K^+$-NPPase cytochemical medium with 0.5 mM ETO for 30 minutes (top) and 60 minutes (bottom). In both figures, electron opaque reaction products are associated with the cytoplasmic surfaces of the labyrinth tubules. Deposition is considerably heavier in the section incubated for 60 minutes. In both figures, mitochondrial membranes are virtually free of deposits.

Figure 15 - 30,000X
Figure 16 - 25,000X
Figure 17 (seawater-adapted)
Figure 18 (seawater-adapted)

Portions of chloride cells from tissue incubated for 60 minutes in the complete cytochemical medium with 0.5 mM BTO. Unstained sections. In figure 17, a common apical crypt shared by at least three chloride cells is shown. Reaction products are associated with the labyrinth tubules, with vesicles in the crypt region, and with the crypt membrane. In each case, precipitates are seen only on the cytoplasmic membrane surfaces. The pavement cells that flank the crypt are devoid of precipitates, except for minor deposits along their lateral membranes. In figure 18, these extracellular deposits are abundant along the interdigitating extensions of two adjacent pavement cells. Precipitation is heavy on the labyrinth membranes of the underlying chloride cell.

Figure 17 - 18,000X
Figure 18 - 15,000X
Figure 19 (seawater-adapted)

In this detail of the crypt region, association of granular precipitates with the cytoplasmic surfaces of vesicles is readily apparent. Reaction products are also associated with both surfaces of the crypt membrane. Those precipitates associated with the extracellular surface appear to be of a more amorphous nature. Note that deposition is much lighter along the labyrinth tubules of the accessory cell at right. Incubated for 60 minutes in the complete medium with 0.5 mM BTO. Unstained section.

24,000X
Figure 20 (seawater-adapted)

A chloride cell from a filament incubated in the complete K⁺-NPPase medium lacking ETO for 60 minutes. Unstained section. Reaction product deposition on the labyrinth membranes in this cell is fairly light, but relatively uniform along the baso-apical cell axis. Most mitochondria are free of precipitates, although the nucleus exhibits significant deposition. The granular precipitates within the nucleus appear to be associated with heterochromatic regions, the nucleolus in particular being intensely reactive. Amorphous extracellular deposits are also abundant within the crypt.

17,000X
Figure 21 (seawater-adapted)  
Figure 22 (seawater-adapted)

Details at the apical and basal poles of chloride cells incubated in the complete medium lacking BTO for 60 minutes. Deposition of reaction precipitates in the apical crypt region (fig. 21) is similar to that seen in figures 17 and 19. Precipitates are associated with many of the vesicles in the cytoplasm subjacent to the crypt (arrows) and with the membranes of the crypt and the labyrinth. At the basal cell surface (fig. 22), the thin basal lamina displays a few scattered precipitates. The endothelium, however, is devoid of reaction products. Localization of precipitates exclusively to the cytoplasmic surfaces of labyrinth tubules is clearly illustrated in this figure.

Figure 21 - 23,500X  
Figure 22 - 27,500X
The nuclear area of a chloride cell incubated in the complete cytochemical medium lacking BTO for 60 minutes. Precipitation on the labyrinth membranes is light, particularly in the area immediately surrounding the nucleus. Fine granular deposits are associated with nuclear chromatin, although the nuclear envelope contains no precipitates.

28,500X
Figure 24 (seawater-adapted)

Figure 25 (seawater-adapted)

Cytoplasmic details from chloride cells in filaments incubated in the KCl-deficient medium with 0.5 mM ETO for 30 minutes (fig. 24) and 60 minutes (fig. 25). Very few depositions of reaction products are observed after 30 minute incubations. A few focal deposits are visible in the chloride cell at left, but the labyrinth tubules are essentially unreactive. In chloride cells incubated for 60 minutes in the absence of K⁺, fine precipitates line the luminal surfaces of labyrinth tubules. The precipitates associated with the cytoplasmic surfaces of membranes in cells incubated in the complete medium are absent, however. Note the deterioration of morphology following the longer incubation.

Figure 24 - 29,000X

Figure 25 - 27,000X
Figure 26 (seawater-adapted)

The crypt region of a chloride cell incubated in the KCl-deficient cytochemical medium with 0.5 mM BTO for 60 minutes. Focal deposits are associated with the extracellular surface of the crypt membrane. A few precipitates are also seen in association with cytoplasmic vesicles. The majority of the vesicles are unreactive, however, as are the tubules of the labyrinth.

