

LOCALIZATION OF Na^+ , K^+ -ATPase AND OTHER ENZYMES IN TELEOST PSEUDOBRANCH

I. Biochemical Characterization of Subcellular Fractions

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ABSTRACT

In an effort to determine the subcellular localization of sodium- and potassium-activated adenosine triphosphatase (Na^+ , K^+ -ATPase) in the pseudobranch of the pinfish *Lagodon rhomboides*, this tissue was fractionated by differential centrifugation and the activities of several marker enzymes in the fractions were measured. Cytochrome *c* oxidase was found primarily in the mitochondrial-light mitochondrial (M+L) fraction. Phosphoglucosmutase appeared almost exclusively in the soluble (S) fraction. Monoamine oxidase was concentrated in the nuclear (N) fraction, with a significant amount also in the microsomal (P) fraction but little in M+L or S. Na^+ , K^+ -ATPase and ouabain insensitive Mg^{2+} -ATPase were distributed in N, M+L, and P, the former having its highest specific activity in P and the latter in M+L. Rate sedimentation analysis of the M+L fraction indicated that cytochrome *c* oxidase and Mg^{2+} -ATPase were associated with a rapidly sedimenting particle population (presumably mitochondria), while Na^+ , K^+ -ATPase was found primarily in a slowly sedimenting component. At least 75% of the Na^+ , K^+ -ATPase in M+L appeared to be associated with structures containing no Mg^{2+} -ATPase. Kinetic properties of the two ATPases were studied in the P fraction and were typical of these enzymes in other tissues. Na^+ , K^+ -ATPase activity was highly dependent on the ratio of Na^+ and K^+ concentrations but independent of absolute concentrations over at least a fourfold range.

INTRODUCTION

The transport of the monovalent cations Na^+ and K^+ , both at the level of the individual cell's plasma membrane and across the epithelial cell layers of many secretory organs, apparently underlies many basic functions in living organisms (37, 48). A major clue in the study of the mechanism of such ion transport by animal cells has been provided by the evidence (30, 42) linking it with the enzyme Na^+ - and K^+ -activated adeno-

sine triphosphatase (Na^+ , K^+ -ATPase). If Na^+ , K^+ -ATPase is all or part of the actual ion pump, rather than a nonspecific component of transporting cells which is involved in the maintenance of the metabolic state needed for transport, a plasma membrane localization might be expected. Much effort has been spent on localizing Na^+ , K^+ -ATPase; the fact that localization attempts are still being made indicates that a

thoroughly satisfactory procedure has yet to be developed. ATPase cytochemistry has been applied to numerous tissues, including teleost pseudobranch (33), but a number of special difficulties (18) have greatly hampered the specific localization of ouabain-sensitive Na^+ , K^+ -ATPase. Cell fractionation techniques have provided evidence which is consistent with localization of Na^+ , K^+ -ATPase in the plasma membrane (see, e.g., 30, 42), but the difficulty of distinguishing fragments of plasma membrane from those of other membranous organelles makes such evidence good only for mammalian erythrocytes (30, 42) and rat liver (e.g., 4, 15).

Data concerning localization is particularly limited for salt-secreting epithelia such as certain invertebrate glands, elasmobranch rectal gland, teleost gills, and reptile and bird nasal salt glands. These ultrastructurally similar tissues have the highest known Na^+ , K^+ -ATPase activities and are specialized for transcellular ion transport. To facilitate understanding of the transport mechanisms of these tissues, subcellular localization of the Na^+ , K^+ -ATPase is required. A new cytochemical approach to this problem has been presented by Ernst (17, 18), who developed a procedure for localizing ouabain-sensitive K^+ -dependent phosphatase in duckling salt gland. This enzyme is believed to be part of the Na^+ , K^+ -ATPase complex. Most of the enzyme activity is inhibited in this procedure, but the uninhibited part was found primarily in the basal infoldings of the plasma membrane.

The tissue studied in the present investigations was the pseudobranch of the teleost *Lagodon rhomboides* (pinfish). Pseudobranchs, or false gills, are small paired organs adjacent to the gills, to which they are closely related in embryological origin, in gross and microscopic appearance, and in the presence of a high Na^+ , K^+ -ATPase activity which increases with adaptation of the fish to greater salinities (16, 24, 28, 40). Unlike gills, the pseudobranch is very homogeneous in structure (9, 28) and thus well suited to cell fractionation studies. Its predominant cell type, characterized by numerous mitochondria and tubular invaginations of the plasma membrane but very little endoplasmic reticulum or other cytoplasmic organelles, is nearly identical to the chloride cells of gill, which are currently thought by many to be the site of salt secretion in a seawater environment (36).

The present paper will consider biochemical evidence for the localization of Na^+ , K^+ -ATPase in pseudobranch and kinetic properties of this enzyme. The morphological characteristics of pinfish pseudobranch and of subcellular fractions obtained from it by differential centrifugation will be presented in the following paper.

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 seawater, made with Seven Seas Marine Mix, usually for periods less than or equal to 1 wk. The fish had no difficulty adapting to full-strength seawater directly, and they could be kept healthy in captivity at least several months.

Tissue Homogenization

Pseudobranchs, averaging 10 mg each in weight, were excised from pinfish after severing the spinal cord. They were blotted and trimmed 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 pseudobranchs were weighed and homogenized in ice-cold 0.3 M sucrose with three slow strokes of a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle.

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.

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. Inorganic phosphate was measured by the method of Fiske and Subbarow (22), with minor modifications.

ATPases: The final concentrations of reagents in the reaction mixtures were typically 3mM ATP (disodium or Tris salt), 3 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 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 octahy-

drate. Activity without ouabain is referred to as total ATPase, that with ouabain as Mg^{2+} -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 min. Samples were kept cold until the start of the phosphate assay to minimize acid hydrolysis of ATP. The ATPases were found to be stable for many months when frozen.

