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STUDIES ON GILL ATPASE OF RAINBOW TROUT (*SALMO GAIRDNERI*)

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SUMMARY

1. Microsomal ATPase activities from gills of fresh water- and salt water-adapted rainbow trout (*Salmo gairdneri*) were examined at temperatures of 13 °C and 37 °C.

2. The alkali metal-stimulated activity measured at 13 °C was enhanced by preincubating the reaction mixture, *minus* ATP, for 30 min at 37 °C. This procedure nearly eliminated "baseline" Mg^{2+}-ATPase activity. The salt water "baseline" showed a greater temperature sensitivity than that of fresh water.

3. The enzyme from salt water fish required both Na^+ (100 mM) and K^+ (20 mM) for maximal activation at 13 °C. The K_m for Na^+ was 7 mM and for K^+, 0.8 mM. $5 \cdot 10^{-4}$ M ouabain completely inhibited the alkali metal stimulation ($K_i = 1.3 \cdot 10^{-5}$ M). Na^+ alone was ineffective in stimulating activity at 13 and 37 °C.

4. The enzyme from fresh water fish required only Na^+ (200 mM) for maximal stimulation at 13 °C, although a small amount of K^+ stimulation was sometimes seen. The K_m for Na^+ was 25 mM. This activity was unaffected by $5 \cdot 10^{-4}$ M ouabain; $5 \cdot 10^{-3}$ M ouabain inhibited only about 30%. At 37 °C, K^+, in addition to Na^+, was required for maximal activity. This K^+ stimulation was inhibited by $5 \cdot 10^{-4}$ M ouabain while the Na^+ stimulation remained relatively insensitive to the inhibitor.

5. The fresh water enzyme required 2.5 mM Mg^{2+} ($K_m = 0.9$ mM) for optimal activity at 13 °C and the salt water enzyme required $[Mg^{2+}] = 5$ mM ($K_m = 1.0$ mM).

6. The fresh water enzyme showed maximal activity over wide ranges of pH (6.6–8.0) whereas the salt water enzyme showed a distinct optimum at pH 7.1.

7. The alkali metal-activated ATPase activity was greater in the gills of salt water fish than those adapted to fresh water.

INTRODUCTION

The presence of $Na^+ + K^+$-activated ATPase has now been demonstrated in gill tissue preparations of a wide variety of teleost fishes[1–7]. It has become evident that the specific activity of this enzyme is greater in the gills of sea water fish than those of fresh water fish, and this difference has been attributed to the increased load of Na^+ in marine forms which must be pumped across the gills from the blood into

Abbreviations: MES, 2-(*N*-morpholino)ethanesulphonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

the external environment. That the $Na^+ + K^+$-activated ATPase is involved in active Na^+ transport in a number of epithelia concerned with absorption has been argued for a number of years. But problems arise when one attempts to translate increased enzyme activity in marine teleosts into a model for Na^+ extrusion across gills. Some of these have been described by Kirschner[4] and Motais[6]. They will be discussed further below.

Motais[6] suggested that two different $(Na^+ + K^+)$-ATPases are involved in Na^+ transport in the fresh water and salt water-adapted European eel, *Anguilla anguilla*, because actinomycin D abolished the increase in ATPase activity noted during adaptation to salt water but had little effect on the level normally seen in fresh water. The purpose of the present paper is to describe some characteristics of gill ATPase activity in the euryhaline rainbow trout, *Salmo gairdneri* Richardson, and to offer further evidence that ATPases with markedly different characteristics may be involved in fresh and salt water adaptation. A preliminary report on some of these results has already appeared[8].

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*S. gairdneri*), weighing 150–300 g, were obtained from a commercial hatchery and kept unfed in large holding tanks. Fresh water animals were held at 8 °C for periods up to two months. Several days before use they were transferred to a holding tank at 13 °C. Fish were adapted to sea water in two steps. They were first placed in 50% sea water (Instant Ocean from Aquarium Systems, Inc., Eastlake, Ohio) at 13 °C for a period of 7 days and then transferred to 100% sea water also at 13 °C for at least one week before use.

