from FW to SW. This finding, therefore, cannot be used to establish a connection between SCN inhibition of ATPase and active chloride transport by the gill.

1973 #47

AUTORADIOGRAPHIC LOCALIZATION OF ^{3}H-OUABAIN BINDING BY Na-K-ATPase IN PERFUSED GILLS OF *Fundulus heterclitus*

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Since the activity of sodium-potassium-activated adenosine triphosphatase (Na-K-ATPase) is especially high in tissues specialized for high level electrolyte transport and its activity appears to be correlated with the osmotic work load of the gland or tissue it has frequently been suggested that this enzyme plays an important role in transepithelial electrolyte transport. Clearly one of the first steps in evaluating this role is localization of the enzyme in the epithelial layers and cells responsible for transport. Our approach to the localization of Na-K-ATPase has taken advantage of the specific binding and inhibitory action of the cardiac glycoside, ouabain. We have examined a most important effector organ of teleost osmoregulation, the branchial epithelium, which in euryhaline fish adapted to varying concentrations of sea water exhibits clearly different rates of sodium transport and activities of Na-K-ATPase (Maetz, J., Phil. Trans. B, 262, 209, 1971).

Specimens of *Fundulus heteroclitus* were adapted for two to five weeks to varying concentrations (2X, 1X, and 0.1X) of artificial sea water (SW) maintained at 18-22°C. For experimentation, specimens were pithed, and mounted in a tray and the perfusion fluid, with or without ^{3}H-ouabain, was introduced at a rate of about 0.1 ml/minute via a cannula in the bulbous arteriosus. The liver was cut to provide for drainage. The gill preparation was maintained near 20 °C and kept moist by irrigation with saline solution, usually 280 mM NaCl. The perfusion fluid consisted of a modified Forster's fish medium (Science, 108, 65, 1948) with 135 mM NaCl, 7.5 mM $NaHCO_3$ and one to two percent albumin. Blanching of the gill filaments and tracer dye, Lissamine green, served as visual checks on the effectiveness of the perfusion. During the course of the perfusion (generally 70 minutes) individual gill arches were removed at various intervals and processed for: a) liquid scintillation counting by standard methods, b) Na-K-ATPase assay by a modification of the technique of Epstein *et. al.* (Science, 156, 1245, 1967) or c) autoradiography by the rapid freezing, freeze-drying, and plastic-embedding technique of Stirling (J. Cell Biol. 53, 704, 1972).

Differences in the external salinity are reflected in the significantly different plasma sodium concentrations of adapted fish (Table 1). Assays of homogenates of non-perfused gill tissue established that the greater the external salinity, the greater the specific activity of Na-K-ATPase. The fact that these fresh tissue values were essentially identical to the values from non-ouabain perfused fish indicates that the Na-K-ATPase survived the perfusion procedure well. Most perfusion experiments were run with 50 µM ouabain, which apparently saturated the tissue binding sites (Table 1, 2nd footnote). In pilot experiments the ouabain distribution reached a steady state within 45 minutes and only a volume approximating extracellular space could be washed out in the next 25 minutes. Thus ouabain present after 25 minute washout was considered bound to gill tissue. The extensive inhibition of Na-K-ATPase by 50 µM ouabain (Table 1) suggests that the perfusion technique is effective in exposing ouabain to at least 85 percent of the Na-K-ATPase binding sites. Finally, and most important