CYTOCHROME *c* OXIDASE: The assay method of Cooperstein and Lazarow, as applied by Appelmans et al. (1), was used with minor modifications.

PHOSPHOGLUCOMUTASE: Samples were preincubated about 20 min at 0°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°C for 60 min in a total volume of 1 ml; with standard reaction conditions phosphoglucosmutase activity remained constant for at least 60 min. The final concentrations of reagents were 4 mM glucose-1-phosphate, pH 7.5, 5×10^{-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 5 N H_2SO_4 , and hydrolysis of acid-labile phosphate (including glucose-1-phosphate) was completed by placing the tubes in a boiling bath for 5 min. 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.

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, pH 7.6. The reaction was carried out at 37°C for 6 h, and stopped by precipitation with $Ba(OH)_2$ and $ZnSO_4$ (46). The optical densities of the supernatants were measured at 250 nm as a measure of the concentration of product benzaldehyde.

PROTEIN: The Lowry method (34) was used, with bovine serum albumin as a standard.

HEMOGLOBIN: The assay was based on the method of Snell and Snell (43). 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 min.

Fractionation by Differential Centrifugation

All procedures were performed at 2°–4°C. The basic centrifugation scheme was similar to that described by Deter (10). The fractions obtained are designated E (cytoplasmic extract), N (nuclear),

M + L (mitochondrial-light mitochondrial), P (microsomal), and S (soluble). Fresh fractions were used for assay of cytochrome *c* oxidase. Samples of fractions were deep frozen at approximately –70°C 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 effects of repeated freeze thaws.

Rate Sedimentation Centrifugation

All procedures were carried out near 2°C. Linear sucrose gradients were prepared with a Beckman Density Gradient Former (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.), the mixing chamber of which was modified to be similar to that designed by H. Beaufay (personal communication). Cell fraction material was suspended in both starting solutions so that it would be distributed homogeneously throughout the final gradient. Shallow linear gradients, approximately 1.038–1.076 g/ml at 2°C and about 4.5 ml in volume, were used. A SW 65L Ti swinging-bucket rotor was used in a Beckman L2-65B Ultracentrifuge which was modified to allow slow manual acceleration and deceleration at speeds below 2,000 rpm.¹ A Beckman ω^2t Integrator Accessory and a Photovolt recorder for continuous monitoring of slow speeds were also attached. Centrifugation conditions were designed to produce a total $W = \int_0^t \omega^2 dt$ (12) of 24×10^7 rad²/s. Total time in the centrifuge was approximately 60 min.

Fraction Collection

A collection system was developed (Fig. 1) as a modification of the ISCO Fractionator (Instrumentation Specialties Co., Lincoln, Neb.), in order to fractionate the pellet-containing gradients. After the gradient tube is positioned under the cone (A), the knife blade (E) is pushed forward to sever the tube and separate the pellet from the rest of the gradient. Leakage around the knife is prevented with Beckman vacuum grease. The gradient is displaced upward through the cone by a pushing fluid which is introduced via a needle inserted through a side port (F), rubber seal (G), and the tube wall into the bottom of the gradient. Trichlorotrifluoroethane (Freon 113, E. I. Du Pont de Nemours and Co., Wilmington, Del.) was used as the pushing fluid since it is immiscible with water, has a low viscosity, and has a density of 1.579 g/cm³. Gradient fractions were collected in preweighed vials from the top of the cone. The pellet was removed from

¹ The authors are indebted to Mr. V. M. Cummings, Beckman Instruments Co., Houston, Texas, for suggesting the appropriate modifications.

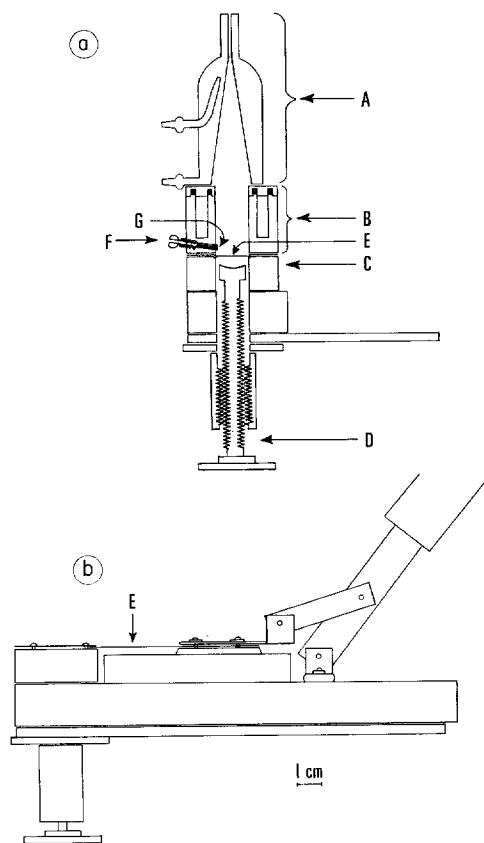


FIGURE 1 Gradient collection system, designed for use with gradients containing pellets. (a) Cross section of entire apparatus. (b) Side view of cutting device. A, glass collecting cone; B, water jacket; C, block in which gradient tube is placed; D, screw for lifting gradient tube; E, pointed knife blade; F, needle port; G, rubber seal. See text for details of operation. Scale, 1 cm.

below the knife and suspended in 0.3 M sucrose. Fraction densities were determined as described by Beaufay et al. (6). 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.