Preparation of enzyme

Experimental animals were anesthetized with 0.1% tricaine methanesulfonate. The gills were immediately perfused through the ventral aorta with a solution containing 0.1 M Tris–HCl, pH 7.8 at 4 °C, 5.0 mM $MgCl_2$, and 5.0 mM Na_2EDTA to remove blood from the gills. Filaments were then cut from the gill arches and homogenized in the perfusing solution (1 g of gill/10 ml) to which approximately 3 mg of Cleland's Reagent (dithiothreitol; Calbiochem) had been added. The homogenate was centrifuged at 9750 × *g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 70000 × *g* for 30 min at 0 °C. The microsomal pellet was then treated with NaI using a modification of the method described by Nakao *et al.*[9]. The pellet was suspended in 10 ml of 2.0 M NaI containing 50 mM Tris–HCl, 2.5 mM Cleland's Reagent, 3.7 mM $MgCl_2$, 2.5 mM ATP (disodium salt), 2.5 mM Na_2EDTA, pH 7.7 at 0 °C. After 30 min the solution was diluted with 15.0 ml H_2O (NaI concentration = 0.8 M) and centrifuged at 70000 × *g* for 30 min. The pellet was washed twice, once with the perfusing solution and once with water, and finally suspended in water containing 1 mM Cleland's Reagent. The final protein concentration was approximately 1–2 mg/ml. One aliquot was assayed immediately; the rest was stored at 0 °C. Under these conditions the $(Na^+ + K^+)$-enzyme retained over 80 % of its activity for 7 days, although activity decreased much more rapidly in the absence of Cleland's Reagent.

Preparation of buffers

In order to determine pH optima a series of buffers from pH 5.2 to 9.8 were prepared. The buffers included 2-(*N'*-morpholino)ethanesulfonic acid (MES) (pH 5.2–6.1), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 6.6–7.8) and Tris–HCl (pH 8.0–9.8). MES and HEPES buffers were made to the desired pH with Tris. All pH determinations were made at 13 °C.

Assay of enzyme

Maximum specific activity was obtained in a reaction mixture which contained 30 mM HEPES, pH 7.1, 5.0 mM $MgCl_2$, 3.4 mM Tris–ATP, 100 or 200 mM NaCl, 20 mM KCl, and an appropriate amount of enzyme (usually 0.1–0.2 mg/ml). Both Na^+ and K^+ were omitted in the determination of Mg^{2+}-ATPase activity, and K^+ was omitted when stimulation by Na^+ alone was to be measured. The final volume was 1.0 ml for all mixtures. The reaction system, minus ATP, was equilibrated at the temperature of the run for 10 min, and the reaction was started by adding the ATP.

After 30 min the reaction was stopped by the addition of 1.0 ml of 15% trichloroacetic acid. The reaction mixture was then centrifuged at 27000 × *g* for 5 min and a 1.0 ml aliquot of the supernatant taken for determination of inorganic phosphate by the method of Fiske and SubbaRow[10]. Protein was determined by a slightly modified method of Lowry *et al.*[11] using bovine plasma albumin as a standard. Vallejo and Lagunas[12] have shown that sulfhydryl compounds will interfere with this method, and we found that dithiothreitol acted as they described. Therefore protein values were corrected for the presence of Cleland's Reagent, or protein was measured before it was added.

Most of the measurements were made at 13 °C because the fish had been adapted to that temperature. It has become apparent that the kinetic parameters of enzymes from poikilothermic animals are sensitive to both adaptation and assay temperature[13]. In addition, one of our objectives was to compare enzymic with transport data, and our flux measurements have all been made at 13 °C. However, no other laboratory employs such low temperatures, and to compare our preparation with others some experiments were run at 37 °C.

