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Subcellular Localization of Na\(^+\), K\(^+\)-ATPase; Biochemical, Morphological, and Cell Fractionation Studies of the Pinfish Pseudobranch

by

Leslie Ann Dendy

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

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ABBREVIATIONS

ATP  adenosine-5'-triphosphate
DNA  deoxyribonucleic acid
E    cytoplasmic extract
g    gravitational acceleration at surface of earth, 2
      980 cm/sec
h    hour
H    homogenate
2+
Mg  -ATPase  magnesium-activated adenosine triphosphatase
ML   mitochondrial, light-mitochondrial fraction
N    nuclear fraction
++
Na+, K-ATPase sodium- and potassium-activated adenosine triphosphatase
P    microsomal fraction
rpm  revolutions per minute
S    soluble fraction
TCA  trichloroacetic acid
Tris tris (hydroxymethyl) aminomethane
ω    angular velocity
INTRODUCTION

Role of Na+, K+-ATPase

It appears likely from a survey of the literature that the enzyme sodium- and potassium-activated adenosinetriphosphatase (Na+, K+-ATPase) is ubiquitous in animal cells (62). Related enzymes may well serve similar purposes in plant cells (107). The name of the enzyme refers to its special requirement for Na+ and K2+ ions; like most other known ATPases, it also requires Mg2+ for activity. Unlike other enzymes activated by monovalent cations, including a halophilic cell membrane ATPase activated by Na+ or K+ (53), Na+, K+-ATPase is thought to act on the ions which activate it. Its basic function is believed to be the movement of monovalent cations across the cell membrane against electrical and chemical gradients. Energy for this process, an example of "active transport", is probably derived from the hydrolysis of the substrate ATP (62, 121). The underlying molecular mechanisms are unknown, although many models have been proposed.

There are two general purposes believed served by the transport of monovalent cations across cell membranes. The first is the creation and maintenance of ionic gradients; all types of animal cells are known to have much lower concentrations of Na+ and much higher concentrations of K+ within the cell than are found in the outside environment or extracellular fluids (109, 133). Many cellular functions are dependent on these gradients, including osmotic regulation, protein synthesis and other enzymatic systems, excitation in its many forms, and the uptake of metabolites such as sugars and amino acids (109, 112,
131, 140, 54). The second general function of ion transport involves the secretion of salt solutions by specialized epithelial tissues (140, 97). These include osmoregulatory organs, such as certain invertebrate glands, elasmobranch rectal gland, fish gills, amphibian skin, reptile and bird salt glands, and vertebrate kidneys. Also included are non-osmoregulatory organs such as mammalian gall bladder and the ciliary body of the eye. Clearly, if Na, K-ATPase is the agent underlying the ion movements, it is a vital enzyme on several counts.

Evidence for ion transport function of Na, K-ATPase

Considering the apparent importance of the enzyme, it is unfortunate that the evidence that Na,K-ATPase is in itself the agent which transports the Na and K (or, in some cases, possibly just Na) is rather indirect. This is understandable, since ion transport is a complicated phenomenon probably dependent on the arrangement of molecules within and adjacent to the cell membrane. This arrangement is itself poorly understood and it may well vary with time and conditions. In addition, it is exceedingly difficult to analyze the components of such a complex system without destroying the functional integrity of the system and introducing artifacts. Likewise, Na,K-ATPase activity is apparently dependent on the organized lipoprotein structure of the particulate preparations in which it is studied (121). At present one can only compare the properties of Na,K-ATPase in cell fragments with those of ion transport by intact tissues. There are three voluminous groups of evidence along these lines:
1) Parameters of ion transport can be measured under varying experimental conditions in several biological systems, particularly the mammalian red blood cell ghost, and to a lesser extent squid axon and multicellular tissues such as kidney, toad bladder, brain, and avian salt gland. Studies of these systems have shown that their ion transport displays a number of biochemical properties in common with $\text{Na}^+, \text{K}^+$-ATPase from any source (62, 121). Both the enzyme and transport require the presence of both $\text{Na}^+$ and $\text{K}^+$ for maximal activity, and in some cases it has been shown that $\text{Na}^+$ must be inside the cell and $\text{K}^+$ outside. Both specifically require ATP; other nucleotides are much less effective. Both are inhibited fairly specifically by cardiac glycosides such as ouabain; the similarities are extended to the concentrations which inhibit, the variable effects of different glycosides on different tissues, and the protective effects of $\text{K}^+$. Both ion transport and $\text{Na}^+, \text{K}^+$-ATPase are inhibited by $\text{Ca}^{2+}$. This sort of evidence is considered convincing, or at least highly suggestive, where it exists. The red blood cell, most easily studied, unfortunately cannot be considered a very "typical" system for study, since it has extremely low ion transport rates and $\text{Na}^+, \text{K}^+$-ATPase activity, has no nucleus and few other organelles, and has a cell membrane which is unusual at least in its resistance to breakage.

2) There is some correlation between the ion transport rates and $\text{Na}^+, \text{K}^+$-ATPase activities of different tissues. Tissues with greater transport rates tend to have greater $\text{Na}^+, \text{K}^+$-ATPase activities, and this correlation holds over a range of activities whose endpoints differ by several orders of magnitude (14, 15). There is also a
correlation between Na⁺, K⁺-ATPase activity and transport rate in individual tissues whose transport rates can vary in response to environmental or hormonal conditions, particularly osmoregulatory tissues (see, e.g., 34, 62). For example, adaptation of teleost fish to greater salinities is accompanied by parallel increases in salt secretion by and Na⁺, K⁺-ATPase activity of the gills (33, 60, 56).

The principal objection to such evidence is that increased ion transport may require a stepped-up metabolism in general, involving increases in a number of different enzymes rather than just the transport agent.

3) It is generally believed that ion transport occurs at the cell membrane (61, 17), and there is a good deal of evidence consistent with the localization of Na⁺, K⁺-ATPase in the cell membrane. (The alternative hypothesis, that ion distributions are the result of selective adsorption to macromolecules throughout the cell, still has adherents but no longer can account for all the evidence nearly as well (133, 61, 17)). It should be recognized that the enzyme does not necessarily have to be found entirely in the cell membrane for it to be equated with the transport agent; it might have intracellular roles too, and at the time of its synthesis it could easily be located elsewhere. Nevertheless, one would expect the transport enzyme to be largely in the cell membrane. On the other hand, one could not assume that it would be distributed homogeneously through the entire cell membrane. It is especially easy to visualize asymmetric distributions of ion pumps in the case of secretory epithelia, which move monovalent cations primarily in one direction across the cell layers, and which
have asymmetric ultrastructure to match. Much effort has been put into the localization of Na, K$^+$-ATPase in various tissues by various methods; the fact that localization attempts are still being made indicates that a thoroughly satisfactory procedure has yet to be developed. The two general methods used to study enzyme localization are cytochemistry and cell fractionation. Ideally cytochemistry should provide more conclusive results, since the organization of subcellular components is left intact and the sites of reaction products should be unambiguous. ATPase cytochemistry has been applied to numerous tissues, including teleost pseudobranch (71). However, attempts to localize Na, K$^+$-ATPase cytochemically have encountered numerous special difficulties (see 35) in addition to the usual ones such as product diffusion and variable inhibition of enzyme activities by fixatives. Reaction deposits have been seen over cell membranes and almost everywhere else, and one cannot be certain whether any of these represent Na, K$^+$-ATPase rather than some other Mg$^{2+}$-ATPase.

Fractionation is more reliable in the sense that one can be sure one is measuring Na, K$^+$-ATPase, but it has never been possible to identify all the components of a fraction with absolute certainty. The simplest type of fractionation is the removal of cytoplasm from intact cell membranes of isolated cells either osmotically, as with mammalian erythrocytes, or mechanically, as with squid axons and other nerve cells. Red blood cell ghosts (see references in 121), squid axon sheaths (13), and microdissected nerve cell membranes (20) all contain at least part of the cellular Na, K$^+$-ATPase; but at least in the case of the
axon, it is known that the technique does not produce a free cell membrane, since some cytoplasmic material remains adhered to the inside and the sheath also includes an outer layer of Schwann cells (120a). More evidence has been obtained from centrifugational fractionation of homogenized tissues. Distributions of Na$^+$, K$^+$-ATPase among nuclear, mitochondrial, microsomal, and soluble cell fractions have been obtained for brain (15, 66, 25, 114, 139), liver (15, 1b), intestine (100), and kidney (66, 137). There is little or no enzyme in the soluble fractions, so one can conclude that it is particulate, or at least tightly adsorbed to particulate material. The distribution of Na$^+$, K$^+$-ATPase between the three (or more) particulate fractions varies markedly with different tissues, but it is usually considered consistent with the distribution of cell membrane fragments.

Incomplete fractionations have demonstrated Na$^+$, K$^+$-ATPase in microsomal fractions from many tissues (see, e.g., references in 62), including specialized transport epithelia (33, 87, 124). The contents of these fractions are uncertain at best, especially when obtained from such heterogeneous tissues as kidney or teleost gill. Several plasma membrane-rich fractions, usually derived from nuclear fractions, have been shown to contain Na$^+$, K$^+$-ATPase in high specific activity, although low recovery. The most reliable such fractions have been obtained from rat liver (36, 6, 32); fragments of liver cell plasmalemma can be definitely identified in the fractions by the presence of desmosomes and microvilli. Other sources of "plasma membrane" fractions with Na$^+$, K$^+$-ATPase activity have been rat intestine (100) and kidney (40), rabbit kidney (137), calf thyroid and adrenal glands (132), and dog
heart (126). In summary, evidence for localization of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in the plasma membrane is reasonably good for mammalian erythrocytes and liver. Elsewhere the evidence can only be considered consistent with cell membrane localization, and this includes the salt-secreting epithelia, which have the highest known Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activities.

**Use of pseudobranch**

The tissue studied here was the pseudobranch of the pinfish, *Lagodon rhomboides*. Pseudobranch is an unusually homogeneous tissue, and it was anticipated that this homogeneity would facilitate interpretation of the results of fractionation. The pseudobranch ("pseudo gill") is a small organ located anterior to the gills, behind the dorsal edge of the operculum or gill cover. Like gills, pseudobranchs are paired, with one on each side of the head. Pseudobranchs are found in most teleosts and also some elasmobranchs and other non-teleost fish (45).

Pseudobranch tissue is closely related to gill in a number of ways, although it is much simpler in construction. For example, the two tissues have common embryological origins: the pseudobranch is derived from the branchial arch just anterior to the first "functional" gill arch (45, 52). The gross appearance of the pseudobranch varies with species (45), but it frequently has gill-like filaments, and it is always as richly vascularized as gill. The predominant cell type of all forms of pseudobranch has a fine structure highly similar to that of the "chloride cells" of gill (52, 19). This is characterized by many mitochondria closely associated with numerous tubular invaginations.
of the cell membrane. Minute projections have been seen on the extracellular surfaces of these tubules in both gill and pseudobranch by Ritch and Philpott (106). The chloride cells are thought by many to be the site of salt secretion by gills in a sea water environment (94, 118), despite past controversy on the subject (reviewed in 41, 88, 118, 94). Like gill also, pseudobranch has a very high $\text{Na}^+$, $\text{K}^+$-ATPase activity, which increases with adaptation of the fish to greater salinities (33). It thus seemed reasonable to study the pseudobranch as a kind of model for the chloride cell component of gill, avoiding the structural and functional complexities of gill, as well as in its own right. It was also hoped that the tiny projections on the cell membrane extensions could be investigated further, since these could conceivably have a role in ion transport.

The actual function of the pseudobranch is still uncertain. It has not been studied extensively, as it tends to be overshadowed by the gills because of their vital respiratory and osmoregulatory functions (and sheer size). The pseudobranch probably does not share the respiratory function of the gills, since blood reaching it is already oxygenated (19, 89). It may share the ion transport function, since it shares two properties with known salt-transporting tissues, such as elasmobranch rectal gland, teleost gill, reptile and bird nasal salt glands, mammalian kidney, and certain insect organs (97). Second, the pseudobranch cells show remarkable extension of their surface area by plasma membrane invaginations and an abundance of mitochondria, which are presumably
needed to supply the energy for ion transport by such cells (52, 19). This type of ultrastructure is characteristic of the salt-transporting tissues (93, 97). The form of the cell membrane extensions varies with different tissues; as discussed by Diamond and his colleagues, the shapes and dimensions may regulate the concentration of the secreted salt solution (26, 27). One problem with the idea that pseudobranch has an ion-transport function is the fact that some pseudobranchs apparently lack a free surface to pump salt to after removing it from the blood (90), but this could be an oversimplified view. Also removal of pseudobranchs seems not to impair the ability of fish to pump out excess salt (89, 90), but the gills of these fish could have adjusted to keep the transport rate normal.

Other possible functions which have been suggested for the pseudobranch include the secretion of carbonic anhydrase (19, 89), regulation of oxygen tension in blood supplying the retina (2), synthesis of a chromatophore-contracting hormone (89), and sensory monitoring of concentrations of CO₂ or O₂ (70) or ions in the blood. Naturally, the pseudobranch could have more than one function. Since the roles of + + Na, K -ATPase are already known to be many, this uncertainty in function was not considered a real handicap in the use of pseudobranch for localization studies.