RESULTS

Preliminary Experiments

A significant amount of blood was trapped in the pseudobranchs when dissected and homogenized. Hemoglobin assays of pinfish blood and pseudobranch indicated that approximately 20% of the weight of dissected pseudobranchs consisted of blood. Therefore, pinfish blood was as-

sayed for the particulate enzymes of uncertain origin, namely the ATPases and monoamine oxidase, to check the possibility that they were localized in blood elements, especially the nuclear and plasma membranes of red blood cells. Negligible if any Na^+ , K^+ -ATPase, Mg^{2+} -ATPase, or monoamine oxidase was detected in fresh or frozen blood.

Pseudobranch Fractionation-Enzyme Distributions

Biochemical assay results for two complete fractionations are presented in Table I and Fig. 2. 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 N and E fractions. The E fraction consists of all the components destined for M+L, 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 in the initial wash steps and/or to actual material loss by sample transfers. (Material losses are implied by the protein recovery values.)

CYTOCHROME *c* OXIDASE (E.C. 1.9.3.1): The localization of cytochrome *c* oxidase in mitochondria exclusively has been demonstrated for a wide variety of tissues and organisms (14). More specifically, the electron transfer chain, including cytochrome *c* oxidase, has been located in the inner membrane (cristae) of the mitochondria (26). The distribution of cytochrome *c* oxidase in pseudobranch fractions was nearly identical to those obtained for rat liver by the same fractionation procedures, both by the authors and by de Duve et al. (13). Most was in the M+L fraction, with a small but significant amount in N, very little in P, and none in S. The specific activity was greatest by far in the M+L fraction. 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.

PHOSPHOGLUCOMUTASE (E.C. 2.7.5.1): Phosphoglucomutase has been localized in the soluble cytoplasm of a number of tissues, including rat liver (14). It was found almost exclusively in the S fraction of pinfish pseudobranch.

MONOAMINE OXIDASE (E.C. 1.4.3.4): Monoamine oxidase is considered a marker en-

TABLE I
Enzyme Distributions in Fractions of Pinfish *Pseudobranch*

Enzyme	Exp. no.	Activity in H (U/g)	Activity in fractions (%)				Recovery (%)	
			N	M+L	P	S	%(E+N)	%H
Cytochrome <i>c</i> oxidase	1	14.8	16.1	80.8	3.0	0.0	78.7	65.5
	2	16.1	9.1	86.9	4.0	0.0	79.2	59.0
Phosphoglucomutase	1	8.2	1.0	1.8	0.5	96.7	87.4	84.8
	2	10.2	0.8	1.6	1.1	96.5	97.4	86.8
Monoamine oxidase	1	0.031	56	13	31	0	77	62
	2	0.059	47	13	28	12	94	74
Na ⁺ , K ⁺ -ATPase	1	27.6	31.2	42.2	26.6	0.0	92.4	105.4
	2	30.0	20.2	44.9	31.8	3.1	111.7	114.9
Mg ²⁺ -ATPase	1	42.5	12.4	66.4	14.3	6.8	86.7	74.4
	2	45.1	11.7	61.6	19.2	7.5	90.2	80.2
Protein	1	125	20.5	22.4	8.7	48.5	94.5	84.8
	2	127	18.2	23.6	9.4	48.8	96.3	81.0

Enzyme activities were measured in the homogenate (H) and in N, M+L, P, S, and E fractions. Results are given for two complete fractionations. Enzyme recoveries were determined in two ways, the significance of which is discussed in the text. Percent activities in the fractions are normalized to 100% recovery. One unit of enzyme activity is the amount of enzyme which converts 1 μ mol of substrate/min, except in the case of cytochrome *c* oxidase. For this latter enzyme, one unit is the amount of enzyme which will reduce the decadic logarithm of the optical density of 100 ml of reaction mixture by one unit per minute. Protein is given in milligrams using bovine serum albumin as the standard. All activities in H are given as units per gram wet weight of pseudobranch.

zyme for mitochondrial outer membranes in rat liver (27), although there have also been reports of high specific activities of the enzyme in nuclear and plasma membrane fractions from liver (25). Monoamine oxidase has been found in mitochondrial and microsomal fractions of other tissues in variable proportions (e.g., 8, 21, 44). A soluble monoamine oxidase has also frequently been observed (5). The distribution of monoamine oxidase in pseudobranch fractions was unusual in two respects: the percent of activity in M+L was quite low, while the percent in N was surprisingly high. The distribution was completely different from that obtained for cytochrome *c* oxidase. Another striking observation regarding monoamine oxidase was the fact that the 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. We have no ready explanation for this.

Na⁺, K⁺-ATPase (E.C. 3.6.1.3): As shown

in Table I, significant amounts of this enzyme were found in all three particulate fractions, with little or none in S. The specific activity was greatest in P. The distributions of Na⁺, K⁺-ATPase in other tissues fractionated similarly have usually consisted of concentrations in nuclear and/or microsomal fractions (see, e.g., 30). A large concentration of the enzyme in a mitochondria-rich fraction, such as was observed here for pseudobranch, has been found infrequently (e.g., 29, 38), the Na⁺, K⁺-ATPase being separable from the mitochondria by subsequent fractionation of the mitochondrial fraction.