Preparation of Tris–ATP

1.6 g of ATP, disodium salt (Calbiochem) was dissolved in 5 ml of water and placed on a 0.9 cm × 9.5 cm column of Bio Rad AG 1-X4 (200–400 mesh; chloride form) at 4 °C. Impurities of ADP and inorganic phosphate were eluted with 80 ml of 0.02 M NH_4Cl in 0.02 M HCl. The column was then washed with 80 ml of water to remove the ammonium ions and the ATP eluted with 50 ml of 0.3 M HCl. The solution was neutralized with 2.2 g Tris, and the ATP concentration was measured in a Beckman DB spectrophotometer at 259 nm.

RESULTS

Preparations of gill homogenates from fresh water fish showed no alkali metal stimulation at 13 °C or 37 °C. The same was true for salt water fish at 13 °C, but some $Na^+ + K^+$ activity was noted at 37 °C. Specific activities for fresh water Mg^{2+}-ATPase and (Mg^{2+},Na^+,K^+)-ATPase were, respectively, 6.1 and 5.9 μmoles·mg^{-1}·h^{-1} at

13 °C and 8.4 and 8.7 at 37 °C. For salt water fish they were 4.6 and 3.7 μmoles·mg^{-1}·h^{-1} at 13° and 5.7 and 9.6 at 37 °C. Similar observations have been made on eel gill homogenates using a 25 °C assay temperature[2]. We found that alkali metal stimulation could be demonstrated in fresh water fish, as well as those from salt water, by using NaI-treated microsomes, and the following results were obtained with this type of preparation.

Initially all reaction mixtures were incubated without ATP for 10 min at the experimental temperature before beginning the measurements. The Mg^{2+}-baseline activity was high at both 13 °C and 37 °C, and alkali metal stimulation was small, especially at the lower temperature. One notable feature was that baseline activity had a temperature coefficient less than unity as can be seen by comparing the appropriate values in Tables I and II (for fresh water fish 10.4 μmoles·mg^{-1}·h^{-1} at 13 °C and 7.2 μmoles·mg^{-1}·h^{-1} at 37 °C; for salt water fish 6.8 and 4.2 μmoles·mg^{-1}·h^{-1} at the same two temperatures). It appeared that the enzyme was becoming inactivated at the higher temperature, and this was confirmed as follows. Aliquots of the reaction mixture were preincubated at 37 °C for different times up to 60 min. The temperature was then lowered to 13 °C and 10 min later ATP was added to all tubes. The results for fresh water and salt water preparations can be seen in Fig. 1. With no 37 °C

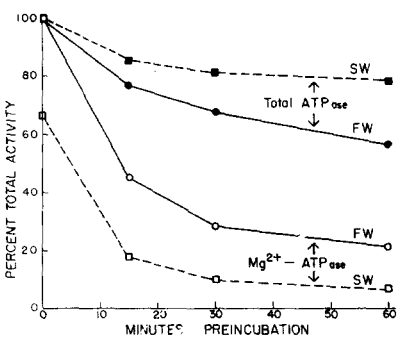

Fig. 1. Effect of preincubation at 37 °C on gill ATPase activity from fresh water (circles) and salt water (squares) -adapted trout. Assay temperature was 13 °C. The values are expressed as percent of total unpreincubated activity. Total ATPase (solid characters) represents activity with 5 mM Mg^{2+}, 100 mM Na^+ and 20 mM K^+ (salt water) or 5 mM Mg^{2+} and 200 mM Na^+ (fresh water). Mg^{2+}-ATPase (open characters) represents activity with 5 mM Mg^{2+} in the reaction mixture. The values are means of two experiments.

treatment baseline activity comprised the total activity in fresh water and 66% of the total in salt water. Incubation at 37 °C for 15 min reduced the salt water Mg^{2+}-ATPase by more than 70%, and after an hour it was negligible. In contrast, the $Na^+ + K^+$-stimulated activity (total minus Mg^{2+}) increased after 15 min at the higher temperature, then remained nearly constant. Similar data were obtained with microsomes from fresh water fish, although Mg^{2+}-ATPase activity was less severely reduced by the preincubation. Whatever the cause, thermal lability of the Mg^{2+}-ATPase affords a means of reducing or nearly eliminating the baseline activity. Hence, all subsequent experiments were run after 30 min preincubation at 37 °C.