TABLE 1

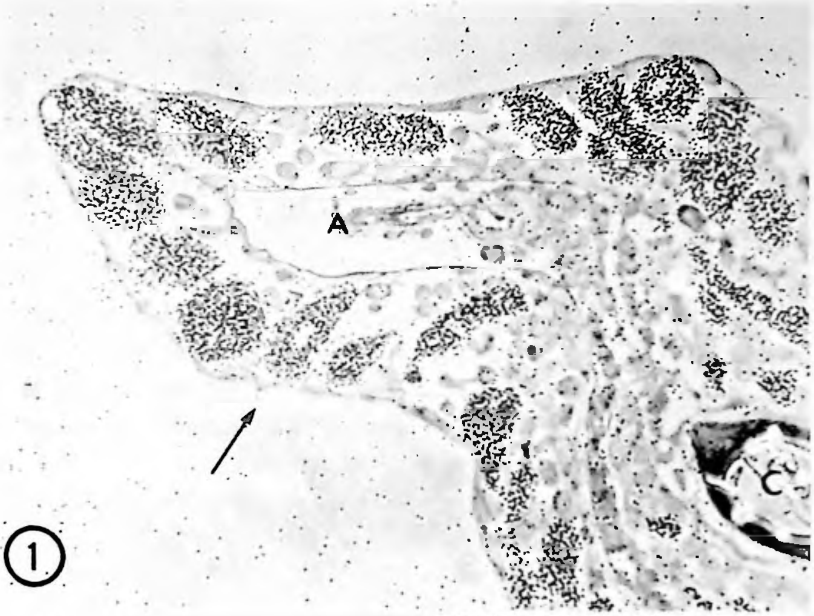
CORRELATION BETWEEN TRANSPORT ATPase AND ^{3}H-OUABAIN BINDING IN GILL TISSUE OF KILLIFISH ADAPTED IN ARTIFICIAL SEA WATER (SW) MIXTURES OF VARYING SALINITY*

		Gill Na-K-ATPase Activity				
Fish Adaptation		Fresh	Perfused (70 min total)		Gill Ouabain**	
Environment	Plasma Na^+	Tissue	Forster's Medium	50 µM** ouabain	Washout† Space	Tissue Binding
(times SW)	(meq/1)	(µmoles P_i/mg protein X hr)			(% tissue)	(umoles/kg)
2X	207 ± 6 (6)	29 ± 3 (6)	32 (2)	5 ± 1 (4)	16 ± 2 (7)	12 ± 1 (7)
1X	175 ± 5 (6)	14 ± 2 (6)	17 (2)	2 (2)	13 ± 2 (8)	8 ± 1 (8)
0.1X	158 ± 4 (6)	8 ± 1 (6)	9 (2)	2 (2)	11 ± 2 (5)	5 ± 1 (5)

*Values generally expressed as mean ± SE (n), where n is number of fish. Plasma Na^+, fresh gill ATPase, and tissue binding of ouabain in 2X and 0.1XSW fish differed significantly from 1XSW values ($P < 0.01$, except <0.025 for binding in 0.1XSW).

**Based on *in situ* vascular perfusion with 50 uM ouabain for 45 minutes followed by washout with ouabain-free Forster's medium for 25 minutes. Higher ouabain concentrations did not greatly alter ATPase inhibition, ouabain washout space, or tissue binding, e.g., binding in 1XSW fish with 100 uM ouabain remained 8 ± 1 (4).

†Difference between steady state ouabain distribution volumes before and after 25 minute washout with ouabain-free medium.



①

Figure 1. ^{3}H-ouabain autoradiograph from 2×SW fish: portion of a gill filament near afferent blood vessel (A) and cartilaginous shaft (C). Grain density (above background) occurs almost exclusively over chloride cells. Note absence of grains over nucleus of chloride cell (arrow). 600X