Mg²⁺-ATPase (E.C. 3.6.1.3): The assay for ouabain-insensitive ATPase (Mg²⁺-ATPase) almost certainly measures more than one enzyme in most tissue homogenates. There is a Mg²⁺-ATPase associated with oxidative phosphorylation in mitochondria, probably located in the projections on the inner membrane (32). There is also a Mg²⁺-ATPase activity which apparently appears wherever Na⁺, K⁺-ATPase does, and

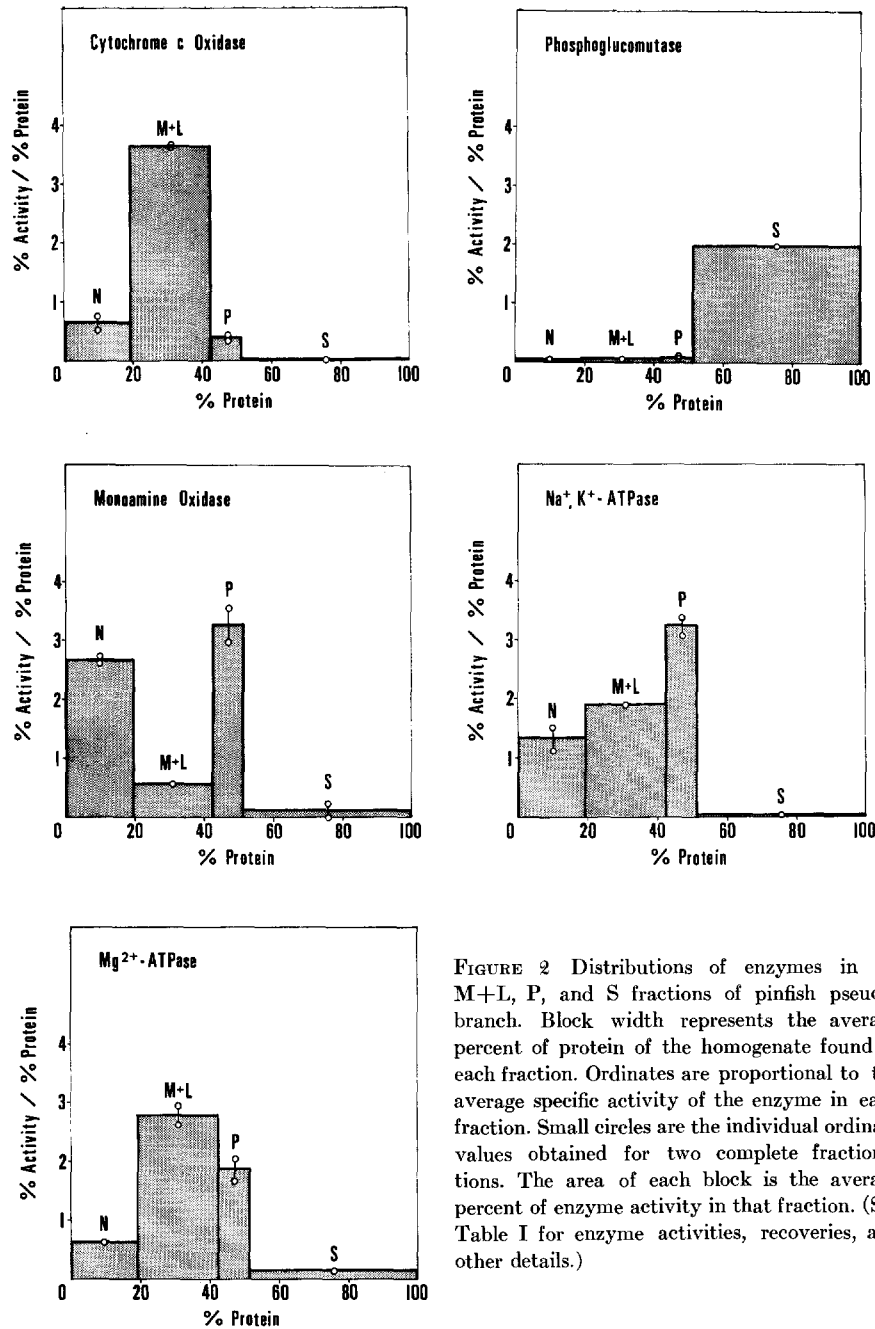


FIGURE 2 Distributions of enzymes in N, M+L, P, and S fractions of pinfish pseudobranch. Block width represents the average percent of protein of the homogenate found in each fraction. Ordinates are proportional to the average specific activity of the enzyme in each fraction. Small circles are the individual ordinate values obtained for two complete fractionations. The area of each block is the average percent of enzyme activity in that fraction. (See Table I for enzyme activities, recoveries, and other details.)

may or may not be functionally related to it (see, e.g., 2, 19, 42). Both of these are ubiquitous in animal cells so that one would expect to find them in pseudobranch, and there might well be other Mg²⁺-ATPases. Most of the ouabain-insensitive ATPase of pseudobranch was recovered in M+L, with significant amounts also in N and P.

Rate Sedimentation Analysis of Enzymes in M+L Fraction

Although the distribution of Na⁺, K⁺-ATPase did not parallel that of mitochondrial cytochrome *c* oxidase (or that of monoamine oxidase), the finding of an unusually high percentage of Na⁺,

K^+ -ATPase in M+L left open the possibility of a dual localization of the enzyme, in both mitochondria and nonmitochondrial particles. For this reason the M+L fraction was subjected to rate sedimentation analysis. The principles of this type of centrifugation have been discussed by de Duve et al. (12) and Deter (10). Initially the fraction material is suspended homogeneously in a fairly shallow gradient. Centrifugation is interrupted before complete sedimentation and the distribution of enzymes in the gradient is determined. This method produces boundaries, rather than bands, of subcellular constituents.

The distributions of cytochrome *c* oxidase, Mg^{2+} -ATPase, and Na^+ , K^+ -ATPase obtained in a typical rate sedimentation study of the pseudobranch M+L fraction are presented in Fig. 3. The extrapolated starting and ending densities of the centrifuged gradient, 1.0375 and 1.0745 g/ml at 2°C, were quite close to those planned, namely 1.038 and 1.076, respectively. The final 7.8% of the recovered volume corresponds to the pellet fraction. The volume recovery after fraction collection was approximately 99%. The gradient was very nearly linear over its entire range. Enzyme activities are expressed as percents of the initial activities, which were constant throughout the gradient before centrifugation. The initial activities were determined from samples of the heavy and light sucrose-M+L fraction mixtures used to make the gradient. Enzyme activities in the pellet fraction are not shown, since they were about two to five times as great as the initial activities. The recoveries of the enzymes in the 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.