Scope of study

The principal object of this study was to determine the subcellular + + localization of the enzyme Na, K -ATPase in pinfish pseudobranch. In a larger sense, the results were intended to contribute to the pool of
evidence regarding localization of Na⁺, K⁺-ATPase in animal cells in general. This enzyme is implicated in many vital cell functions involving ion transport, and its localization is of prime importance in the analysis of its specific role. An especially important deficiency in such evidence has been the lack of precise information on the localization of Na⁺,K⁺-ATPase in a number of epithelial tissues specialized for transport of salt solutions on a tissue level. The simultaneous occurrence in these tissues of high ion transport rates, high levels of Na⁺, K⁺-ATPase activity, and highly amplified cell membranes is suggestive, but inconclusive in itself. The results of the present study were intended to help remedy this deficiency.

The major difficulty with localization of enzymes by cell fractionation is the ambiguity of the cellular origin of fraction components, particularly membrane fragments. Consequently, the first step in this study was selection of a tissue with the simplest possible construction: one major cell type consisting of only a few kinds of subcellular components, whose fragments were readily identifiable. The Na⁺, K⁺-ATPase of this tissue needed to be biochemically typical to help justify extension of localization results to other tissues. Study of this tissue was then to involve centrifugational fractionation of homogenates, determination of fraction contents by electron microscopy and assays of marker enzymes, and analysis of the distribution of Na⁺, K⁺-ATPase among the fractions. If necessary, attempts to resolve ambiguities in the results would be made by density gradient centrifugation of fractions.
In the following it will be shown that:

1. Pinfish pseudobranch is unusually simple in structure, and thus well suited to cell fractionation studies.

2. The biochemical properties of pseudobranch Na, K-ATPase are quite similar to those of Na, K-ATPases from many other sources, including salt-secreting epithelia.

3. Fragments of pseudobranch cell membranes can be readily identified in fractions on the basis of three criteria. First, they are very numerous, as expected from the abundance of cell membrane invaginations in situ. Second, they are largely tubular, as expected from electron microscopy of intact tissue. Third, when negatively stained they show the characteristic regular projections previously seen on the cell membrane extensions in situ (106). These fragments can be easily distinguished from most other fraction components, including mitochondria and their fragments, which are the other major components of fractions.

4. The distributions of marker enzymes are consistent with the conclusions about fraction contents based on electron microscopy.

5. The distribution of Na, K-ATPase parallels that of cell membrane fragments, and its specific activity is greatest in that fraction which comes closest to being a pure cell membrane fraction.

6. Mitochondrial-linked enzymes and Na, K-ATPase activity of the mitochondrial fraction behave very differently in density gradient centrifugation. The possibility that the high Na, K-ATPase activity of this fraction is associated partly or entirely with the mitochondria is thus effectively eliminated.
MATERIALS AND METHODS

Fish

Pinfish (*Lagodon rhomboides*) were caught individually in the brackish water of Offat's Bayou at Galveston, Texas. The fish ranged from 10 to 20 cm. in length. They were maintained in aerated pools of full-strength artificial sea water, made with Seven Seas Marine Mix, usually for periods less than or equal to one week. The fish had no difficulty adapting to full-strength sea water directly, and it was observed that they could be kept in captivity in good condition for at least several months.

Chemicals

Most chemicals were obtained from Sigma Chemical Co., Fisher Scientific Co., or J.T.Baker Chemical Co. Cytochrome c was Sigma Type III from horse heart. The ATP usually used was the "sigma" grade of disodium salt from Sigma; Tris ATP from Sigma was used as noted in experiments where the exact sodium concentration was critical.

Light and Electron Microscopy

1) Whole tissue

Pseudobranchs, either nearly whole or cut into pieces a few millimeters on a side, were fixed in 3% glutaraldehyde in Millonig's phosphate buffer pH 7.4 (80). For light microscopy, nearly whole pseudobranchs were fixed for 3-1/2 hours at room temperature and then about 4 days in the cold, and rinsed with Millonig's buffer. They were dehydrated overnight in 1:1 methylcellosolve: absolute ethanol, 2 x 12 hours in n-propanol, 2 x 12 hours in n-butanol, and 3 x 3 days in Ruddell's methacrylate solution A (110). Then the tissue samples were placed
under pressure (200 p.s.i. N₂) for 20 hours in Ruddell's methacrylate solution B (110), placed in the complete Ruddell mixture at -10°C for 6 hours, thawed, and polymerized, according to the unpublished procedure of Dr. E. Jay Wheeler. For light and electron microscopy, small pieces were fixed in the buffered glutaraldehyde for 90 minutes, rinsed with buffer, postfixed in buffered 1% OsO₄ for 90 minutes, rinsed with water, dehydrated with ethanol and propylene oxide, and embedded in Epon (74). An LKB Pyramitome was used to cut thick sections, and a Sorvall Porter-Blum MT-2 microtome for both thick and thin sections. Thick sections were stained with various common light microscopic stains, including 1:1 methylene blue - azure II (105a), periodic acid-Schiff (73a), and Harris hematoxylin (73a). Thin sections, about 500 Å thick, were stained with uranyl acetate and lead citrate (104). Preparations were examined with RCA EMU-3 and Philips EM-200, Type PW6000 electron microscopes.

2) Fraction pellets

Thin uniform pellets of fraction material, fixed with phosphate-buffered 1.5% glutaraldehyde, were formed by filtration under pressure on millipore filters, as described by Bauduin, et al. (8). This method produces pellets in which the distribution of different types of particles is much more uniform than is the case for pellets formed by centrifugation. The pellets, protected with overlaying pellets of blood cells, were rinsed in sucrose-phosphate buffer, postfixed with buffered 1% OsO₄, rinsed, and dehydrated in a graded series of ethanol solutions. The millipore filters were dissolved in propylene oxide,
and the pellets embedded in Epon. They were sectioned and stained as above for electron microscopy.

When ruthenium red was used, purified ruthenium red (75) was added to all the solutions up to and including the osmium tetroxide in a concentration of 0.067%. The buffer used was 0.067 M. cacodylate pH 7.4, and the concentration of OsO₄ 1.67%. Otherwise pellets were prepared as usual. Sections were not stained.

3) Negative staining

Unfixed fractions were stained with 3% ammonium molybdate, pH 7.0 - 7.1, as described by Munn (82) and Muscatello and Horne (83), or with 2 or 3% potassium phosphotungstate pH 7.0, as described by Parsons (92). Several staining techniques were tried. The best results were obtained by transferring the sample with a needle to a small container of stain, placing collodion- and carbon-coated copper specimen grids face down on the resulting surface film, picking the grids up and draining away excess fluid with filter paper. Whole fresh pseudo-branch was prepared for negative staining by briefly mashing it with fine forceps to break open the cells.

Tissue homogenization

Pseudobranchs, averaging 10 mg. each in weight, were excised from pinfish after severing the spinal cord. They were blotted and trimmed at the base to remove connective tissue and the large blood vessels of the branchial arch, leaving the free filaments. For most fractionations pseudobranchs from 30 to 45 fish were pooled; excised pseudobranchs
were kept in ice-cold 0.3 M sucrose during the dissection. The trimmed pseudobranchs were weighed and homogenized in ice-cold 0.3 M sucrose with three slow strokes of a Potter-Elvehjem homogenizer (96) with a motor-driven teflon pestle. The electron microscopic observations suggest that this procedure breaks open most cells (except blood cells), but is gentle enough to keep almost all mitochondria intact. The concentration of homogenate was generally 1/25 gm. pseudobranch/ml.

Gill filaments were homogenized by the same method. Pinfish blood was obtained from the tail, scraped free of scales, and was homogenized in ice-cold 0.3 M sucrose containing 5.26 mM EDTA, pH 7.4, to prevent clotting. The EDTA did not interfere with subsequent enzyme assays.

**Fractionation by differential centrifugation**

All procedures were performed at 0-4°C. The basic centrifugation scheme was that of de Duve, et al. (30). Except in the case of preliminary rat liver studies, the M and L (mitochondrial and light mitochondrial) fractions were collected as one, referred to as ML. An International Centrifuge was used for speeds of 2300 rpm or less. A Beckman Spinco L2-65B Ultracentrifuge with type 65 anglehead rotor was used for greater speeds. Ten milliliters of homogenate were centrifuged at 2300 rpm for 10 minutes (10,000 g-min.); the resulting pellet was washed twice with 0.3 M sucrose and resedimented at 1800 rpm for 10 minutes (6000 g-min.). This pellet constituted the nuclear (N) fraction. The supernatant and washings were centrifuged at 25,000
rpm for 8 minutes 3 seconds (280,000 g-min.). This second pellet, washed once and resedimented at the same speed, constituted the ML fraction. The supernatant and washing from the second stage were centrifuged at 65,000 rpm for 16 minutes 37 seconds (3.3 x 10^6 g-min.). The pellet, washed once, constituted the microsomal (P) fraction. The final supernatant and wash constituted the soluble (S) fraction. Pellets were resuspended in 0.3 M sucrose.

Fresh fractions were used for assay of cytochrome c oxidase and for electron microscopy. Aliquots of fractions were deep frozen for assays of the other enzymes, which were found to be stable with respect to freezing. Samples were frozen and thawed only once to avoid detrimental or non-reproducible effects of repeated freeze-thaws.

Assays

All assays were checked to ensure that the final values obtained, usually as optical densities, were directly proportional to the concentration of the component being assayed. Appropriate blanks and standards were included in each assay. Inorganic phosphate was assayed by the method of Fiske and Subbarow (39), with minor modifications.

1) Adenosinetriphosphatases

The final concentrations of reagents in the reaction mixtures were typically 3 mM ATP (disodium or Tris salt), 3 mM MgCl_2 · 6H_2O, 92 mM Tris buffer pH 7.2 at 37°C. (7.5 at 25°C.), 100 mM NaCl, 20 mM KCl, and 0.25 mM neutralized EDTA, with or without 0.1 mM ouabain octahydrate. Activity without ouabain is referred to as total ATPase, that with 2+ ouabain as Mg ATPase, and the difference (ouabain-sensitive ATPase)
as Na, K-ATPase. Omission of NaCl and/or KCl from the medium reduced the ATPase activity to the same extent as did addition of ouabain. The reaction was run at 37°C for 15 minutes, started by addition of enzyme and stopped by addition of ice-cold trichloroacetic acid to give a final concentration of about 7% TCA. Precipitated material was removed by filtration or centrifugation, and the filtrates were assayed for inorganic phosphate. Samples were kept cold until the start of the phosphate assay to minimize acid hydrolysis of ATP. The ATPases were found to be stable frozen for many months.

2) Cytochrome c oxidase

The assay method of Copperstein and Lazarow, as applied by Appelmans, et al. (36), was used with minor modifications. The oxidation of cytochrome c was followed by continuous monitoring of the decadic logarithm of the optical density at 550 nm. by a Beckman-Gilford spectrophotometer coupled to a Photovolt Linear-Log Recorder. Three milliliters of cytochrome c solution, containing 0.054% (w/v) horse cytochrome c, 0.03 M phosphate buffer pH 7.4, and 1 mM. EDTA pH 7.4, were placed in each of two cuvettes. This solution was almost completely reduced just before use with sodium dithionite. The cytochrome c in the blank cuvette was oxidized with potassium ferricyanide. One-tenth milliliter of enzyme preparation, diluted just before use with 1 mM phosphate buffer containing 1 mM. EDTA and 0.01% (w/v) Triton X-100, was added to both cuvettes. The reaction was allowed to continue for about 2-5 minutes. The slope of the plot of log O.D. vs. time,
proportional to $-d \ln [S] \ dt$ (where $[S]$ is the substrate concentration), was used as a measure of enzyme activity.

3) Phosphoglucomutase

The assay was modified from that of Najjar (84). Samples were pre-incubated about 20 minutes at $0^\circ$C. in a solution with final concentrations of 40 mM imidazole buffer pH 7.5 and 1 mM MgCl$_2$. The reaction was carried out at $37^\circ$C. for 60 minutes in a total volume of 1 ml. The final concentrations of reagents were 4 mM glucose-1-phosphate pH 7.5, 5 x $10^{-6}$ M glucose-1,6-diphosphate, 4 mM MgCl$_2$, and 40 mM imidazole buffer pH 7.5. The reaction was stopped with 1 ml. 5N H$_2$SO$_4$, and hydrolysis of acid-labile phosphate (including glucose-1-phosphate) was completed by placing the tubes in a boiling bath for 5 minutes. The solutions were filtered, and inorganic phosphate was determined. Since the product, glucose-6-phosphate, is not acid-labile, enzyme activity was obtained from the decrease in acid-labile phosphate. With standard reaction conditions and enzyme concentration the activity of phosphoglucomutase was found to remain constant for at least 60 minutes. The activity apparently decreased gradually over a period of weeks in the frozen state; freezing itself did not decrease activity relative to a fresh preparation. Dilution of the enzyme down to 2.5 mg. pseudobranch/ml. before or after freezing had no inhibitory effect.