23,000X
Figure 27 (seawater-adapted)

The apical crypt region in a chloride cell incubated in the K\textsuperscript{+}-NPPase cytochemical medium with 0.5 mM ETO and 10 mM ouabain for 30 minutes. Very little deposition is observed on membranes of the chloride cell labyrinth or on the cytoplasmic surface of the crypt membrane. Amorphous deposits are, however, localized to the extracellular surfaces of the pavement cell lateral and basal plasma membranes.

12,500X
Cytoplasmic details from chloride cells incubated for 30 minutes (fig. 28) and 60 minutes (fig. 29) in the K⁺-NPPase cytochemical medium with 0.5 mM BTO and 10 mM ouabain. In all filaments incubated in the presence of ouabain, no precipitates were observed on the cytoplasmic surfaces of the labyrinth tubules. As in the K⁺ controls, a fine peppering of deposits covered mitochondria and membranes after 60 minute incubations, and the quality of morphological preservation was reduced.

Figure 28 - 24,000X
Figure 29 - 26,000X
Figure 30  (seawater-adapted)

Filament incubated for 60 minutes in the K⁺-NPPase cytochemical medium lacking NPP. Fine granular precipitates are associated with the endothelial cell plasma membranes, the basal lamina, and the nuclear envelope and endoplasmic reticulum cisternae of the basal cell. The tubules of the chloride cell labyrinth and associated mitochondria also display a fine peppering of deposits.

20,500X
Figure 31 (seawater-adapted)
Figure 32 (seawater-adapted)

Figure 31 illustrates the major site of precipitation in filaments incubated in the complete cytochemical medium containing B-glycerophosphate instead of NPP. Discrete foci of reaction products are observed primarily along the basal lamina and associated with connective tissue collagen. In filaments incubated in the complete cytochemical medium without BTO (fig. 32), the appearance and localization of these precipitates is identical. The deposits in both cases are much reduced, although not eliminated entirely by inclusion of BTO in the incubation media.

Figure 31 - 17,500X
Figure 32 - 14,500X
Figure 33 represents the appearance of density gradients of cell suspensions from the branchial epithelium of seawater-adapted pinfish. The tube at left diagramatically illustrates the appearance of the Ficoll step gradient prior to centrifugation. Following centrifugation (top right), three cell bands are obtained. Figures 34 and 35 are light micrographs of 0.5 μm sections from pellets derived from gradient bands B and C, respectively. Most cells in band B are less than 10 μm in diameter. Band C contains many chloride cells, identifiable by their large size and many mitochondria.

Figure 34, 1125X

Figure 35, 1125X
Representative cell types recovered from gradient band B. The granule-containing cell at top is probably a mast cell. The cell in the bottom figure is tentatively identified as a squamous pavement cell.
Figure 38 (seawater-adapted)

Figure 39 (1/3 seawater-adapted)

Mucous cells (top) and erythrocytes (bottom) are commonly recovered in band C. Mucous cells appear to be "sticky" and are usually observed adhering to other cells, in this image a chloride cell. A few erythrocytes are usually seen in band C pellets, the quantity depending on the success of the initial perfusion.

Figure 38 - 14,000X

Figure 39 - 18,000X
Figure 40 (1/3 seawater-adapted)

An isolated chloride cell recovered from band B of the density gradient. The cell displays a rounded profile and a thin but well defined rim of cortical cytoplasm. Inset: In a few of the isolated chloride cells, the cortical cytoplasmic zone was not observed. Continuity of labyrinth tubules and the plasma membrane in one of these cells is shown at several points (arrowheads).

Figure 40 - 22,500X
Inset - 19,000X
Figure 41 (seawater-adapted)

An isolated chloride cell recovered from band C. Preservation of morphological features is excellent with little swelling of membranes or disruption of mitochondria observed. Note the absence of continuity between the plasma membrane and tubules of the labyrinth.

11,500X
Figure 42 (seawater-adapted)

An isolated chloride cell recovered from gradient band C that exhibited a well defined cortical zone around the cell periphery. Inset: Cisternae of granular endoplasmic reticulum are commonly observed in the cortical zone.

Figure 42 - 18,500X
Inset - 37,000X
Cytoplasmic details from isolated chloride cells. These micrographs illustrate the excellent preservation of organelle morphology in the isolated cells. In addition, comparison of the two figures emphasizes that the appearance of mitochondria and labyrinth tubules in chloride cells from fish adapted to both salinities is similar.

Figure 43 - 30,500X
Figure 44 - 34,500X