It is immediately apparent that the distributions of cytochrome *c* oxidase and Mg^{2+} -ATPase were quite similar 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 in rat liver cytoplasmic extract fractions treated similarly, while the distribution of Na^+ , K^+ -ATPase resembled that of the liver microsomal enzyme glucose-6-phosphatase (11). The Na^+ , K^+ -ATPase distribution differed from those of cytochrome *c* oxidase and Mg^{2+} -ATPase both in the amount of completely unsedimented enzyme (75% vs. 10% and 0%) and in the position of the end of the boundary (25% of the gradient

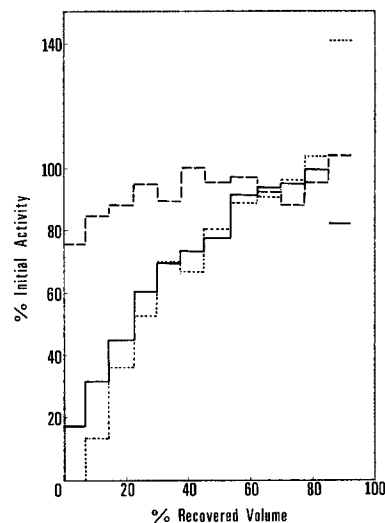
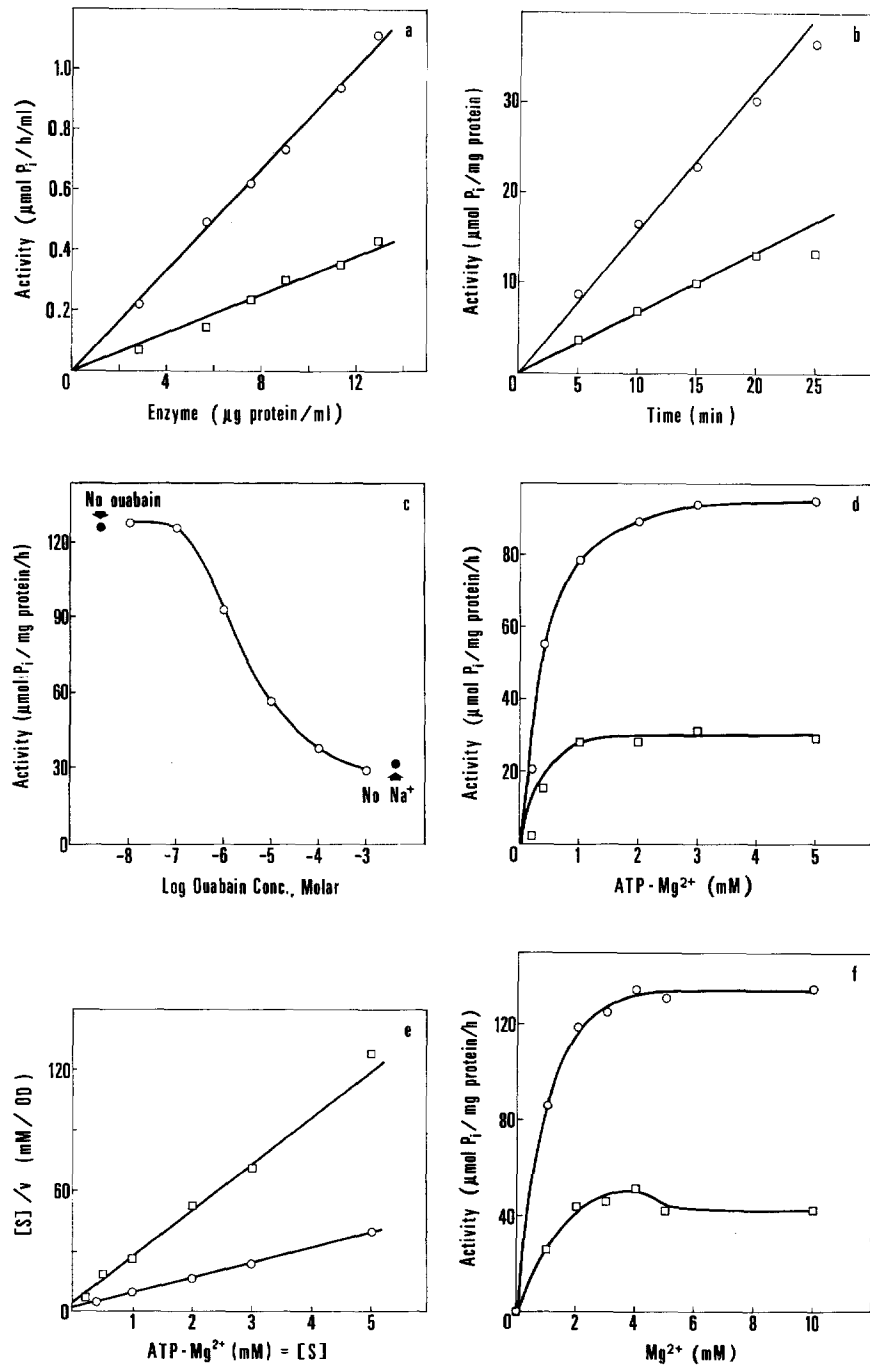


FIGURE 3 Rate sedimentation study of M+L fraction. Distributions of cytochrome *c* oxidase (—), Na^+ , K^+ -ATPase (---), and Mg^{2+} -ATPase (---) in gradient fractions. The top and bottom of the gradient correspond to 0% and 100% volume, respectively. Enzyme activities are expressed as percents of initial activities in the fractions, which were constant throughout the gradient before centrifugation. Activities were normalized for enzyme recoveries. Pellet values are not plotted (see text).

volume vs. about 80 or 90%). The simplest explanation for these results is that most of the Mg^{2+} -ATPase of the M+L fraction was associated with mitochondria, and that most or all of the Na^+ , K^+ -ATPase belonged to a completely different class of particles with a smaller average sedimentation constant. 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 implies that at least 75% of the Na^+ , K^+ -ATPase activity was localized in particles containing no measurable Mg^{2+} -ATPase.