4) Monoamine oxidase

The final concentrations of reagents in the 1 ml. reaction volume were 3 mM benzylamine HCl and 0.05 M sodium phosphate buffer at
pH 7.6. The reaction was carried out at 37°C. for 6 hours, and stopped by precipitation with Ba (OH)\textsubscript{2} and ZnSO\textsubscript{4} (136). The optical densities of the supernatants were measured at 250 nm. as a measure of the concentration of product benzaldehyde.

5) Acid phosphatase

This assay was slightly modified from that of Appelmans et al. (36). Final concentrations in the 2 ml. reaction volume were 0.05 M. \(\beta\)-glycerophosphate pH 5, 0.05 M. acetate buffer pH 5, and 0.1% Triton X-100 detergent (the latter for releasing the enzyme from lysosomes). The reaction was carried out at 37°C. for 30 minutes, and stopped with 10 ml. of 8% trichloroacetic acid. Solutions were filtered and assayed for inorganic phosphate.

6) Protein

The Lowry Method (73b) was used, with bovine serum albumin as a standard.

7) Deoxyribonucleic acid

The assay of Bonting and Jones (16) was modified. Fractions were extracted with cold trichloroacetic acid, ethanol, and ether. They were then dried and redissolved with NaOH at 37°C. for one hour. The indole reaction was carried out, and the optical densities of the final solutions were measured at 490 nm., using micro-cuvettes.

8) Glucose-6-phosphatase

The assay was basically that of de Duve et al. (30). The 1 ml. reaction volume contained 0.04 M. glucose-6-phosphate pH 6.5; the substrate acted as its own buffer. The reaction was run at 37°C. for
20 minutes and stopped with 5 ml. 8% TCA. Phosphate was determined in the filtrates.

9) Hemoglobin

The assay was based on the method of Snell and Snell (125). Tissue homogenates were hemolyzed with water to put the hemoglobin into solution, and particulate material was removed by centrifugation at 65,000 rpm for 16.5 minutes. Two milliliters of diluted blood or tissue samples were mixed with the standard benzidine reagent. Then 0.5 ml. of 1% \( \text{H}_2\text{O}_2 \) was added to the samples, the tubes were put in an ice bath, and the optical densities at 660 nm. were read after 35 minutes.

**Density gradient centrifugation**

All procedures were carried out near \( 2^\circ \text{C} \). Linear continuous sucrose gradients were prepared with a Beckman Density Gradient Former with a modified mixing chamber (23b). Shallow gradients, ranging approximately from 1.038 to 1.076 gm/ml at \( 2^\circ \text{C} \), were used. Fraction material was suspended in the heavy and light starting solutions in equal concentrations so that it would be distributed homogeneously throughout the final gradient. About 4.5 ml. of gradient was made in 1.27 cm. \( \times \) 5.08 cm. polyallomer centrifuge tubes.

Rate sedimentation centrifugation was performed with a SW65 L Ti swinging-bucket rotor in a Beckman 12-65B Ultracentrifuge modified (23b) to allow slow manual acceleration and deceleration at speeds below 500 rpm. A Beckman \( \omega^2 t \) Integrator Accessory and a Photovolt Linear-Log Recorder for continuous monitoring of slow speeds were also
attached. Acceleration and deceleration below about 300 rpm were 
maintained at about 10 rpm/min., between 300 and 500 rpm at about 
50 rpm/min. Above 1000 rpm automatic acceleration and deceleration 
without brake were employed. With a plateau speed of 7000 rpm, the 
timing of centrifugation was designed to produce a total \[ W = \int_0^T \omega^2 dt \] (29) of \( 24 \times 10^7 \) rad /sec. Total time in the centrifuge was 
approximately 50-60 minutes.

After centrifugation, fractions were collected from gradients 
with an Isco Model D Density Gradient Fractionator adapted to cut off 
the bottom of the centrifuge tube which contains the pellet (23b). 
The gradient above the knife was pushed up through a glass cone from 
below with a slow stream of (liquid) Freon 113 and collected in pre-
weighed vials from a tube connected to the top of the cone. The pellet 
and remaining gradient were removed from below the knife and suspended 
in 0.3 M. sucrose.

Densities of fractions were determined by placing sample drops in 
a vertical column containing a continuous gradient of petroleum ether 
and o-dichlorobenzene. This gradient was made with a Glenco gradient 
maker, kept at 2 C., and calibrated with drops of standard KI 
solutions. Positions of drops in the column were measured with a 
Gaertner Cathetometer. Volumes of fractions were determined from 
fraction weights and densities. The initial volume of the pellet 
fraction was determined by measuring the capacity of the severed tube 
bottom with a pipette.
RESULTS

Microscopy of pseudobranch

The pinfish pseudobranch is about 1 cm. in length and has the appearance of a comb, with thin gill-like filaments ("teeth") extending from a base of vascular and connective tissue. The histology of pseudobranchs from other teleosts has been described (45, 52, 19, 106), and the present observations on pinfish pseudobranch are quite similar. Preliminary observations on pseudobranchs from three other marine teleosts were also entirely similar, so that there is no reason to consider the pinfish organ atypical.

Figure 1 shows portions of several filaments and their connection to the vascular base. This base was always trimmed away before homogenization and discarded. Each filament has a thin cartilage supporting rod and blood vessels in its core. Around this core are arranged layers of pseudobranch cells and sinusoidal blood spaces, forming "leaflets" or "platelets" homologous to the respiratory leaflets of gill filaments. The leaflets may project freely from the filament core or may be fused to one another, so that pinfish pseudobranch is apparently intermediate between two of the distinct types of pseudobranch described by Goodrich (45). Both fused and unfused areas are visible in Figure 1. The overall appearance of the filaments is homogeneous, the major components being pseudobranch cells and red blood cells. A small number of mucus cells were observed in certain areas.

Figure 2 shows a portion of a filament at higher magnification. The large, granular pseudobranch cells, which dominate the epithelium,
are covered at the outer edges of leaflets by squamous "pavement" epithelial cells. The blood space is supported by "pilaster" cells, whose thin extensions form the endothelial lining of the blood space. This space is continuous with blood vessels in the filament core.

The fine structure of the pseudobranch cells can be seen in Figures 3-8. The most prominent features of the cells are the large number of mitochondria and the tubular membrane arrays in close proximity to them. These tubules have been shown to be extensions of the cell membrane (19). Aside from nuclei, other organelles such as lysosomes, Golgi complexes, and granular endoplasmic reticulum are rare. Nuclear membrane fragments would be expected to contribute to particulate fractions, since it is difficult to keep nuclei intact during homogenization and fractionation, but it is clear from the micrographs that the contribution of such fragments would necessarily be very much less than that of cell membrane fragments, simply because of the much greater surface area of the latter. Glycogen deposits exist in pseudobranch cells in variable amount; they appear after meals and disappear during fasting. The pilaster cells contain little besides their nuclei. The squamous epithelial cells contain a few vesicles and membranous organelles, possibly involved in synthesis of the glycocalyx. Thus the major subcellular components of the pseudobranch are mitochondria and the infolded plasma membranes of pseudobranch cells.

Abundant membrane extensions and mitochondria are also found in the cells of a number of epithelial organs which have in common the
ability to transport electrolyte solutions across the cell layers against concentration and/or electrical gradients. These include plant salt glands, blowfly rectal papillae, elasmobranch rectal gland, teleost gill, reptile and bird nasal glands, and vertebrate kidney. The cells of the pseudobranch are most like the "chloride cells" of the gills, buccal epithelium, and surface epithelium of teleost fish. Harb and Copeland (52) concluded that there were two distinct cell types in flounder pseudobranch, both related to chloride cells. The more numerous "pseudobranch-type" cells had no free surface exposed to the environment, and possessed very regular parallel arrays of the tubular membrane extensions. A smaller number of "chloride-type" cells each had an exposed apical crypt filled with granular material, and their tubules were irregularly branched, so that these cells apparently did not differ at all from their namesakes in gill.

The situation in pinfish pseudobranch appears to be more complex. The organization of tubules is much more variable, as shown particularly in Figure 7, and it is not possible to divide cells into two distinct types on this basis. In fact, tubule form often varies considerably within single cells. An example of a cell with the apical crypt and sub-crypt vesicles characteristic of chloride cells is shown in Figure 8; it too shows both irregular and ordered arrays of tubules.

Negative staining of fresh whole pseudobranch, slightly mashed to break open the cells, provided information of a different sort about the fine structure. This technique gives more of a three-dimensional view of cell components than thin sections can, it is well adapted to
the illustration of surface structures which are not stained by positive stains, and it avoids the uncertainties of chemical fixation. On the other hand, surface tension and other forces acting during the drying-down of the negative stain could introduce artifacts of other sorts. For example, membranes could be disrupted or their component parts could be rearranged into unnatural conformations. The two major components of negatively stained pseudobranch (Figure 9) are mitochondria and long, branching membrane tubules almost certainly identical to the tubular extensions of the cell membrane seen in sectioned material. Lollipop-shaped projections from mitochondrial inner membranes are clearly visualized here (Figure 9a and c). These were first seen by Fernandez-Moran in beef heart mitochondria (37), and have since been seen in mitochondria from many other sources (see, e.g., 128, 91, 21, 138). They are believed by a number of workers to be the site of mitochondrial ATPase (102, 65). The diameter of the "headpieces" is approximately 80-100 Å in all cases. The outer mitochondrial membrane has been reported to be free of projections, and this seems to be the case for pseudobranch mitochondria.

The tubular membranes also have a surface structure which was not seen in the fixed, sectioned material described above. On the "outside" of the tubules (intracellular or cytoplasmic side) there appears a layer of particulate matter about 40 Å thick. Its appearance is somewhat variable, probably depending on how well the stain penetrated next to different areas of the membrane; details are significantly more distinct in samples negatively stained with molybdate than with phospho-
tungstate. As seen in Figure 9, it frequently appears to consist of rows of individual projections. These will be discussed further below. Here it is sufficient to note that this surface structure is characteristic of the cell membrane, including its tubular extensions, and was not observed on other recognizable types of membranous components. In Figure 9c it can be seen that the plasma membrane projections (right) are readily distinguishable from the larger, distinctly spherical mitochondrial projections (left).

Functional Properties of the ATPase

1) Relation of enzyme activity to salt water adaptation

The positive correlation between the salinity of the environment \( + \) \( + \) \( 2+ \) and the \( \text{Na}^+, \text{K}^+ \text{-ATPase} \) (and sometimes \( \text{Mg}^{2+} \text{-ATPase} \)) activity of fish gills and pseudobranch has been studied previously by a number of workers (see, e.g., 33, 60, 56, 134; exceptions are 64 and 69). It was not intended to repeat such a study on pinfish in a systematic way, but results consistent with such observations were obtained as a result of keeping the brackish water-adapted fish in pools of full-strength sea water for different periods of time before use. Table 1 shows pertinent figures. Fish kept in sea water for moderately long periods of time (a week or more) had consistently greater activities of \( \text{Na}^+, \text{K}^+ \text{-ATPase} \) in homogenates of pseudobranch than those used the day after they were caught. Some batches of fish also showed greater \( \text{Mg}^{2+} \text{-ATPase} \) activities. Since the microsomal \((P)\) fraction was the fraction with the highest specific activity of \( \text{Na}^+, \text{K}^+ \text{-ATPase} \), as discussed below, it is interesting to note the large increases in the \( \text{Na}^+, \text{K}^+ \text{-ATPase} \) specific activity of this fraction associated with increases in salt water adaptation time.
<table>
<thead>
<tr>
<th>Time in SW tanks</th>
<th>$\text{Na}^{+,\text{K}^+}$-ATPase in H$\mu$mole Pi/mg $\cdot$ h</th>
<th>$\text{Mg}^{2+}$-ATPase in H$\mu$mole Pi/mg $\cdot$ h</th>
<th>$\text{Na}^{+,\text{K}^+}$-ATPase in P$\mu$mole Pi/mg protein $\cdot$ h</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sim$ 18 hours</td>
<td>1.65</td>
<td>2.50</td>
<td>-</td>
</tr>
<tr>
<td>$\sim$ 18 hours</td>
<td>1.66</td>
<td>2.55</td>
<td>40.4</td>
</tr>
<tr>
<td>$\sim$ 40-45 hours</td>
<td>2.12</td>
<td>2.56</td>
<td>88.0</td>
</tr>
<tr>
<td>1 week</td>
<td>2.76</td>
<td>3.50</td>
<td>134.</td>
</tr>
<tr>
<td>1 week</td>
<td>2.92</td>
<td>3.55</td>
<td>-</td>
</tr>
<tr>
<td>2 weeks</td>
<td>2.70</td>
<td>2.21</td>
<td>-</td>
</tr>
</tbody>
</table>

Table I
<table>
<thead>
<tr>
<th></th>
<th>Na^{+},K^{+}-ATPase</th>
<th>Mg^{2+}-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity, μmole Pi/mg·h</td>
<td>Specific activity, μmole Pi/mg protein·h</td>
</tr>
<tr>
<td>Pseudobranch</td>
<td>2.76</td>
<td>19.9</td>
</tr>
<tr>
<td>Gill</td>
<td>0.74</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Table 2
2) Comparison of gill and pseudobranch

Table 2 gives activities of the ATPases of gill and pseudobranch homogenates from a single group of fish, kept in salt water one week. The specific activity of Na+, K+-ATPase of gill is about half that of pseudobranch. This is consistent with the idea that the Na+, K+-ATPase of the chloride cells in gill and the structurally similar pseudobranch cells is greater than that of other cells in these organs, on account of their amplified surface membrane area. Chloride cells are only one of a number of different cell types prevalent in gill, whereas the bulk of pseudobranch consists of the cells with invaginated cell membranes. The pseudobranchs of a pinfish weigh only about 1/20 as much as the gills, but their Na+, K+-ATPase activity is so concentrated that the total Na+, K+-ATPase activity of the pseudobranchs amounts to about 1/5 or 1/6 of that of the gills. Thus the percent of the total Na+, K+-ATPase activity of the gills and pseudobranchs of a pinfish which is contributed by the pseudobranchs is about 15%. Such considerations are of interest in the evaluation of possible ion transport roles for the pseudobranch.