Table I shows mean specific activities of gill ATPase from fresh water and salt water-adapted trout assayed at 13 °C, and the effects of preincubation at 37 °C are obvious. In fresh water fish assayed without preincubation baseline activity

TABLE I

ACTIVITY OF FRESH WATER AND SALT WATER TROUT GILL ATPase MEASURED AT 13° C

Reaction mixture contained 100 mM Na^+ for Na^+-ATPase determinations and 100 mM Na^+ and 20 mM K^+ for $(Na^+ + K^+)$-ATPase determinations. The Mg^{2+} column represents "baseline" Mg^{2+}-ATPase activity. The Na^+ column represents the increase (or decrease) from the baseline when Na^+ is added (*i.e.* $(Mg^{2+} + Na^+)$-ATPase *minus* the baseline). The $Na^+ + K^+$ column represents the increase in activity when both Na^+ and K^+ are added (*i.e.* $(Mg^{2+} + Na^+ + K^+)$-ATPase *minus* the baseline). Values represent the means ± S.E. (n) = number of enzyme assays.

Adaptation medium	*Specific activity* (μmoles $P_i \cdot mg^{-1}$ *protein* $\cdot h^{-1}$)					
	(n)	Mg^{2+}	(n)	Na^+	(n)	$Na^+ + K^+$
Fresh water	(6)	10.4 ± 0.9	(4)	0.4 ± 0.5	(6)	1.3 ± 0.8
Fresh water*	(10)	1.1 ± 0.1	(5)	3.8 ± 0.6	(10)	3.7 ± 0.4
Salt water	(12)	6.8 ± 0.7	(4)	−0.9 ± 0.7	(12)	3.4 ± 0.5
Salt water*	(10)	0.5 ± 0.1	(5)	0.9 ± 0.2	(10)	6.3 ± 0.6

* Reaction mixture, *minus* ATP, preincubated for 30 min at 37 °C.

was high and stimulation, either by Na^+ alone or by $Na^+ + K^+$, was not significant. However, when the microsomes were preincubated the baseline was reduced, and a significant Na^+ stimulation was observed. Addition of K^+ had little further effect. In a few experiments some K^+ activation occurred but was less than 25% of the Na^+-stimulated activity. In most, ATPase activity was maximal in the presence of Na^+ alone, and the mean values with and without K^+ reflect this fact. Baseline activity in salt water preparations was reduced nearly to zero by preincubation, but Na^+ alone was ineffective in stimulating ATPase activity; both Na^+ and K^+ were required. The data also show that alkali metal stimulation in salt water fish is about twice that in fresh water fish assayed at this temperature.

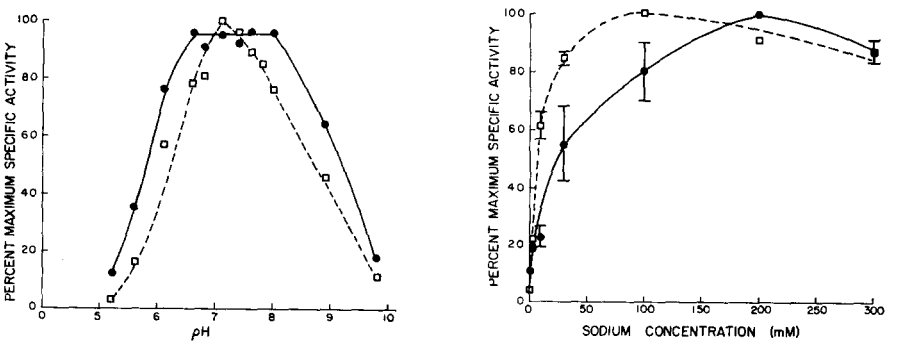

Fig. 2. Effect of pH on the fresh water Na^+-ATPase (●) and the salt water $(Na^+ + K^+)$-ATPase (□). Activity was measured at 13 °C following a 30 min preincubation at 37 °C. The reaction mixture contained 200 mM Na^+ for fresh water determinations and 100 mM Na^+ *plus* 20 mM K^+ for salt water determinations. The values are means of two experiments. Representative maximum specific activities (± S.E.) for Figs 2–6 are 6.3 ± 0.6 μmoles $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ (n = 10) for salt water and 4.6 ± 0.5 (n = 6) for fresh water.