Kinetic Properties of ATPases in P Fraction

The major reason for studying the kinetic properties of the Na^+ , K^+ -ATPase of pseudobranch was to compare it with Na^+ , K^+ -ATPases from other sources. Since Na^+ , K^+ -ATPase cannot be measured without measuring ouabain-insensitive Mg^{2+} -ATPase, the properties of the latter were



FIGURES 4 a-f

also determined. Unless otherwise stated, reaction conditions were as given in Materials and Methods. The P fraction was used for these experiments because of its high specific activity of Na⁺, K⁺-

ATPase. Contamination of the Mg²⁺-ATPase activity in this fraction with mitochondrial ATPase is not likely to be significant. The reaction temperature of 37°C was chosen to facilitate com-

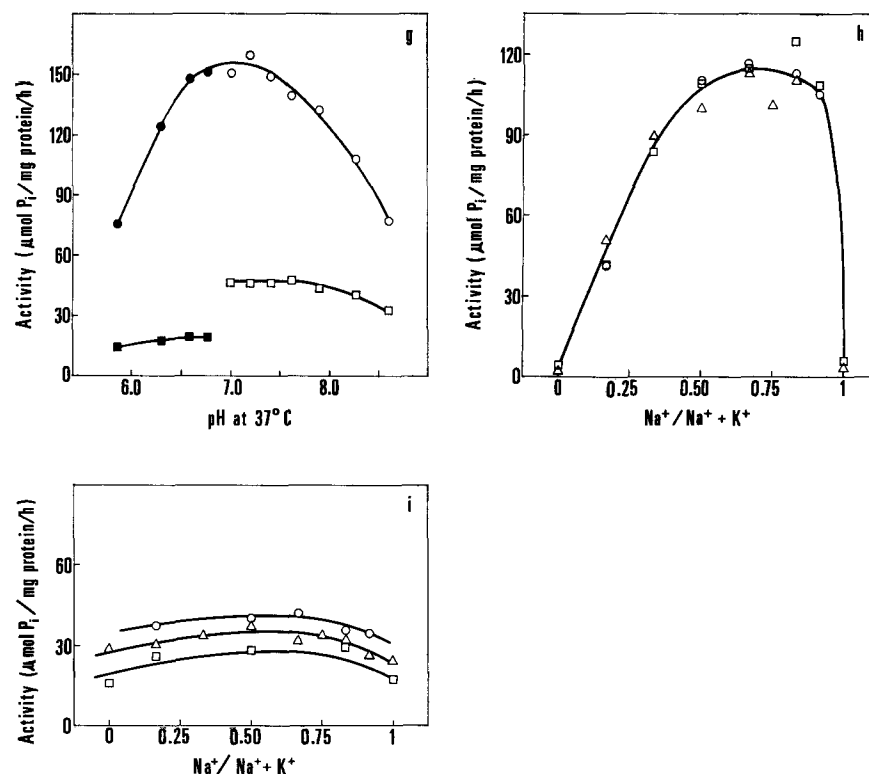


FIGURE 4 Kinetic properties of Na^+ , K^+ -ATPase and Mg^{2+} -ATPase in P fraction. Unless specified otherwise, reaction conditions were as given in Materials and Methods and curves for Na^+ , K^+ -ATPase (\circ) and Mg^{2+} -ATPase (\square) are drawn together. Except in *e*, ordinates are all enzyme activity, given in the units shown. (a) Dependence of activities on tissue concentration. Abscissa gives concentration of total P fraction protein in reaction mixture. (b) Dependence of activities on reaction time. (c) Dependence of total ATPase activity on concentration of the inhibitor ouabain. For comparison ATPase activities without ouabain, either with standard concentrations of both Na^+ (100 mM) and K^+ (20 mM) or with 120 mM K^+ (no Na^+), are given (\bullet). (d) Dependence of activities on substrate concentration. Concentrations of ATP and Mg^{2+} were varied together. (e) Graphical determination of K_m and V_{max} (see text). (f) Dependence on Mg^{2+} concentration. (g) Dependence of Na^+ , K^+ -ATPase (\circ or \bullet) and Mg^{2+} -ATPase (\square or \blacksquare) on pH. Buffers used were either 92 mM Tris (\circ , \square) or 92 mM imidazole (\bullet , \blacksquare). (h, i) Dependence of Na^+ , K^+ -ATPase (h) and Mg^{2+} -ATPase (i) on Na^+ and K^+ concentrations. (Na^+ + K^+) concentrations were 60 mM (\circ), 120 mM (Δ), and 240 mM (\square). The abscissa is the fraction of the (Na^+ + K^+) concentration contributed by Na^+ . Tris-ATP was used.

parison with results from other tissues. Although the water in which the pinfish live is probably never as warm as 37°C, it is unlikely that this temperature had any adverse effect on the pseudobranch Na^+ , K^+ -ATPase, since its activity was extremely high in comparison to activities in other tissues (7).

TISSUE CONCENTRATION: Fig. 4 *a* shows that the amount of product formed in a 15 min interval was proportional to the amount of P fraction in the reaction mixture for both ATPases,

over the range of concentrations used in other experiments.