Pseudobranch fractionation - preliminary tests

Because of the unusual structure of pseudobranch cells, especially with regard to the invaginated cell membrane, the fractionation procedure was checked first on rat liver, the tissue on which it has been used most frequently. Distributions obtained for cytochrome oxidase, glucose-6-phosphatase, acid phosphatase, and protein agreed closely with those published by de Duve, et al. (30).
A significant amount of blood was trapped in the pseudobranchs when dissected and homogenized, since pseudobranch is a highly vascularized tissue. Hemoglobin assays of pinfish blood and pseudobranch indicated that approximately 20% of the weight of dissected pseudobranchs consisted of blood. Therefore, pinfish blood was assayed for the particulate enzymes of uncertain origin, namely the ATPases and monoamine oxidase, to check the possibility of their localization in particulate elements of blood, especially the nuclear and cell membranes of the numerous red blood cells. Negligible if any ATPase, Mg$^{2+}$ or Na$^+$, K$^-$, was detected in fresh or frozen hemolyzed blood. This is consistent with the very low activities found in mammalian red blood cells as compared to other tissues (14, 15). Likewise, no measurable monoamine oxidase was found in fresh or frozen blood.

**Pseudobranch fractionation-enzyme distribution**

Biochemical assay results for two complete fractionations are presented in Table 3, and graphical presentation of the same data is also presented in Figures 28 to 32 to facilitate comparison of values. The recovery of each activity was determined in two ways, as percent of the activity in unfractionated homogenate (H), and as percent of the sum of activities in the nuclear (N) and cytoplasmic extract (E) fractions. Cytoplasmic extract refers to the combined supernatant and washings from the first set of centrifugations, which sediment the N fraction, and therefore contains all the components destined for ML, P and S fractions. Ideally, the activities in E + N should be equal to those in H; the differences between them reflect enzyme activity losses due to dilution of components in the two initial
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity in H</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st fractionation</td>
<td>2nd fractionation</td>
</tr>
<tr>
<td>cytochrome c oxidase</td>
<td>14.8 units/gm</td>
<td>16.1</td>
</tr>
<tr>
<td>phospho-glucomutase</td>
<td>0.49 units</td>
<td>0.61</td>
</tr>
<tr>
<td>monoamine oxidase</td>
<td>1.84 x 10⁻³ units</td>
<td>3.54 x 10⁻³</td>
</tr>
<tr>
<td>Na⁺, K⁺-ATPase</td>
<td>1.66 units</td>
<td>1.80</td>
</tr>
<tr>
<td>Mg⁺-ATPase</td>
<td>2.55 units</td>
<td>2.71</td>
</tr>
<tr>
<td>Protein</td>
<td>125 mg/gm</td>
<td>127</td>
</tr>
</tbody>
</table>

Table 3. Enzyme distributions in fractions of pinfish pseudobranch.

Per cents are given as the average from two fractionations ± deviations. A unit of cytochrome c oxidase is defined (3) as the amount of enzyme which will reduce by one unit per minute the decadic logarithm of the optical density of 100 ml. of reaction mixture. Other enzyme activities are given in units of μ moles substrate converted per gm. of pseudobranch per minute. Protein is given as mg. protein per gm. pseudobranch.
<table>
<thead>
<tr>
<th>N</th>
<th>ML</th>
<th>P</th>
<th>S</th>
<th>%H</th>
<th>%E + N</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.6±3.5</td>
<td>83.9±3.0</td>
<td>3.5±0.5</td>
<td>0.0±0.0</td>
<td>62.3±3.2</td>
<td>79.0±0.2</td>
</tr>
<tr>
<td>0.9±0.1</td>
<td>1.7±0.1</td>
<td>0.8±0.3</td>
<td>96.6±0.1</td>
<td>85.8±1.0</td>
<td>92.4±5.0</td>
</tr>
<tr>
<td>51.7±4.4</td>
<td>13.1±0.1</td>
<td>29.4±1.6</td>
<td>5.8±5.8</td>
<td>68.1±6.1</td>
<td>85.3±8.2</td>
</tr>
<tr>
<td>25.7±5.5</td>
<td>43.6±1.4</td>
<td>29.2±2.6</td>
<td>1.5±1.5</td>
<td>110.1±4.8</td>
<td>102.0±9.6</td>
</tr>
<tr>
<td>12.0±0.4</td>
<td>64.0±2.4</td>
<td>16.8±2.4</td>
<td>7.1±0.4</td>
<td>77.3±2.9</td>
<td>88.5±1.8</td>
</tr>
<tr>
<td>19.4±1.2</td>
<td>23.0±0.6</td>
<td>9.0±0.3</td>
<td>48.6±0.2</td>
<td>82.9±1.9</td>
<td>95.4±0.9</td>
</tr>
</tbody>
</table>
wash steps and/or to actual material loss by sample transfers occurring in the separation of N from E. (Material losses are implied by the protein recovery values).

A significant factor limiting the accuracy of the assays was the small amount of starting material available. The combined wet weight of pseudobranchs pooled from 30-45 fish (two each) was approximately 0.7 gm., and the fractions obtained from this tissue were split into aliquots for at least six biochemical assays and for electron microscopy. Taking this into consideration, the recovery values and reproducibility compare favorably with results of fractionations reported by de Duve, et al. (30).

1) Cytochrome c oxidase (E.C. 1.9.3.1)

Cytochrome c oxidase is an enzyme complex which catalyzes the terminal reaction in the electron transfer chain of mitochondria, namely the oxidation of reduced cytochrome c and the reduction of O₂ to H₂O (72). Its localization in mitochondria exclusively has been demonstrated for a wide variety of tissues and organisms (31). More specifically, there is good evidence that the electron transfer chain, including cytochrome c oxidase, is located in the inner membrane (cristae) of the mitochondria (48). The distribution of cytochrome c oxidase in pseudobranch fractions corresponded well with the distribution of mitochondria, as determined by electron microscopy and described below. Most was in the ML fraction, with a small but significant amount in N, and very little in P. The activity in N and ML was presumably associated with whole mitochondria, and that in P with small fragments.
There was no cytochrome c oxidase in the soluble fraction, since the enzyme is not easily removed from the mitochondrial membrane. The specific activity was greatest by far in the ML fraction. The results were nearly identical to those obtained for rat liver by the same fractionation procedures, both by the author and by de Duve, et al. (30), indicating similarities in the sedimentation properties of the mitochondrial populations of the two tissues. The fact that the recoveries of cytochrome c oxidase were lower than those of the other enzymes probably reflects the recognized sensitivity of this enzyme to dilution and long handling times. In addition, fractions E, N, P and S were diluted with less of the activating buffer than H and ML because of their lower activities, and thus may not have been fully activated.

2) Phosphoglucomutase (E.C. 2.7.5.1)

This glycolysis-associated enzyme catalyzes the reaction

\[
\text{glucose-1-P} \rightleftharpoons \text{glucose-1,6-diP} \rightleftharpoons \text{glucose-6-P},
\]

which is important in the utilization of stored glycogen for energy production. Phosphoglucomutase has been localized in the "soluble" cytoplasm of a number of tissues, including rat liver (31). The high concentration of the enzyme in the S fraction of pinfish pseudo-branch confirmed its non-particulate localization for this tissue, and showed that the particulate fractions were well washed. Assuming that phosphoglucomutase is a typical soluble enzyme, this result minimizes the possibility that enzymes found in the particulate
fractions were "soluble" in the cell and became adsorbed to particulate components during fractionation.

3) Monoamine oxidase (E.C. 1.4.3.4)

The reaction catalyzed by this enzyme is the oxidative deamination of a number of monoamines to the corresponding aldehydes, utilizing \( \text{O}_2 \) and releasing \( \text{NH}_3 \) and \( \text{H}_2\text{O}_2 \). Monoamine oxidase is widespread in nature and may serve a variety of functions, including detoxification of ingested amines in liver, metabolism of neural transmitter substances and hormones, plant alkaloid synthesis (12), and regulation of iodine metabolism in thyroid (38). The function in pseudobranch is unknown. This enzyme was chosen as a marker for the outer mitochondrial membrane, since it was not known in advance whether the inner and outer membranes would stay together well in fractionation. Free fragments of outer mitochondrial membranes could have been a significant and morphologically unidentifiable impurity in the microsomal fraction.

The evidence for localization of monoamine oxidase in the outer membranes of rat liver mitochondria is considered good (49). Beyond that, it has been localized in mitochondrial fractions of a number of other tissues (31, 38, 57, 68), but it has also been observed in other membranous fractions. These include microsomal fractions (121, 7, 38, 18, 57, 68), a nuclear membrane fraction from rat liver (47), and plasma membrane fractions from rat liver and thymus (2); the specific activity was high in the nuclear and plasma membrane fractions. Since detachment of the outer mitochondrial membrane can not be detected by assay of inner-membrane mitochondrial markers such as
cytochrome c oxidase, and since morphological identification of outer membrane fragments in membrane fractions is difficult, the possibility remains that the localization of monoamine oxidase in these non-mitochondrial fractions resulted from contamination of the fractions with mitochondrial outer membrane. A lesser soluble component of monoamine oxidase activity has also been frequently observed (7). It is possible that this has resulted from release of the enzyme from the outer mitochondrial membrane. Thus, although the localization evidence for monoamine oxidase lacks consistency, it cannot be considered proved that monoamine oxidase is localized in subcellular components other than outer mitochondrial membranes. On the other hand, the possibility of multiple localizations in some tissues should not be ignored. There is evidence that a single tissue may possess multiple forms of monoamine oxidase (63,46). Different localizations for isoenzymes of other enzymes such as malic dehydrogenase (see 119a) and α-glycerophosphate dehydrogenase (141) have been demonstrated, and a similar situation could hold true for monoamine oxidase.

The distribution of monoamine oxidase in the subcellular fractions from pinfish pseudobranch was unusual in two respects: The percent of the activity in the mitochondrial fraction was quite low, while the percent in the nuclear fraction was surprisingly high. In addition, the distribution was completely different from that obtained for cytochrome c oxidase in the same set of fractions. If pseudobranch monoamine oxidase is localized only in mitochondria, and if the inner and outer mitochondrial membranes remained together during fractionation, then
one would expect the two distributions to be similar. The remarkable differences indicate that at least one of these conditions did not hold.

It is possible to estimate the proportion of mitochondria which would have to have lost their outer membranes in order for the monoamine oxidase distribution to be attributable to outer membrane fragments. One needs to assume that there is a one-to-one correspondence between cytochrome c oxidase activity and inner membrane protein and that the relative proportions of cytochrome c oxidase and monoamine oxidase are constant in all intact mitochondria. If all the monoamine oxidase in ML were associated with outer membranes of intact mitochondria, only $13.1/83.9 = 15.6\%$ of the mitochondria in ML could be intact, and the other $84.4\%$ would have lost their outer membranes; if some of the monoamine oxidase in ML were associated with outer membrane fragments, even fewer of the mitochondria could have been intact. At most $12.6/51.7 = 24.4\%$ of the monoamine oxidase in N and $3.5/29.4 = 11.9\%$ of that in P could have been associated with intact mitochondria; the rest would have to have been contributed by outer membrane fragments.

Such large-scale removal of mitochondrial outer membranes seems unlikely for three reasons. First, in subfractionation studies with rat liver mitochondria, special disruptive techniques such as enzymatic digestion have been required to remove the outer membranes (65). Second, some special reason would be required to explain why such a large proportion of the broken outer membranes sedimented in N since a large concentration of monoamine oxidase in the nuclear fraction has apparently
never been previously observed for any tissue. Third, electron microscopic examination of the pseudobranch fractions, described below, suggested that very few of the mitochondria lost their outer membranes. Consequently, it does not seem reasonable to evaluate the distribution of outer membrane fragments in the fractions on the basis of the monoamine oxidase distribution.

Another striking observation was the fact that the monoamine oxidase activity in the homogenate and all the fractions was about twice as great in the second fractionation as the first, while the activities of the other enzymes varied little.