Fig. 3. Na^+ concentration dependence of the fresh water Na^+-ATPase (●) and the salt water $(Na^+ + K^+)$-ATPase (□). Activity was measured at 13 °C following a 30 min preincubation at 37 °C. The salt water reaction mixture contained 20 mM K^+. Vertical lines represent standard errors of the mean for three experiments.

The pH dependence of the fresh water Na^+-ATPase and the salt water ($Na^+ + K^+$)-ATPase assayed at 13° is shown in Fig. 2.

The fresh water enzyme showed a broad pH optimum with maximal activity from pH 6.6–8.0. The pH activity curve for the salt water enzyme differed from that seen in fresh water in showing a distinct optimum around pH 7.1. This is in close agreement with values reported for the ($Na^+ + K^+$)-ATPase from other species[14]. However, the response to pH seen in the fresh water enzyme does not agree with the optimum of 6.4 found with the Na^+-ATPase of calf brain microsomes[15]. As will be seen these two preparations, although both activated by Na^+, also differ in their sensitivity to ouabain.

The Na^+ concentration dependence of the fresh water Na^+-ATPase and the salt water ($Na^+ + K^+$)-ATPase assayed at 13 °C is shown in Fig. 3. Maximum activity of the fresh water enzyme was obtained with $[Na^+]$=200 mM with a small, but reproducible decrease at 300 mM. Half-maximal activity was obtained at about 25 mM (K_m = 25 mM). Optimal activation of the salt water enzyme occurred at 100 mM ($[K^+]$=20 mM). The K_m for this enzyme was about 7 mM. Inhibition was observed at Na^+ concentrations above 100 mM.

Fig. 4 shows the effect of varying $[K^+]$ on both fresh water and salt water preparations. Maximal activation of the fresh water enzyme required no added K^+, and the endogenous K^+ concentration in these reaction mixtures was shown, by atomic absorption spectrophotometry, to be less than 0.1 mM. The apparent increase in activity going from no K^+ to 0.2 mM K^+ is due to one low value (73%) obtained in the absence of this ion. High concentrations of K^+ were slightly inhibitory. In contrast, the salt water enzyme required K^+. Optimum concentration ($[Na^+]$ = 100 mM) was 20 mM and K_m = 0.8 mM. Such differences in K^+ dependence suggested that the two preparations might show different sensitivities to the cardiac glycosides

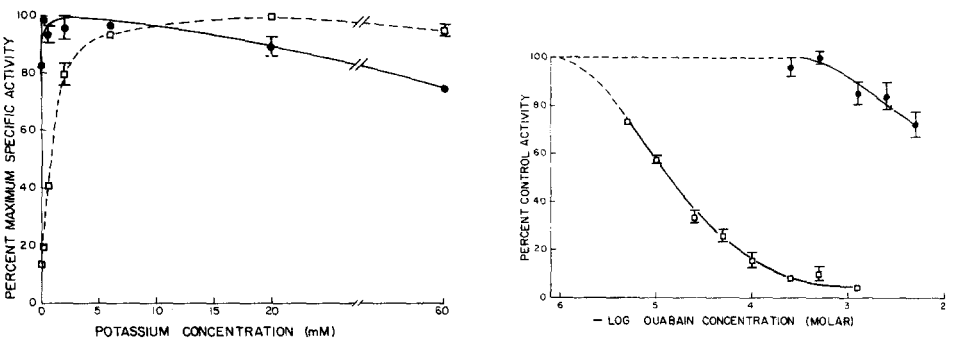

Fig. 4. K^+ concentration dependence of the fresh water Na^+-ATPase (●) and the salt water ($Na^+ + K^+$)-ATPase (□). Activity was measured at 13 °C following a 30 min preincubation at 37 °C. Reaction mixtures contained 200 mM Na^+ for fresh water and 100 mM Na^+ for salt water determinations. Vertical lines represent ranges of two experiments for fresh water and standard errors of the mean (n = 3) for salt water.