TIME OF REACTION: Using standard reagent and enzyme concentrations at 37°C, the rate of reaction of both ATPases remained constant for about 20 min, as seen in Fig. 4 *b*. The standard assay time was 15 min, so that problems related to depletion of substrate, accumulation of inhibitory reaction products, and decrease in enzyme activity were avoided.

OUABAIN CONCENTRATION: Fig. 4 *c* 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. 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.

SUBSTRATE CONCENTRATION: Concentrations of ATP and MgCl_2 were varied together. The Na^+ , K^+ -ATPase activity leveled off around 2 or 3 mM ATP-Mg, and Mg^{2+} -ATPase around 1 mM ATP-Mg. Fig. 4 *e* is a plot of the data in one of the linear forms (47) of the Michaelis-Menten equation:

$$\frac{[S]}{v} = \frac{1}{V_{\max}} [S] + \frac{K_m}{V_{\max}},$$

where $[S]$ is the substrate concentration, v the reaction rate, V_{\max} the maximum reaction rate, and K_m the substrate concentration at half-maximal reaction rate. The values of K_m obtained from the graphs were 0.27 mM for Na^+ , K^+ -ATPase and 0.22 mM for Mg^{2+} -ATPase. Values of V_{\max} were 98.7 and 32.6 $\mu\text{mol P}_i/\text{mg protein per h}$ for Na^+ , K^+ -ATPase and Mg^{2+} -ATPase, respectively.

Mg^{2+} CONCENTRATION: As seen in Fig. 4 *f*, both ATPases were completely dependent upon Mg^{2+} . With 3 mM ATP, the Na^+ , K^+ -ATPase had optimal activity at about 3 or 4 mM Mg^{2+} , and slightly lower activity at higher concentrations.

pH: The values of pH plotted in Fig. 4 *g* are those 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. There was apparently some inhibition of the Mg^{2+} -ATPase by imidazole buffer. Aside from this, the activity exhibited a broad range of optimal values of pH from about 6.6 to 7.7, with little decrease in activity as low as pH 5.8 or as high as 8.6.

Na^+ AND K^+ CONCENTRATIONS: Three sets of points are plotted together in Fig. 4 *h*. In each set the total concentration of $\text{NaCl} + \text{KCl}$ was kept constant while the relative concentrations of Na^+ and K^+ were varied. 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 $\text{Na}^+ + \text{K}^+$ concentration in the range 100–150 mM, designed to approximate in vivo intracellular conditions (e.g., 39, 45). 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 fourfold 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 relative concentrations of the two ions. The range of optimal ionic concentration ratios was rather wide, from about 1 Na^+ : 1 K^+ ($\text{Na}^+/\text{Na}^+ + \text{K}^+ = 0.5$) to 11 Na^+ : 1 K^+ ($\text{Na}^+/\text{Na}^+ + \text{K}^+ = 0.9$). Absolute dependence on both Na^+ and K^+ was demonstrated. It may also be noted that the left and right halves of Fig. 4 *h* closely resemble curves obtained by varying Na^+ or K^+ alone, with the concentration of the other ion kept constant at the standard assay concentration (e.g., 3, 19, 39, 41).

The Mg^{2+} -ATPase was much less affected by Na^+ and K^+ , as shown in Fig. 4 *i*. The small increase in activity in the presence of both ions, and its slight variation with ionic ratio, can probably be accounted for by the small amount of Na^+ , K^+ -ATPase left uninhibited by 10^{-4} M ouabain, although variable inhibition by ouabain at different ionic ratios may also be important (35). More significant was the moderate inhibition of activity by greater $\text{Na}^+ + \text{K}^+$ total concentrations.

Transport-Related Properties of Na^+ , K^+ -ATPase

RELATION OF ENZYME ACTIVITY TO SALT WATER ADAPTATION: A positive correlation between the salinity of the environment and the Na^+ , K^+ -ATPase activity of fish gills and pseudobranch has been observed previously by a number

of workers (16, 36). 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 seawater for different periods of time before use. Fish kept in seawater for a week or more consistently had approximately 70% greater activities of Na^+ , K^+ -ATPase in homogenates of pseudobranch than those used the day after they were caught. Since the P fraction was the fraction with the highest specific activity of Na^+ , K^+ -ATPase, it was interesting to note that large increases (up to 200%) in the Na^+ , K^+ -ATPase specific activity of this fraction were associated with increases in salt water adaptation time.

COMPARISON OF GILL AND PSEUDOBRANCH: The ATPase activities of pinfish gill and pseudobranch homogenates were compared for evaluation of the possible significance of an ion transport role for the pseudobranch. These measurements indicate that, of the total Na^+ , K^+ -ATPase activity found in the gills and pseudobranchs, approximately 15–20% is in the pseudobranchs and 80–85% in the gills. The activity per unit wet weight was four times greater in pseudobranch than in gill, while the activity per unit protein of pseudobranch was twice that of gill. These results correlate with the observation that pseudobranch tissue contains a considerably larger proportion of cells having highly invaginated plasma membranes (chloride cells and variants). They also indicate that the pseudobranchs could make a significant contribution to any transport process dependent on Na^+ , K^+ -ATPase, despite their small size.

DISCUSSION

Localization of Na^+ , K^+ -ATPase

Although one would expect the enzyme(s) responsible for transport of Na^+ and K^+ to be localized largely in the plasma membrane, it should be kept in mind that such an enzyme does not necessarily have to be found entirely in the plasma membrane for it to be equated with the transport agent. It might have intracellular roles as well, and at the time of its synthesis it could easily be located elsewhere. On the other hand, it is not necessary to assume that the transport enzyme is distributed homogeneously in all parts of the plasma membrane. It is especially easy to

visualize asymmetric distributions of ion pumps in the case of secretory epithelia, since monovalent cations are moved primarily in one direction across the cell layers and the configurations of the plasma membranes at the basal and apical ends of the cells are distinctly different.