4) Na\(^+\), K\(^+\)-ATPase (E.C. 3.6.1.3)

As shown in Table 3, significant amounts of this enzyme were found in all three particulate fractions, with little or none in S.

(The high recovery values are a result of Na\(^+\), K\(^+\)-ATPase activities being obtained as the difference between total and ouabain-insensitive ATPase). The distributions of Na\(^+\), K\(^+\)-ATPase in other tissues fractionated similarly have usually consisted of concentrations in nuclear and/or microsomal fractions, presumably depending on the harshness of homogenization and the relative fragility of the cell membranes. (See, e.g., 15, 1b, 66, 137, 62). A large concentration of the enzyme in a mitochondrial fraction, such as was observed here for pseudobranch, has been found only infrequently (11, 100, 99); in one case the Na\(^+\), K\(^+\)-ATPase could be separated from the mitochondria by subsequent fractionation of the mitochondrial fraction (99). Since the pseudobranch mitochondrial fraction was also unusual in the large amount of non-mitochondrial
tubular membrane material it contained, as described below, the simplest explanation is that the Na⁺, K⁺-ATPase in ML was located in this component of the fraction. Further experiments designed to establish this will be described below.

The highest specific activity of Na⁺, K⁺-ATPase was observed in the microsomal fraction. Since this fraction contained very little cytochrome oxidase or phosphoglucomutase, it probably consisted primarily of non-mitochondrial particulate material. Judging from the electron microscopic observations of pseudobranch, it is likely that the major component of this fraction consisted of small fragments of the highly amplified pseudobranch cell membranes.

5) Mg⁺-ATPase (E.C. 3.6.1.3)

The assay for ouabain-insensitive ATPase (Mg⁺-ATPase) almost certainly measures more than one enzyme in most tissue homogenates. There is a Mg⁺-ATPase associated with oxidative phosphorylation in mitochondria, probably located in the headpieces of the repeating units of the inner membrane (59). There is also a Mg⁺-ATPase activity which apparently appears wherever Na⁺, K⁺-ATPase does, and may or may not be functionally related to it (see, e.g., 121, 34, 108). Both of these are ubiquitous in animal cells, so that one would expect to find them in pseudobranch, and there might well be other Mg⁺-ATPases. The distribution obtained for ouabain-insensitive ATPase in pseudobranch is suggestive of multiple localizations; such a distribution could be approximated, for example, by superimposing the distributions of cytochrome c oxidase (mitochondria) and Na⁺, K⁺-ATPase, and adding a small
amount of soluble ATPase.

6) Other assays

Acid phosphatase activity was measured in the fractions, but its activity was too low to obtain a reliable distribution, (about 0.1 μmoles Pi/mg pseudobranch · h in the homogenate), considering the small amounts of fractions available for assay. This is consistent with the rare observations of lysosomes in electron micrographs of whole pseudobranch or fractions. The results suggested that there were both particulate and soluble components of the little acid phosphatase activity present.

Since some nuclei probably ruptured during fractionation, a micro-method for assay of DNA was used to attempt to determine possible contamination of fractions with nucleoprotein. This assay was carried out with H, NML, P and S fractions from two incomplete fractionations used to obtain P fractions for kinetic studies. Although the optical density was proportional to DNA concentration when either DNA standard solutions or dilutions of pseudobranch homogenate were assayed, the recoveries obtained for sets of fractions were too high, as shown in Table 4. The values obtained for P and S are suspect, primarily because the optical densities on which they are based were so low; the values for H and NML are probably much more reliable. Thus, the results suggest that 80% of the DNA was located in NML (in N or ML for complete fractionation), and about 20% was distributed between P and S.
<table>
<thead>
<tr>
<th>Fractionation</th>
<th>% DNA (not normalized)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NML</td>
<td>P</td>
</tr>
<tr>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4

To test the possibility that apparently particulate enzymes such as monoamine oxidase and the ATPases might be soluble enzymes which became adsorbed to particulate matter during fractionation due to a decrease in the ionic strength of their environment by dilution with sucrose, attempts were made to solubilize them. To approximate intracellular conditions, KCl was added to samples of N and ML fractions to give a final concentration of 0.15 M and then they were recentrifuged. There was no indication at all of release by HCl of monoamine oxidase $+^+$ or of Na $^+$, K $^-$-ATPase or Mg $^+$-ATPase from N or ML fractions. There was some indication that agglutination of particulate matter resulted, which may mean that such tests were not meaningful. The tendency of membrane fragments to aggregate upon addition of salts such as KCl has been observed by others, and has been analyzed in detail by Wallach, et al. (135).

Pseudobranch fractionation-electron microscopy

Figure 19 shows sections through a nuclear fraction pellet. This fraction was obviously quite heterogeneous, as implied by the alternate
term "cell debris" fraction, and it is consequently impossible to show a single representative micrograph. All large cell fragments sedimentioned in this fraction, including nuclei, whole blood cells, bunches of collagen fibrils, clumps of cytoplasm containing various organelles, large mitochondria, sections of tubular membrane networks, and long sections of the smooth portions of cell membranes of adjacent cells, held together by the basement layer underlying the pseudobranch cells. A likely source of the amorphous flocculent material was nucleo-protein from ruptured nuclei; apparently many of the nuclei were ruptured during resuspension procedures. On the other hand, most of the blood cells probably remained whole, since they are less fragile and could probably slip between the homogenizer tube wall and pestle without being broken by pressure or shear forces. (They presumably withstand similar forces in the circulatory system of the fish). The appearance of some mitochondria in N is consistent with the fairly low but significant activity of cytochrome c oxidase in this fraction. The observations of large, easily identifiable sections of pseudobranch cell membranes (Figure 19), help support the proposition that the Na⁺, K⁺-ATPase of the nuclear fraction was localized in cell membrane fragments.

A mitochondrial fraction pellet is shown in Figure 20. The mitochondria of the fraction appeared to be at least morphologically intact; a few swollen mitochondria were observed but even these apparently retained their outer membranes. The configuration of the cristae was orthodox rather than condensed, which is somewhat unusual for mitochondria
isolated in sucrose (50b). The reason for this is not known. Organ-
elles which could be lysosomes were rarely observed in the fraction, 
as predicted from the microscopy of whole pseudobranch. However, the 
fraction did contain a significant non-mitochondrial membranous com-
ponent, in the form of tubules, sheets, and vesicles. The major source 
of these was probably the invaginated cell membranes of pseudobranch 
cells. Such a component has not usually been observed in such large 
amount in comparable fractions from tissues whose cell membranes are 
not invaginated, such as liver (8). Abundant membranous material 
has been seen in mitochondrial fractions from tissues with more 
structurally complex cell membranes (117, 42, 100).

The microsomal fraction pellet was the most homogeneous in 
appearance, as seen in Figure 21. It consisted primarily of small 
membrane vesicles with a few clearly tubular fragments, small granules 
which were probably glycogen, and some amorphous flocculent material. 
Because of the huge area of the cell membranes of pseudobranch cells, 
this was probably the major source of the membrane vesicles. Minor con-
tributions could have come from cell membranes of other cell types, 
nuclear membranes, and the few elements of endoplasmic reticulum and 
Golgi membranes present in the pseudobranch. The flocculent material 
could have been cell-surface-associated polysaccharide or nucleoprotein 
from ruptured nuclei.

A section from a microsomal pellet containing ruthenium red is 
shown in Figure 22. Ruthenium red is a cationic complex which was ex-
pected to bind to negatively charged membrane fragments and stain them;
it was hoped that this procedure would provide further information about the microsomal membranes. Such pellets appeared quite homogeneous. The apparent absence of glycogen can be attributed to the fact that the fish used had been fasted for a week. (Incidentally, the presence or absence of glycogen in the fractions had no noticeable effect on enzyme distributions, so that glycogen particles can be eliminated as a possible location for Na, K-ATPase). The most striking characteristic of microsomal pellets containing ruthenium red was the sheetlike appearance of the membrane fragments, which was completely unlike the vesicular appearance of microsomes fixed and embedded without the stain.

Still another configuration of the microsomal membranes was observed when unfixed samples were negatively stained. As seen in Figures 23 and 24, the membrane fragments were largely tubular. When stained with potassium phosphotungstate (Figure 23), the tubular fragments were rather long. Short tubular fragments and spherical vesicles were seen in molybdate-stained samples (Figure 24). The tubular fragments and most of the spherical ones showed the surface projections characteristic of the cell membrane, particularly when stained with molybdate. Some uncoated vesicles were also observed, which could have been fragments of nuclear membranes, mitochondrial outer membranes, etc. There were also some very small mitochondrial fragments displaying the unmistakable large knob-like projections (Figure 24). If the negative staining techniques used gives reasonably random sampling of the fraction contents, then the relative infrequency
of these knobbed fragments suggests a low degree of contamination of the P fraction with pieces of broken mitochondria. This is consistent with the very low cytochrome c oxidase activity observed in P. The apparent predominance of cell membrane fragments in P is in accord with predictions made on the basis of the enzyme distribution and electron microscopy of whole pseudobranch and supports the contention that the Na⁺, K⁺-ATPase of the microsomal fraction is located in these fragments.

Negative staining of the mitochondrial fraction (Figures 25 and 26) demonstrated the same two major components as pellet sections, namely mitochondria and non-mitochondrial membrane material. In this case it was much easier to conclude that the latter were not fragments of broken mitochondria but rather fragments of cell membrane extensions, since they were distinctly tubular and had the characteristic projecting layer. Distinction between these and the smooth or large-knobbed mitochondrial membranes was clear-cut. The impression was gained of roughly equal amounts of mitochondrial and tubular membrane in the negatively stained samples; this is consistent with the picture presented by pellet sections, whose uniformity is more certain. The observation of both an unusually large Na⁺, K⁺-ATPase activity and an unusually prominent cell membrane component in the mitochondrial fraction suggests localization of the Na⁺, K⁺-ATPase in the cell membrane fragments, but further evidence is required to rule out a mitochondrial localization. In rare instances collagen fibrils were observed, along with a small amount of unidentifiable material.
In a number of negatively stained preparations of mitochondrial and microsomal fractions the interior of membrane tubule fragments was intermittently filled with stain in such a way as to demonstrate another superficial layer on the inside (extracellular side) of the tubules. As seen in Figures 24, 26 and 27, this was similar to but smaller than the projecting layer on the intracellular side. With fortuitous interaction between the stain and membranes, the intracellular layer could be resolved to individual projections about 30-40 Å in diameter with a center-to-center separation of about 50-100 Å, and the extracellular layer to about 20 Å projections separated by 40-50 Å. The intracellular projections generally appeared more irregular and filamentous, while the extracellular ones usually appeared more like well-defined spherical particles. The latter probably correspond to the projections seen in situ by Ritch and Philpott (106) along the extracellular sides of cell membrane tubules in gill and pseudobranch, with lanthanum acting as a negative stain during the fixation. The two-dimensional arrangements of the projections on the membrane surfaces could not be determined; the occasional appearance of regular parallel rows of particulate material in the places where the tubule membranes were seen face-on (Figures 24a and 26) suggested regular geometric arrays rather than random placement, but it could not be determined whether these patterns arose from the outside or inside surfaces, or both. Very similar patterns of parallel rows were observed by Ritch and Philpott.

The present observations apparently represent the first time projections have been seen on both sides of a type of membrane by any
technique. Negative staining has been used to demonstrate membrane projections in fractions of a number of different tissues, but it has not generally been possible to decide on which side of the membranes the projections occurred. The membrane vesicles could conceivably have formed with either side out, and the projections could not be correlated with in situ observations. In the present case, the two different types of projections were observed on long, branched tubular fragments, which could hardly have turned inside-out during cell fractionation, as well as on smaller vesicles; in addition, the results correlate well with the in situ observations of Ritch and Philpott (106). Thus it can be stated with certainty that the larger projections were observed on the intracellular side of cell membrane fragments and the smaller ones on the extracellular side. It should be recognized that although the "extracellular" side of the tubular membranes is topologically outside the cell, the environment of the extracellular projections is not necessarily identical to the intercellular fluid, and the membrane of the tubular extensions may differ from the smooth portions of plasma membrane which face adjacent cells.

**Kinetic properties of ATPases in P fraction**

The major reason for studying the kinetic properties of the Na\(^+\), K\(^+\) -ATPase was to characterize it in comparison with the enzyme from other sources. Since Na\(^+\), K\(^+\) -ATPase cannot be measured without measuring 2+ ouabain-insensitive Mg\(^2+\) -ATPase, the properties of the latter were also determined. They are presented because the possibility still exists that the particular Mg\(^2+\) -ATPase which is almost always found together
with Na⁺, K⁺-ATPase and is separable from it only with difficulty may be structurally and/or functionally related to it (121).