Fig. 5. Effect of ouabain on the fresh water Na^+-ATPase (●) and the salt water ($Na^+ + K^+$)-ATPase (□). Activity was measured at 13 °C following a 30 min preincubation at 37 °C. Ouabain was included in the reaction mixture during both preincubation and assay. The values are expressed as percent of control (ouabain-free) activity. The fresh water system contained 200 mM Na^+ and the salt water system 100 mM Na^+ *plus* 20 mM K^+. Vertical lines represent standard errors of the mean for three experiments.

Fig. 5 shows that ouabain (strophanthin G) inhibited the salt water ($Na^+ + K^+$)-ATPase. With these assay conditions half-maximum inhibition was noted at $1.3 \cdot 10^{-5}$ M and the enzyme was completely inhibited at $5 \cdot 10^{-4}$ M. In contrast, the fresh water activity was unaffected by ouabain at $5 \cdot 10^{-4}$ M and 70% of the control activity was obtained at $5 \cdot 10^{-3}$ M.

The Mg^{2+} dependence of both preparations is shown in Fig. 6. The fresh water enzyme had an optimum concentration of 2.5 mM and its activity was little changed at twice this concentration. K_m was 0.9 mM. The salt water enzyme showed optimal activity at $[Mg^{2+}] = 5$ mM with a K_m of 1.0 mM. Both enzymes were inhibited at higher concentrations, but the fresh water system was considerably more sensitive. These results are similar to those obtained for crab nerve where a doubling of the optimum Mg^{2+} concentration (3 mM to 6 mM) was found when K^+ was added to a Na^+-ATPase preparation[16].

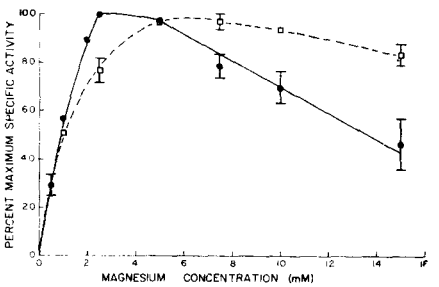

Fig. 6. Mg^{2+} concentration dependence of the fresh water Na^+-ATPase (●) and the salt water ($Na^+ + K^+$)-ATPase (□). Activity was measured at 13 °C following a 30 min preincubation at 37 °C. The reaction mixture contained 200 mM Na^+ for fresh water determinations and 100 mM Na^+ *plus* 20 mM K^+ for salt water determinations. Vertical lines represent ranges of two experiments for fresh water and standard errors of the mean ($n = 3$) for salt water.

TABLE II

ACTIVITY OF FRESH WATER AND SALT WATER TROUT GILL ATPase MEASURED AT 37 °C

All conditions same as in Table I.

Adaptation medium	*Specific activity* (μmoles $P_i \cdot mg^{-1}$ *protein* $\cdot h^{-1}$)					
	(*n*)	Mg^{2+}	(*n*)	Na^+	(*n*)	$Na^+ + K^+$
Fresh water	(7)	7.2 ± 0.9	(5)	7.5 ± 1.1	(7)	12.6 ± 1.7
Fresh water*	(5)	3.2 ± 0.5	(5)	7.4 ± 0.6	(5)	10.2 ± 0.6
Salt water	(10)	4.2 ± 0.8	(5)	1.1 ± 0.5	(10)	23.6 ± 2.1
Salt water*	(3)	0.6 ± 0.3	(3)	1.1 ± 0.4	(3)	19.8 ± 2.1

* See footnote to Table I.