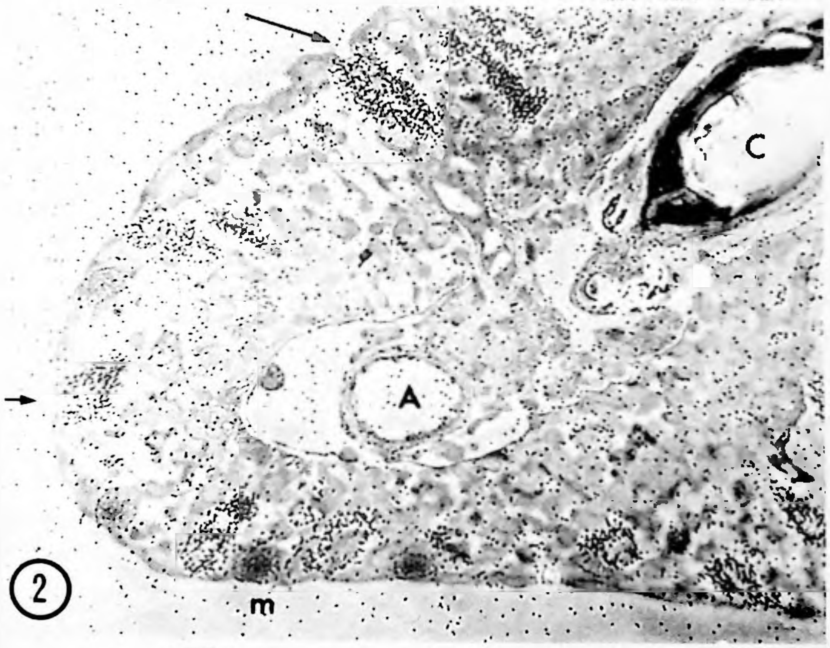


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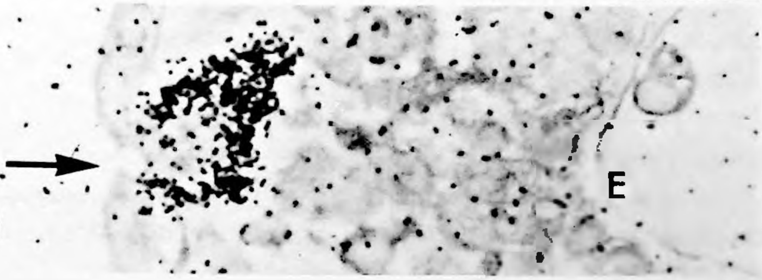
Figure 2. ^{3}H-ouabain autoradiograph from 0.1×SW fish. Chloride cells (arrows) appear to be smaller and less numerous in this environment, but the grain density and distribution is similar to that in 2×SW fish. Numerous mucous cells (m) are found only in 0.1×SW fish. 600X



③

Figure 3. Enlargement of tip of gill filament in Figure 2 showing conspicuous absence of silver grains in area adjacent to the apical crypt membrane. The basal portion of this chloride cell (not in the plane of the section) presumably contacts the extravascular space (E). 1300X

differences in tissue binding for the three environments parallel the Na-K-ATPase activity, i.e., the ouabain appears to be bound specifically by this enzyme.

Autoradiographs from 1 µm plastic sections of gills perfused with 3 µM ^{3}H-ouabain (20 µCi/ml) exhibit adequate morphological perservation for light microscopy (Figures 1-3). In the three environments virtually all of the silver grains (above background) were localized to the chloride cells. Moreover the largest and most numerous chloride cells were observed in 2X SW fish which also possess the highest ouabain binding and Na-K-ATPase activity. These results and Kamiya's recent demonstration of Na-K-ATPase in chloride cells with cell fractionation techniques (Comp. Biochem. Physiol. 43B, 611, 1972) provide strong evidence for most of the enzyme being located in these cells. Although the question of the intracellular location of the enzyme cannot be definitively answered with the resolution of light microscope autoradiography the grain pattern follows closely the distribution of the tubular system (cell surface invaginations), i.e., both are uniformly distributed throughout the cell except for the nucleus and the area adjacent to the apical crypt (Figures 1-3).

Of final interest is the preliminary autoradiographic observation that when the outside of the gill in 2X and 1X SW fish is exposed to ^{3}H-ouabain via the irrigation fluid there is no heavy labelling of the apical crypt membrane of chloride cells. This finding does not support the view of Maetz (*op. cit.*) that sodium extrusion from the gill into sea water involves a Na-K-ATPase located at the chloride cell apical membrane.

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1973 #48

SINGLE NEPHRON FILTRATION RATE IN THE HAGFISH *Myxine glutinosa*

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Because of its discrete anatomical structure the nephron of the hagfish lends itself to studies of pressure-flow relationships across the glomerular membrane. We have begun such a study and report here some basic parameters of the filtration process hoping that this simple model may eventually further our understanding of the more complex relationships of glomerular filtration in higher forms.

Hagfish (80 - 140 g) freshly caught in Frenchman Bay were prepared for kidney micropuncture as described in a previous report (Bull. MDIBL 11:11, 1971). Aortic and cardinal vein pressures were measured with a simple water column device attached to indwelling needles. Microperfusion of single glomeruli was performed by catheterization of afferent arterioles via segmental branches of the aorta. Perfusion pressure was monitored through an L-shaped glass tubing in parallel with the perfusion device. Quantitative collection of the perfused glomerular filtrate was accomplished by retrograde cannulation of the archinephric duct. The perfusion fluid contained (mM/L) sodium 530, potassium 9.0, calcium 6.0, magnesium sulfate 18 and 30 mg percent glucose. Inulin ^{14}C was added as a marker for fluid reabsorption.

The hydrostatic pressure in the aorta under control (non-perfused) conditions was 5.4 ± 0.11