Unless otherwise stated, reaction conditions were as given in Materials and Methods. The microsomal fraction was used for these experiments because of its high specific activity of Na⁺, K⁺-ATPase and its apparent homogeneity. The Mg²⁺-ATPase activity in this fraction is more likely to be a single enzyme, the Mg²⁺-ATPase usually associated with Na⁺, K⁺-ATPase, without significant contamination from the mitochondrial ATPase. The reaction temperature of 37°C. was chosen to facilitate comparison with results from other tissues, although it does not correspond with the body temperature of the fish. This is the standard temperature at which Na⁺, K⁺-ATPase from any source is studied, corresponding to the normal body temperature of most mammals (22); in any case, it would not be possible to choose a single body temperature for poikiloithermic animals such as fish. Since the activity of Na⁺, K⁺-ATPase measured at 37°C. in pseudobranch was extremely high in comparison to activities in other tissues (14, 15), it is unlikely that this rather high temperature had any adverse effect on the Na⁺, K⁺-ATPase.

1) Enzyme concentration

Figure 10 shows that the amount of product formed in a 15-minute interval was proportional to the concentration of enzyme (P fraction) in the reaction mixture for both ATPases, over the range of concentrations used in other experiments. Since the enzymes have not been purified, their actual concentrations of course cannot be determined. This is typical enzyme behavior under conditions where the enzyme is saturated.
with substrate and the reaction rate is constant throughout the reaction.

2) Time of reaction

Using standard reagent and enzyme concentrations at $37^\circ C$, the rate of reaction of both ATPases remained constant for about 20 minutes, as seen in Figure 11. The standard assay time was 15 minutes, so that problems related to depletion of substrate, accumulation of inhibitory reaction products, and decrease in enzyme activity were avoided.

3) Ouabain concentration

Figure 12 shows the total ATPase activity as a function of concentration of the inhibitor ouabain. The curve is sigmoidal, ranging from no inhibition at approximately $10^{-8}$ M. ouabain to apparently maximal inhibition at about $10^{-3}$ M. Inhibition by the omission of Na $^+$ (when no ouabain was present) was nearly the same as that produced by $10^{-3}$ M. ouabain. Na $^+$, K $^+$-ATPase is defined as that part of the total ATPase which is inhibited by either of these conditions, and the remaining ATPase is the ouabain - insensitive Mg $^{2+}$-ATPase. The slightly greater ATPase activity which was observed with no Na $^+$ than with $10^{-3}$ M. $^+$ ouabain probably resulted from traces of Na $^+$ present in the P fraction or various reagents, particularly the ATP. The ouabain concentration used in most experiments, $10^{-4}$ M., inhibited at least 92% of the Na $^+$, K $^+$-ATPase activity. Half-maximal inhibition occurred somewhere between $10^{-5}$ and $10^{-6}$ M. ouabain. The sigmoid shape of the curve is quite typical for inhibition by cardiac glycosides. The details vary significantly for Na $^+$, K $^+$-ATPases of different tissues and for different glycosides (44). The details of the response of the pinfish pseudobranch enzyme
are similar to those of preparations from calf heart (103) and rat brain (111).

4) Substrate concentration

Concentrations of ATP and MgCl$_2$ were varied together since the substrate for Mg$^{2+}$-activated ATPases is believed to be a one-to-one complex of ATP and Mg$^{2+}$ (28, 76). As seen in Figures 13 and 14, both ATPases showed typical Michaelis-Menten kinetics (76). The Na$^+$, K$^+$-ATPase activity leveled off around 2 or 3 mM ATP-Mg$^{2+}$; at this stage the enzyme becomes saturated with substrate and the enzyme concentration becomes rate-limiting. The plateau for Mg$^{2+}$-ATPase was reached at approximately 1 mM ATP. Figure 14 is a plot of the data in a form similar to a Lineweaver-Burke double-reciprocal plot. Plotting $\frac{[S]}{v}$ vs. $\frac{[S]}{v}$ gives a more even spread of points than $\frac{1}{v}$ vs. $\frac{1}{v}$. The pertinent equation, obtained from the basic Michaelis-Menten equation $v = \frac{V_{\text{max}} [S]}{k_m + [S]}$, is $\frac{[S]}{v} = \frac{1}{V_{\text{max}} [S]} + \frac{k_m}{V_{\text{max}}}$. Here $[S]$ is the substrate concentration, $v$ the reaction rate, $V_{\text{max}}$ the maximum reaction rate, and $k_m$ the substrate concentration at half-maximal reaction rate.

Thus $k_m$ and $V_{\text{max}}$ for each enzyme under these experimental conditions can be determined from the slopes and y-intercepts of the straight lines. The values obtained are shown below:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_m$ (mM)</th>
<th>$V_{\text{max}}$ (μmole Pi/mg protein - h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$, K$^+$-ATPase</td>
<td>.27</td>
<td>98.7</td>
</tr>
<tr>
<td>Mg$^{2+}$-ATPase</td>
<td>.22</td>
<td>32.6</td>
</tr>
</tbody>
</table>
The $k_m$ for Na, K-ATPase is similar to those for preparations from calf heart (78) and turtle bladder (116). Both larger and smaller values have been observed, for example with duck salt gland (34) and rat intestine (101), respectively. The value of ATP-Mg concentration at which Na, K-ATPase activity began to level off, 2 or 3 mM, is similar to that observed for ox brain (113), but much higher than for a rat intestine preparation (0.5 mM, 101). Parameters for Mg-ATPases of other tissues vary considerably more, probably due to enzyme multiplicity. (This holds true for all the kinetic properties discussed.

5) Mg concentration

As seen in Figure 15, both ATPases were completely dependent upon Mg. With 3 mM ATP, the Na, K-ATPase activity reached a plateau at about 3 or 4 mM Mg. The Mg-ATPase had optimal activity at about 3 or 4 mM Mg, and slightly lower activity at higher concentrations. This type of behavior is one kind of evidence that the substrate of Mg-activated ATPases is a complex of one ATP molecule and one Mg ion. The leveling off of Na, K-ATPase at Mg concentrations beyond equimolar ATP:Mg has also been observed for preparations from rat intestine (101) and crab nerve (122), whereas in other cases the activity has shown some inhibition at higher concentrations of Mg (34, 103, 5, 113, 4).

6) pH

The values of pH plotted in Figure 16 are the pH's of reaction mixtures at the reaction temperature of 37°C; these are lower by
0.2-0.3 pH units than the values at room temperature for both buffer systems. The pH change during the course of the reaction was negligible. The Na, K-ATPase showed a moderately broad peak of activity, from about pH 6.6 to 7.3. Similar pH optima have been observed for Na, K-ATPases from rabbit kidney (130), rat intestine (101) and liver (5), calf heart (78), crab nerve (122), and ox brain (113). In other cases pH optima around 7.5 or 7.6 have been observed (105b, 43, 4, 116, 95); since few authors note the temperature at which the pH's were determined, some of these results may reflect the temperature variance in pH, especially for Tris buffer, rather than real differences in pH optima.

There was apparently some inhibition of the Mg-ATPase by imidazole buffer. Aside from this, the activity exhibited a very broad range of optimal pH's, from about 6.6 to 7.7, with little decrease in activity as low as pH 5.8 or as high as 8.6. Similar results have been obtained for rat intestine (101) and calf heart (78) preparations.

7) Na and K concentrations

Three sets of points are plotted together in Figure 17. In each set the total concentration of NaCl + KCl was kept constant while the ratio of concentrations of Na and K was varied from no Na to all Na. The total monovalent cation concentration was set at 60, 120, and 240 mM. The shape of the curve obtained is quite similar to those obtained for other tissues with the total Na + K concentration in the range 100-150 mM, designed to approximate in vivo intracellular conditions (105b, 130, 101, 111, 123). Comparison of curves obtained for different
total ionic concentrations has apparently not been done previously. Rather unexpectedly, considering the sensitivity of Na, K -ATPase to Na and K, all three sets of points fit the same curve within experimental error. This implies that, at least in the four-fold range of total concentrations studied here, neither the ionic strength nor the specific concentrations of Na and K are important, and that activity is dependent only on the ratio of the two ions. The range of optimal ratios was rather wide, from about 1 Na : 1 K to 11 Na : 1 K. Absolute dependence on both Na and K was demonstrated. It may also be noted that the left and right halves of Figure 17 closely resemble curves obtained by varying Na or K alone, with the concentration of the other ion kept constant at the standard assay concentration (103, 122, 5, 34, 101, 113).

The Mg -ATPase was much less affected by Na and K, as shown in Figure 18. The small increase in activity in the presence of both ions, and its slight variation with Na : K ratio, can probably be accounted for by the small amount of Na, K -ATPase left uninhibited by 10^{-4} M ouabain. More significant was the moderate inhibition of activity by greater Na + K total concentrations. This was likely a nonspecific effect of increasing ionic strength, and may be related to the effectiveness of the NaI treatment employed by many workers to reduce Mg -ATPase activity and thus "purify" the Na, K -ATPase (see, e.g., 85).

Rate sedimentation analysis of enzymes in ML

The ML fraction from pinfish pseudobranch was subjected to rate sedimentation analysis because of the unusual observation that it
contained the largest percentage of Na+, K+-ATPase. Although the
distribution of Na+, K+-ATPase did not parallel that of mitochondria,
and the high specific activity of the enzyme in P was certainly not
consistent with a purely mitochondrial localization, it was still
possible to interpret the results in terms of a double localization
of Na+, K+-ATPase, in both cell membrane fragments and mitochondria.

The principles of this type of centrifugation have been dis-

cussed by de Duve et al. (29). Initially the subcellular particles
are suspended homogeneously in a fairly shallow supporting gradient,
whose purpose is to minimize disruptive factors such as convection
currents and to reduce the variability of particle velocity with
respect to distance from the axis of rotation. (The gradient used
in the present study was designed to approximate constant velocity
for particles having an equilibrium density similar to the median
equilibrium density of rat liver mitochondria (23).) Centrifugation
is interrupted before complete sedimentation and the distribution of
enzymes in the gradient is determined. Ideally a homogeneous class
of particles should move toward the bottom of the centrifuge tube
leaving a zone of increasing length at the top of the gradient free of
particles, the initial particle concentration being maintained through-
out the tube below this boundary down to the pellet. In practice, vari-
ations in the size and density of particles and imperfections in the
gradient itself prevent the attainment of such perfect boundaries, and
the boundaries actually obtained tend to be sigmoidal in shape. The
supporting gradient itself is not affected by the centrifugation.
The principles involved in the separation of different classes of particles in rate sedimentation studies are the same as those involved in ordinary differential centrifugation, which was used to prepare the N, ML, P and S fractions. The differences between the two procedures, which are responsible for the greater resolving power of rate sedimentation analysis, are the use of a stabilizing gradient which results in nearly constant velocity sedimentation, the use of a swinging-bucket rotor rather than an anglehead type to minimize adverse geometric effects, and the separation of the centrifuged sample into multiple fractions for analysis rather than just pellet and supernatant.

Density gradient centrifugation has been used previously to separate Na, K-ATPase activity from mitochondria in mitochondrial fractions from rat intestine (100) and guinea pig brain (66). In the first case Quigley and Gotterer managed to separate Na, K-ATPase from cytochrome c oxidase if the mitochondrial fraction had been aged for 5 days at 0-2°C. In the second case Kurokawa, et al. demonstrated a much lower Na, K-ATPase in a subfraction enriched in mitochondria than in their original "large granule" fraction. In both cases the isopycnic gradient centrifugation technique was employed, in which a sample is layered over a continuous or discontinuous gradient whose density range includes the equilibrium densities of the particles, and the gradient is centrifuged long enough for the particles to reach equilibrium positions.

The rate sedimentation technique was considered to have several advantages over the isopycnic technique for the present study, especially
in view of the unpredictable sedimentation behavior of chunks of long, branched tubular membranes. First, it separates particles on the basis of size as well as density differences. Second, it permits the use of larger amounts of sample material, since homogeneous suspension of the sample avoids the drop sedimentation problem (29), and thus the centrifuged gradient can be separated into a larger number of fractions for improved resolution. In addition, the shorter centrifugation times required may reduce inactivation of sensitive enzymes such as cytochrome oxidase, and the less dense supporting gradients are easier to form accurately because of the lower viscosity of the solutions.

The results of a rate sedimentation study of the pseudobranch mitochondrial fraction are presented in Figures 33 and 34. One complete experiment is represented. Results of a previous experiment were entirely similar with respect to both overall patterns of distribution and all the irregular details described below; however, they were not averaged in because problems in the collection and resuspension of the pellet fraction resulted in poor recoveries of all three enzymes in this fraction, and the overall recoveries in the gradient could not be determined.

Figure 33 shows the density profile of the centrifuged sucrose-ML gradient. The extrapolated starting and ending densities, 1.0375 o and 1.0745 gm./ml. at 2 C., were quite close to those planned, namely 1.038 and 1.076 respectively. The density of the liquid part of the pellet fraction was not determined; the final 7.8% of the recovered volume corresponds to this fraction. (Previous experiments on pure
sucrose gradients indicated that the linearity of such gradients extended into the pellet fraction). The volume recovery after fraction collection was approximately 99%. The gradient was very nearly linear over its entire range; small deviations have been frequently observed at the ends of such gradients. This indicates that the presence of the ML material did not interfere with the formation of the gradient, and that little if any disruption of the gradient occurred during centrifugation and fraction collection.