In the present experiments Na^+ , K^+ -ATPase was found distributed among the three particulate fractions. If this enzyme is found in a single subcellular constituent in pseudobranch, this observation suggests localization in a structure which fragments into particles of widely variable size or degree of agglutination. Pseudobranch cells are characterized by highly complex invaginated plasma membranes (9, 28) which would be likely to fragment into pieces of variable size during homogenization; localization of the Na^+ , K^+ -ATPase in such membrane fragments could explain our results. Of course it is also possible that this enzyme is concentrated in less noticeable membranous components of pseudobranch, such as nuclear membranes, endoplasmic reticulum, or Golgi membranes. (Blood cell membranes were excluded by the negative assay results on whole blood.) The high specific activity of Na^+ , K^+ -ATPase in the P fraction is consistent with a membrane localization, while the virtual absence of a known soluble enzyme, phosphoglucomutase, in the three particulate fractions indicates that soluble enzyme adsorption to, or entrapment within, particles is most unlikely.

The results of the rate sedimentation analysis of the M+L fraction suggest that most of the Na^+ , K^+ -ATPase cannot be localized 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. However, this requires two new assumptions: multiple localizations of Na^+ , K^+ -ATPase and biochemical heterogeneity of the mitochondrial population. Localization in the plasma membrane fragments found in this fraction (9) is a more likely explanation of the observed sedimentation behavior of this enzyme.

Membrane Heterogeneity

The increased specific activity of the enzyme in P fractions after salt water adaptation may indicate that the newly synthesized or activated Na^+ ,

K^+ -ATPase goes first into that part of the plasma membrane which sediments in this fraction. Perhaps the small fragments in P came from newly synthesized sections of membrane which were more susceptible to disruption during homogenization. Further indirect evidence for membrane heterogeneity is provided by the rate sedimentation analysis of the M+L fraction. The results of others (2, 42) have often suggested that a Mg^{2+} -ATPase is localized in plasma membranes along with Na^+ , K^+ -ATPase, and may even be structurally related to it. The present differential centrifugation results are consistent with this hypothesis. However, the sedimentation behavior of the ATPases of M+L indicate that at least 75% of the Na^+ , K^+ -ATPase of this fraction was associated with unsedimented particles containing no Mg^{2+} -ATPase. If plasma membranes do contain a Mg^{2+} -ATPase as well as Na^+ , K^+ -ATPase, such membranes are not homogeneous. The concept of biochemical heterogeneity of plasma membranes is supported by the observations of several other groups of workers (e.g., 20, 31, 23). It is quite possible that the fragments of pseudobranch plasma membrane sedimenting in P contained both ATPases, while most of those which sedimented in M+L contained Na^+ , K^+ -ATPase but little or no Mg^{2+} -ATPase.

Localization of Other Enzymes

The odd distribution of monoamine oxidase remains puzzling. The low activity in M+L suggests either that most of the mitochondria lost their outer membranes during fractionation or that monoamine oxidase is not localized exclusively in mitochondria in pseudobranch. There are two reasons for preferring the latter explanation. First, it would be difficult to explain why such a large proportion of the outer membrane fragments sedimented in N. Second, electron microscope examination of pseudobranch fractions provided no evidence for large-scale loss of mitochondrial outer membranes (9). Perhaps monoamine oxidase has multiple localizations in pseudobranch cells: possibilities include plasma membranes of various cell types (except blood cells), nuclear membranes, Golgi complexes, endoplasmic reticulum, or synaptic vesicles from nerve cells. The low monoamine oxidase activity in M+L reduces the possibility that the enzyme was located in Na^+ , K^+ -ATPase-bearing particles, at least those of M+L. It also reduces the possi-

bility that the monoamine oxidase is a soluble enzyme which was nonspecifically adsorbed to particulate material. Besides multiple localizations of a single enzyme, the possibility that the assay measured two or more amine oxidases with different localizations is certainly worth considering.

Biochemical characterization of pseudobranch fractions was handicapped by a lack of previous enzyme localization work on either pseudobranch or the closely related gills. Extrapolation of the localization of a marker enzyme from other tissues to pseudobranch is reasonable for such a well-documented universal marker as cytochrome *c* oxidase, but risky for enzymes whose localizations are confined primarily to a few mammalian tissues. This was the principal reason for not measuring supposed markers for nuclear and Golgi membranes, endoplasmic reticulum, and other possible components in the fractions. Their localizations in pseudobranch would have had to be evaluated before their distributions could be used to evaluate fraction contents. Electron microscope study of the fractions was carried out to help overcome some of these limitations. These results are discussed in the following paper.

Kinetic Properties of Na^+ , K^+ -ATPase

The kinetic properties of Na^+ , K^+ -ATPase from pinfish pseudobranch fell easily within the range of variability of the Na^+ , K^+ -ATPases from many other sources (e.g., 3, 19, 39, 41). The results thus provided no reason to suspect that this Na^+ , K^+ -ATPase differs from the others in molecular mechanism or 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 relative concentrations of Na^+ and K^+ over a fourfold range of total concentrations. This result was of course obtained for a preparation of membrane fragments 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. The rather wide range of ionic ratios ($Na^+/Na^+ + K^+$) consistent with optimal activity may be mechanistically significant or it may just result from the artificial situation of having the same ionic composition on both sides of the membrane. Nonetheless, the apparently prime importance of the relative concentrations of Na^+ and K^+ should be kept in

mind when evaluating mechanistic models, especially the proposed specificities and interactions of ion-binding sites.

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