Some experiments on the trout gill enzymes were run at 37 °C for purposes of comparison with data from other laboratories. Table II shows specific activities of both enzymes with and without preincubation at 37 °C for 30 min. It can be seen that preincubation again lowered the Mg^{2+} baselines, and that the effect was greater in the salt water preparation. The fresh water enzyme was stimulated by Na^+ and the Na^+-ATPase activity after preincubation was about double that at 13 °C. But at

TABLE III

EFFECT OF OUABAIN ON FRESH WATER MICROSOMES ASSAYED AT 37 °C WITH A 30 min, 37 °C PREINCUBATION

Reaction mixture contained 100 mM Na^+ for Na^+-ATPase determinations and 100 mM Na^+ and 20 mM K^+ for ($Na^+ + K^+$)-ATPase determinations. Values represent the means ± S.E. of four experiments. All other conditions same as in Table I.

	Specific activity (μ*moles* $P_i \cdot mg^{-1}$ *protein* $\cdot h^{-1}$)		
	Mg^{2+}	Na^+	$Na^+ + K^+$
Control	3.2 ± 0.5	7.2 ± 0.9	9.8 ± 0.9
$5 \cdot 10^{-4}$ M ouabain	3.4 ± 0.5	5.8 ± 0.7	5.6 ± 0.8

37 °C full activity required K^+, and the specific activity in the presence of both Na^+ and K^+ was 30–40% greater than that with Na^+ alone. Table III shows that the K^+-activated fraction observed at 37 °C in the fresh water preparation is completely inhibited by $5 \cdot 10^{-4}$ M ouabain while Na^+ stimulation is only slightly depressed. In the salt water fish Na^+ alone had very little effect at 37 °C, and after preincubation $Na^+ + K^+$ activity was about 3 times as high as at 13 °C. As has been reported for several fish, including the rainbow trout[3], total alkali metal stimulation was greater in the salt water than in the fresh water preparation.

DISCUSSION

ATPase activities in fresh water fish

Gill microsomes from trout adapted to fresh water show both Mg^{2+}-ATPase, and ($Na^+ + K^+$)-ATPase activities as has been reported for other species. The former was thermolabile, and its activity was markedly reduced by preincubating the system without ATP for 30 min. The $Na^+ + K^+$-stimulated enzyme appeared to resemble the one described in other, primarily mammalian, preparations. It was activated by 20 mM K^+ in the presence of 100 mM Na^+, and was completely inactivated by $5 \cdot 10^{-4}$ M ouabain (Table III). No activity was demonstrable at 13 °C using the mean value of ten experiments (Table I) although, as mentioned earlier, low levels of activity at 13 °C are sometimes seen. The failure to consistently observe K^+ activation is probably due to the low assay temperature. At 13 °C a K^+ stimulation of 1.0 μmole $\cdot mg^{-1} \cdot h^{-1}$ greater than the Na^+ activity has been observed but an activity this low is difficult to reproduce.

The predominant microsomal ATPase from fresh water gills is activated by Na^+ but does not require K^+ and is little affected by ouabain at concentrations below 10^{-3} M. It is of interest that Zaugg and McLain[17] reported that ouabain only partially inhibited ($Na^+ + K^+$)-ATPase activity in microsomes from fresh water salmon gills. Their assays were conducted at 30 °C and the data resembled those in Table III. This suggests that the Na^+-ATPase may exist in salmon gills. Davis and Wedemeyer[18] have also observed ouabain insensitivity in the gill ($Na^+ + K^+$)-ATPase of rainbow trout and warn against using classical ouabain inhibition in studies of this type. However, the data presented in the present paper indicates they were dealing with the ouabain-insensitive Na^+-ATPase, a fact obscured by their experimental condi-