The distributions of cytochrome c oxidase, Mg$^{2+}$-ATPase and Na$^+$, K$^+$-ATPase in the gradient fractions are presented together in Figure 34 to facilitate comparison. Enzyme activities are expressed as percents of the initial activities, which were constant (at 100%) throughout the gradient before centrifugation. The initial activities were determined from reserved samples of the heavy and light sucrose-ML solutions used to make the gradient. Enzyme activities in the pellet fractions are not shown, since they were about two to five times as great as the initial activities. The recoveries of the enzymes in the 2+ gradient were 76% for cytochrome c oxidase, 108% for Mg$^{2+}$-ATPase, and 112% for Na$^+$, K$^+$-ATPase; the relative activities in the fractions were normalized to these recovery values. The abscissa, percent of recovered gradient volume, may also be thought of in terms of a scale of increasing sedimentation constant s; then for a given sedimentation constant s$_1$, the corresponding ordinate value is proportional to the fraction of total particle volume associated with particles having a sedimentation constant less than or equal to s$_1$ provided that the concentration (or range of
concentrations) of enzyme in the particles is the same for all values of s (23a).

It is immediately apparent that the distributions of cytochrome c oxidase and Mg\(^{2+}\) -ATPase were quite similar to each other and definitely different from the Na\(^+,\) K\(^+\) -ATPase distribution. The distributions of cytochrome c oxidase and Mg\(^{2+}\) -ATPase were much like that of cytochrome c oxidase obtained for a rat liver fraction treated similarly (24), while that of Na\(^+,\) K\(^+\) -ATPase was like the distribution of the liver microsomal enzyme glucose-6-phosphatase. The Na\(^+,\) K\(^+\) -ATPase distribution differed from the other two both in the amount of completely unsedimented enzyme (75% vs. 10% and 0%), and in the position of the plateau or end of the boundary (25% volume vs. about 80 or 90%). The simplest explanation for these results is that most of the Mg\(^{2+}\) -ATPase of the ML fraction was associated with mitochondria, and that most or all of the Na\(^+,\) K\(^+\) -ATPase was in a completely different class of particles, probably the cell membrane fragments. Extrapolation of the curves to the top of the gradient shows that 75% of the Na\(^+,\) K\(^+\) -ATPase activity was unsedimentable by the centrifugation conditions used, while apparently none of the Mg\(^{2+}\) -ATPase was. This result was unexpected; it implies that at least 75% of the Na\(^+,\) K\(^+\) -ATPase activity was localized in particles containing no measurable Mg\(^{2+}\) -ATPase.

Several irregularities were observed in the distributions of cytochrome c oxidase and Mg\(^{2+}\) -ATPase. First, the relative concentrations of both enzymes in the last liquid fraction (81.9% and 140.8%, respectively) appeared abnormal. The most likely explanation for this is that
this last fraction came in contact with both vacuum grease from the
knife and the Freon pushing fluid during fraction collection, either
of which might have altered enzyme activity. Second, an apparent
intermediate plateau was observed in the distribution of cytochrome
c oxidase at about 55% volume. This is more easily seen in Figure 35,
where the center of each fraction interval is plotted. This may
indicate that there are two populations of pseudobranch mitochondria.
Figure 36 is a plot of the slope of the curve in Figure 35; in this
case the abscissa can be considered to correspond directly to sedi-
mentation constant, and the ordinate can be interpreted as the per-
cent of cytochrome c oxidase associated with particles having a given
sedimentation constant. The distribution of Mg$^{2+}$-ATPase may or may
not have contained this irregularity; experimental variability was
too great to resolve this detail. Third, the cytochrome c oxidase
$^{2+}$
distribution differed from that of Mg$^{2+}$-ATPase at the top of the grad-
ient. The 10% cytochrome c oxidase which was unsedimented apparently
$^{2+}$
had no counterpart in the Mg$^{2+}$-ATPase distribution.
DISCUSSION

The sum total of the results discussed above supports the present ideas regarding an active role for Na, K -ATPase in transport of ions across cell membranes and epithelia. Good evidence has been presented for three properties of the Na, K -ATPase of a single tissue, the pseudobranch of the teleost Lagodon rhomboides. The first is its localization in the cell membrane, the site of ion transport. Second, the Na, K -ATPase showed adaptive increases in activity with transfer from brackish to salt water, a change which is known to result in increased salt excretion by epithelial tissues in the branchial region of the fish, such as the gills. Third, the Na, K -ATPase showed the biochemical properties which have linked it to ion transport in other tissues where transport is measurable.

**Kinetic Properties of Na, K -ATPase**

The kinetic properties of Na, K -ATPase from pinfish pseudo-branch fell easily within the range of variability of the Na, K -ATPases from many other sources (121). The results thus provided no reason to suspect that this Na, K -ATPase differs from the others in molecular mechanism or localization and role in the cell. One finding which may well be new was the observation that the total concentration of Na and K made no difference in the variation of activity with the Na :K ratio, at least over a four-fold range of total concentrations. This result was of course obtained for a preparation of fragments of cell membranes, far removed from their native environment, particularly with respect to ionic gradients, and one cannot be certain that it can be applied to analysis of the mechanism of ion transport by intact
cells. Nonetheless, the apparently prime importance of the ratio of $^+$ $^+$ Na and K concentrations should be kept in mind when evaluating mechanistic models, especially the proposed specifications and interactions of ion-binding sites.

**Variable morphology of cell membranes**

As described above, the membrane fragments of the microsomal fraction from pseudobranch appeared vesicular, tubular, or sheetlike, depending upon the technique used for visualization. This is a rather unusual observation, and may indicate unusual flexibility of the pseudobranch cell membranes. Such an interpretation would be consistent with the striking variation observed in sections of whole pseudobranch tissue. The configuration of the membrane fragments in an untreated microsomal fraction, as assayed for enzymes, cannot be ascertained, but it seems likely that they were largely short tubules with some smaller spherical vesicles. Fixation of pellets may have caused tubular fragments to break up into small round vesicles; on the other hand, it is possible that some of the round profiles in pellet sections (Figure 21) were actually cross sections through tubules. The binding of cationic ruthenium red may have disrupted the tubular form and produced sheetlike pieces by altering the surface charge of the membrane. The differences between samples negatively stained with phosphotungstate (large tubular fragments with indistinct surface structure) and with molybdate (shorter tubular fragments and well-defined surface structure) could well have resulted from differences in the interaction between the membrane and the negative stain molecules. All these considerations
suggest that the configuration of the cell membranes in intact tissue may be a very dynamic property, with the exact form determined by a number of interdependent physical and chemical variables.

Cell membrane projections

Tiny projections were observed on both sides of the plasma mem-
brane fragments from the pseudobranch cells. The fact that those on
the extracellular side have been visualized both by conventional
negative staining and by the lanthanum-fixation method of Ritch and
Philpott (106) significantly improves the chances that these are real,
rather than artifact. Likewise, the observations in situ of intra-
cellular projecting layers in several other tissues with cell structure
similar to pseudobranch (3a, 50a, 120b) support the reality of the
intracellular projections on pseudobranch cell membranes.

These other tissues, namely fish gill (120b), blowfly rectal
papilla (50a), and cecropia midgut epithelium (3a), are all thought to
have electrolyte transport functions. This raises the possibility that
such projections have a role in transport, whether they consist of
glycoprotein, enzyme assemblies, or whatever. On the other hand, pro-
jections of various shapes and sizes have been seen with several
techniques on a number of other types of membranes from widely differ-
ent sources, including mammalian intestine (86, 58, 10), rat liver (9,
21, 98), beef heart (21), human erythrocyte ghost (51), frog muscle (83),
and bacteria (55, 119b); this multiplicity suggests a variety of
functions for the projections. The possibilities include transport and non-transport enzymes, antigenic sites, hormone receptors, and subunits of glycocalyx which confer the necessary physico-chemical properties to the cell surface.

There is no direct evidence on the possibility that either or both kinds of projections on the pseudobranch cell membranes are associated with the ion pump, presumably Na⁺, K⁺-ATPase, but it is possible to discuss the plausibility of such a proposition. The size of the projections is consistent with their being complexes of enzyme protein(s), possibly including lipid and/or polysaccharide. Na⁺, K⁺-ATPase has been "solubilized" from a number of membrane preparations (see, e.g., 130, 79), i.e. obtained in membrane fragments too small to be sedimented by the centrifugalational procedures which usually define a "soluble" fraction. Such fragments have even been studied with the electron microscope and found to be particles about the same size as the projections (115). These results are consistent with the localization of Na⁺, K⁺-ATPase in a membrane subunit such as a projection. Of course, such fragments could also come from within the membrane.

From the point of view of theoretical models for ion transport by an enzyme, it would be possible for either or both kinds of projections to be part or all of the ion pump. Many models have been proposed, based on several fundamentally different concepts (reviewed in 17). Although these models have often assumed that the ion pump is extended across the whole thickness of the membrane, this is usually
not a necessary condition, and the pump could be at one side of the membrane or even projecting from it. It is equally easy to visualize ion channels through the pump enzyme complex wherever it is. A model proposed by Skou (123) specifically envisions a pump at one side of the membrane, in the protein layer on the cytoplasmic side, with the ions diffusing passively through the rest of the membrane; he pointed out that the pump could also consist of two different proteins on opposite sides of the membrane. The chemiosmotic hypothesis (81) involves transport of H$^+$ by the ATPase in the mitochondrial inner membrane projections. In this connection, it is interesting to note that the suggestion has been made that Na$^+$ and K$^+$ transport is actually based on chemiosmosis: that the energy for transport is supplied by a pH (H$^+$ concentration) gradient produced by the ATPase (77).

One possible objection to the location of Na$^+$, K$^+$-ATPase on the extracellular side of the membrane might be the inaccessibility with regard to the substrate ATP, which is produced within the cell. However, evidence for the presence of ATP within membranes (1a) suggests that ATP could move through the membrane to reach the ATPase wherever it is situated. Localization of the pump on the outside of the cell might even facilitate some interactions, such as with hormones. At any rate, there is no theoretical barrier to the ion pumps' being located in projections on either or both sides of the cell membrane.

Since it is clear that the precise localization of Na$^+$, K$^+$-ATPase in cell membranes would greatly facilitate the evaluation and extension
of hypotheses of ion transport, it is worthwhile to discuss the methods by which one could determine whether or not the enzyme is located in membrane projections such as those observed in pseudobranch. Experiments designed to identify enzymatic activities of membrane projections are difficult; this is apparent from a comparison of conflicting results obtained in studies of intestinal disaccharidases (86, 58 vs. 10), bacterial ATPases (55 vs. 119), and mitochondrial ATPase (138, review). In these previous experiments the negative staining technique was used to "assay" for the presence of membrane projections before and after disruptive treatments, and the fate of the projections was compared with that of the enzymes in question. It is not clear that the usual methods of negative staining are good enough for such experiments. First, there is no guarantee that the sampling of material is random and representative. Second, the visualization of projections smaller than the mitochondrial ones is too dependent upon fortuitous drying-down of the stain, and there are often indistinct areas where the presence or absence of projections could not be determined with certainty. Present methods would need to be improved to provide really conclusive quantitative results. Perhaps another technique, such as freeze-etching or heavy metal shadowing of membrane fractions, would be more reliable.

The disruptive techniques previously used to try to remove projections and enzymes from membranes have included digestive enzymes, detergents, urea, and sonication. Problems include loss of enzyme activity, additional deterioration of membrane structure beyond
removal of projections, and lack of control over the extent of disruption (e.g., the time of enzymatic digestion). The use of papain covalently bound to cellulose presently seems promising (10). An entirely different alternative of possible use in the study of Na, K -ATPase would involve natural variations in enzyme activity, such as those which occur in adaptation of fish to greater salinities. Relative numbers and densities of membrane projections could be compared in preparations from animals known to have different Na, K -ATPase activities in their pseudobranchs. The pseudobranch P fraction would probably be a good preparation with which to work in such ultra-localization studies, especially if the two different types of projections have different sensitivities to disruptive agents so that they could be analyzed separately.

Even with ideal methods it would be simpler to demonstrate that the cell membrane projections were not Na, K -ATPase than that they were associated with it. For example, a technique which removed many of the projections but not the Na, K -ATPase would provide relatively good evidence for their nonidentity, although it could be argued that the technique just rearranged the membrane components in such a way that the Na, K -ATPase no longer appeared as a projection upon negative staining (or other visualization technique). Similarly, solubilization of the enzyme without the removal of the projections would indicate that they are different, although one might imagine that the Na, K -ATPase was a small subunit within a projection, which was released, leaving the rest of the projection intact. On the other
hand, if a technique removed both from the membrane, very little
argument would be required to dispute the conclusion that the pro-
jections were Na⁺K⁺-ATPase; it is easy to imagine a disruptive
technique removing a number of different components from a membrane
at the same time it removes projections. It would help considerably
to purify the freed particles and Na⁺K⁺-ATPase by chromatographic
or electrophoretic procedures to see if they remain together, and to
attempt to rebind both to the membranes in reconstitution experiments.
If the Na⁺K⁺-ATPase is localized in one or the other types of
cell membrane projections, the mechanism of its synthesis and incor-
poration into the complex structure of the membrane would be of added
interest, particularly since its total activity is easily variable by
changes in environmental salinity. If increases in activity are due
to increased synthesis of enzyme, rather than activation of enzyme
already present, one can imagine at least two basic means of incorpor-
ation of the new enzyme molecules into the cell membrane. The first
consists of synthesis of complete new membrane subunits, containing
all the structural elements of the membrane and also the Na⁺K⁺-
ATPase. These subunits could subsequently aggregate into sections of
entirely new membrane at the cell surface. The second involves synthesis
of the Na⁺K⁺-ATPase (complex) alone, and its incorporation into the
existing cell membrane, possibly by specific enzymes. If the Na⁺K⁺-
ATPase were on the outside of the cell membrane, this latter process
could be compared to the extracellular synthesis of cell walls by
certain bacteria; both the newly synthesized macromolecules and the construction enzymes would probably have to pass through the cell membrane to the outside, either directly or in secretory vesicles.

**Pseudobranch fractionation-enzyme distributions**

As described above, the Na, K-ATPase was found distributed in all three particulate fractions, in relative amounts consistent with the distribution of cell membrane fragments. There were small, but probably significant, increases in the percent of activity in the P fraction associated with the greater total Na, K-ATPase activities which were observed when fish were kept in the salt water longer. This change in distribution probably accounted for part of the variability in the Na, K-ATPase distribution presented in Table 3. Results of incomplete fractionations used to obtain P fractions for kinetic studies were similar. An indication of the increase in specific activity of the P fractions was presented in Table 1. This observation suggests that the newly synthesized or activated Na, K-ATPase appearing during salt water adaptation occurs in that part of the cell membrane which ends up in the P fraction. Perhaps there is some functional difference between the subsets of cell membrane which sedimented in different fractions; for example, the small fragments in P could have come from newly-synthesized sections of membrane which were more easily fragmented by homogenization. More complicated explanations are also possible, of course.

The odd distribution of monoamine oxidase remains puzzling. As discussed above, it appears unlikely that it was associated solely
with mitochondrial outer membranes. Perhaps it has multiple localizations in pseudobranch cells. There are a number of possible locations, including cell membranes of various cell types (except blood cells), nuclear membranes, Golgi complexes, or endoplasmic reticulum. Innervation of the pseudobranch has been described (70); although such components must be minor contributions to fractions, monoamine oxidase could be concentrated in organelles such as synaptic vesicles or nerve cell membranes. Blood cells were excluded by the negative assay results on whole blood. The low monoamine oxidase activity observed in ML reduces the possibility that the enzyme was localized in the tubular fragments of pseudobranch cell membranes, and also the possibility that it is a soluble enzyme which was adsorbed to particulate material or trapped within membrane vesicles. Besides multiple localization of a single enzyme, the possibility that the assay measured two or more amine oxidases with different localizations is certainly worth considering.

There is also no ready explanation for the two-fold difference in monoamine oxidase activities of the homogenates. Since the pinfish used were not an inbred laboratory-raised population, but were obtained "wild" from their native environment, it is difficult to pin down a specific cause. Possible reasons include observed differences in fish size, the number of gill parasites (a few for the first fractionation, none for the second), and the length of time in salt water tanks. In addition, the salinity and pollution content of the source bayou could have varied significantly, e.g., as a result
of weather disturbances.

Rate sedimentation analysis of enzymes in ML

The results of the rate sedimentation experiments were basically incompatible with localization of Na\(^+\), K\(^+\)-ATPase in mitochondria. It is conceivable that the part (25%) of the Na\(^+\), K\(^+\)-ATPase which sedimented slightly was associated with a small, slowly sedimenting subset of the mitochondrial population, but this is unlikely because two new assumptions are involved: multiple localizations of Na\(^+\), K\(^+\)-ATPase and heterogeneity of a mitochondrial population. It is much more likely that the Na\(^+\), K\(^+\)-ATPase of the ML fraction was associated entirely with the abundant non-mitochondrial component of the fraction, namely tubular cell membrane fragments.

It was expected that the distribution of Mg\(^{2+}\)-ATPase would look much like that of cytochrome oxidase, since the enzyme distributions in N, ML; P and S suggested that most of the Mg\(^{2+}\)-ATPase in ML was of mitochondrial origin. However, some of the details of the distribution were unexpected. Both the present fractionation results and the previous results of others suggested that a Mg\(^{2+}\)-ATPase is localized in the cell membrane along with Na\(^+\), K\(^+\)-ATPase. However, the distributions of the ATPases in the gradient indicated that at least 75% of the Na\(^+\), K\(^+\)-ATPase of the ML fraction was associated with (unsedimented) particles containing no Mg\(^{2+}\)-ATPase. Perhaps the cell membranes do contain a Mg\(^{2+}\)-ATPase as well as Na\(^+\), K\(^+\)-ATPase, but the membrane is not homogeneous. That is, fragments from different regions of the cell membrane could differ in their relative concentrations of the two
ATPases, and also in their sedimentation properties.

This interpretation is supported by the observations of Evans (36) and Medzihradsky et al. (79). Evans separated a rat liver plasma membrane fraction into two major subfractions by equilibrium density gradient centrifugation, and observed differences in the distributions of Na\(^+\), K\(^+\)-ATPase and Mg\(^{2+}\) -ATPase. The lighter, vesicular subfraction had both Na\(^+\), K\(^+\)-ATPase and Mg\(^{2+}\) -ATPase activities, while the denser, sheetlike subfraction contained only Na\(^+\), K\(^+\)-ATPase. Medzihradsky et al. showed some separation of the Na\(^+\), K\(^+\)-ATPase and Mg\(^{2+}\) -ATPase of "solubilized" guinea pig microsomes by agarose chromatography. Thus it is possible, for example, that the fragments of pseudobranch cell membranes which sedimented in P contained both ATPases, and those which sedimented in ML contained Na\(^+\), K\(^+\)-ATPase but little or no Mg\(^{2+}\) -ATPase. This would be consistent with the indirect evidence discussed above for functional differences between the cell membrane fragments of ML and P.

A small amount (10\%) of unsedimented cytochrome c oxidase activity was observed in the rate sedimentation analysis of ML. This could have been associated with mitochondrial fragments. If so, this suggests that the mitochondrial Mg\(^{2+}\) -ATPase was not active in the fragments. A less likely explanation is heterogeneity of slow-sedimenting mitochon-
dria with respect to cytochrome oxidase and Mg\(^{2+}\) -ATPase activities.
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Figure 1. Light micrograph of pinfish pseudobranch, stained with periodic acid-Schiff and hematoxylin. X206. FL, fused leaflets; UL, unfused leaflets; C, cartilage; Br, vascular and connective tissue of branchial arch.
Figure 2. Light micrograph of pinfish pseudobranch, stained with P.A.S. and hematoxylin. X700. Ps, pseudobranch cell; S, sinusoidal blood space; Ep, squamous epithelial cells; Pi, pilaster cells.
Figure 3. Pinfish pseudobranch. X5350. Ps, pseudobranch cell; S, sinusoidal blood space; Pi, pilaster cell.
Figure 4. Pinfish pseudobranch. X8830. N, nucleus of pseudobranch cell; S, sinusoidal blood space; Ep, squamous epithelial cell; Ps, pseudobranch cell.
Figure 5. Pinfish pseudobranch. X12,400. T, tubular extensions of pseudobranch cell plasmalemma; M, mitochondrion; Er, erythrocyte; En, endothelial cell lining blood space.
Figure 6. Pinfish pseudobranch. X12,400. Ep, squamous epithelial cell; Ps, pseudobranch cell; T, tubular extensions of pseudobranch cell plasmalemma.
Figure 7. Variations of configuration of pseudobranch cell membrane invaginations. X14,500. T, tubular or sheetlike extensions of pseudobranch cell plasmalemma; M, mitochondria. A somewhat unusual configuration of mitochondrial cristae is shown in d.
Figure 8. Chloride-type cell in pinfish pseudobranch.

X14,500. A, apical crypt; V, subcrypt vesicles; T, tubular extensions of cell membranes; S, blood space.
Figure 10. Dependence of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (○) and Mg\textsuperscript{2+}-ATPase (□) activities on enzyme concentration. Reaction conditions are given in text.

Figure 11. Dependence of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase (○) and Mg\textsuperscript{2+}-ATPase (□) activities on time of reaction.
Figure 12. Dependence of total ATPase activity on concentration of the inhibitor ouabain. Comparison points (○) represent activities in the absence of ouabain, either with standard concentrations of both Na and K or with 120 mM K (no Na).

Figure 13. Substrate concentration curves for Na, K-ATPase (○) and Mg-ATPase (□). Concentrations of ATP and Mg were varied together.
Figure 14. Plots of $\frac{[S]}{v}$ vs. $[S]$ for $\text{Na}_2\text{K}^{+}\text{ATPase}$ (O) and $\text{Mg}^{2+}\text{ATPase}$ (□).

Figure 15. Dependence of $\text{Na}_2\text{K}^{+}\text{ATPase}$ (O) and $\text{Mg}^{2+}\text{ATPase}$ (□) activities on $\text{Mg}^{2+}$ concentration.
Figure 16. Dependence of Na⁺, K⁺-ATPase (O) and Mg²⁺-ATPase (□) activities on pH. Buffers used were 92 mM Tris (open points) and 92 mM imidazole (closed points).
Figure 17. Dependence of Na\textsuperscript{+}, K\textsuperscript{+} -ATPase activity on Na\textsuperscript{+} and K\textsuperscript{+} concentrations. Total Na\textsuperscript{+} + K\textsuperscript{+} concentrations were 60 mM. (O), 120 mM. (Δ), and 240 mM. (□). Tris ATP was used in this experiment.

Figure 18. Dependence of Mg\textsuperscript{2+} -ATPase activity on Na\textsuperscript{+} and K\textsuperscript{+}. Total Na\textsuperscript{+} + K\textsuperscript{+} concentrations were 60 mM. (O), 120 mM. (Δ), and 240 mM. (□). Tris ATP was used in this experiment.
Figure 19. Sections from nuclear fraction pellet.  a. Base line is where pellet rested on filter.  X13,000.  
b,c,d. Plasma membrane fragments in nuclear pellet.  Cell membrane of adjacent cells, held together by the basement layer, are shown in c.  X13,000.  N, nucleus;  Er, erythrocyte;  C, collagen;  M, mitochondrion.
Figure 20.  
Section from mitochondrial fraction pellet.  
X14,000.  M, mitochondria;  T, tubular cell membrane fragments.
Figure 21. Section from microsomal fraction pellet. The two micrographs were taken from adjacent areas and represent the entire thickness of the pellet. Dense small particles are probably glycogen. X9,600.
Figure 22. Section from microsomal pellet fixed in the presence of ruthenium red, unstained. X13,500.
Figure 23. Microsomal fraction, negatively stained with potassium phosphotungstate. Numerous tubular cell membrane fragments are visible. X105,000.
Figure 24. Microsomal fraction, negatively stained with ammonium molybdate. X185,000. PI, projections on intracellular surface of tubular plasmalemma fragments; PE, projections on extracellular surface of tubular fragments; PR, parallel rows of particulate material; M, mitochondrial fragments.
Figure 25. Mitochondria of ML fraction, negatively stained with ammonium molybdate. X79,500.
Figure 26. Tubular plasma membrane fragment in ML fraction, negatively stained with ammonium molybdate. X105,000. PI, projections on intracellular surface; PE, projections on extracellular surface; PR, parallel rows of particulate material; M, mitochondrial fragment.
Figure 27. Cell membrane fragments of ML fraction, negatively stained with ammonium molybdate. a. Projections are visible on smooth cell membrane which is continuous with tubular invaginations. X122,000.
b,c. X163,000. PI, projections on intracellular surface; PE, projections on extracellular surface.
Figure 28. Distribution of cytochrome c oxidase in N, ML, P and S fractions. In Figs 28 to 32, the ordinate is proportional to the specific activity of the enzyme in a given fraction, and the area of a block is the percent of the total activity in that fraction. Small circles represent the original values of % activity/% protein from the two complete fractionations.

Figure 29. Distribution of phosphoglucomutase.
Figure 30. Distribution of monoamine oxidase.

+ +

Figure 31. Distribution of Na, K-ATPase.
Figure 32. Distribution of Mg$^{2+}$ ATPase.
Figure 33. Density profile of the centrifuged, fractionated ML-sucrose gradient. The top and bottom of the centrifuge tube correspond to 0% and 100% volume, respectively. X = planned endpoints of gradient.

Figure 34. Distributions of cytochrome c oxidase (———), $^+ + \text{Na}^+$, $^+ + \text{K}^+$ -ATPase (———), and $^{2+} \text{Mg}^{2+}$ -ATPase (-----) in ML density gradient fractions.
Figure 35. Distribution of cytochrome c oxidase in ML gradient.

Figure 36. Plot of the slope of the cytochrome c oxidase distribution. See text for interpretation.