tions (*i.e.* both Na^+ and K^+ were included in the reaction mixture). An ouabain-insensitive, alkali metal-stimulated ATPase has also been reported for octopus gills[19]. Whether this represents a Na^+- or ($Na^+ + K^+$)-ATPase is uncertain since K^+ was also included in the experiments. There are other reports of Na^+-stimulated ATPase activity[15,16,20–25], but in every case activation by Na^+ alone has been only a small fraction of that in the presence of $Na^+ + K^+$, and where ouabain was used it abolished Na^+ stimulation. It is possible that some aspect of our procedure, not used in other laboratories, is producing an artifact. For example, the 37 °C preincubation may convert the ($Na^+ + K^+$)-enzyme to one with completely altered characteristics. Two observations make us doubt this. First, the same procedure produces no Na^+-stimulated, ouabain-insensitive enzyme in gills from fish adapted to salt water. Secondly, the ($Na^+ + K^+$)-enzyme can clearly be demonstrated in fresh water microsomes by assaying at a sufficiently high temperature, and its characteristics resemble those of other ($Na^+ + K^+$)-ATPase preparations (*i.e.* both Na^+ and K^+ are required for full activity and ouabain inhibits).

ATPase activities in salt water fish

When microsomes are prepared from fish adapted to salt water a different enzymic pattern is observed. Two ATPase fractions are present. One is a Mg^{2+}-ATPase which is almost completely inactivated by preincubating at 37 °C. As noted above the fresh water baseline ATPase, although partly inactivated at high temperature, is less thermolabile than the enzyme from salt water microsomes.

The other enzyme requires both $Na^+ + K^+$ for activity; no appreciable Na^+ stimulation was demonstrable at 13 °C or 37 °C. Quantitative parameters were measured at 13 °C and resembled those of the mammalian enzyme at 37 °C. Maximum activity was obtained with 100 mM Na^+ *plus* 20 mM K^+ whereas Na^+ alone (200 mM) was required in fresh water microsomes. The K_m was about 7 mM for Na^+, which is different from the K_m for Na^+-stimulated activity in the fresh water preparation (25 mM). For K^+ the K_m was 0.8 mM. Ouabain ($5 \cdot 10^{-4}$ M) completely inhibited the enzyme. The salt water ($Na^+ + K^+$)-ATPase is more temperature-sensitive than the fresh water enzyme. The mean specific activity at 13 °C with preincubation (Table I) was about 1/3 that at 37 °C (Table II), whereas with the fresh water Na^+-enzyme, under the same conditions, the 13 °C value was 1/2 that at 37 °C. Our data agree with those of other laboratories[1–3,5–7] in showing that gills from fish adapted to sea water have much higher $Na^+ + K^+$-activated ATPase levels than those from fresh water.

Gill ATPases and sodium transport

What role, if any, the ATPases described here play in ion transport across the gill can only be speculative. As noted above crude gill homogenates and even untreated microsomes show little Na^+ or $Na^+ + K^+$ activation of ATPase at 13 °C. The activities described become measurable only after treatment with NaI and preincubation at 37 °C. Such pretreatment, extending to the use of deoxycholate or lyophilization, is often necessary to obtain maximum enzymic activity. This may be due to the need to disrupt vesicles often formed by membrane fractions in order that cofactors such as Na^+ and K^+ have access to binding sites. Whatever the reason for the need to pretreat the preparation, the resulting rates may not be the same as

those *in vivo*, and this makes it difficult to correlate them with a physiological event like an ion flux. The only relevant points emerging from our data are (1) that two different alkali metal-activated ATPases can be demonstrated in microsomes from trout gills, and (2) that their activities vary greatly depending on whether the fish is adapted to sea water or fresh water. The $Na^+ + K^+$-activated ATPase activity is much higher in sea water than in fresh water while Na^+ activation is noted only in fresh water preparations and seems to disappear when the fish are transferred to sea water. The changes suggest a possible correlation with the different ionic regulatory problems in the two media.

NOTE ADDED IN PROOF (Received July 12th, 1972)

It has come to our attention that thermal inactivation of high baseline ATPase activity with a concomitant increase in alkali metal stimulation has previously been reported in erythrocyte membrane preparations[26].

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