The Nature and Properties of the Inducible Sodium-plus-Potassium Ion-Dependent Adenosine Triphosphatase in the Gills of Eels (*Anguilla anguilla*) Adapted to Fresh Water and Sea Water

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1. Gill tissue from eels adapted to fresh water or to sea water was disrupted in 0.32 M-sucrose containing 0.1% (w/v) sodium deoxycholate and the subcellular distribution of $(\text{Na}^+ + \text{K}^+)$ -dependent adenosine triphosphatase was determined. 2. About 70% of the recovered enzyme was in a fraction sedimenting between 225 000 $g_{av.}$ -min and 6000 000 $g_{av.}$ -min; the specific activities of enzymes from tissues of freshwater and seawater eels were 16 and 51 μmol of phosphate/h per mg of protein respectively. 3. The enzymes from gills of freshwater and seawater eels were indistinguishable on the basis of a number of parameters. These included phosphorylation by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the binding of $[\text{H}^3]\text{jouabain}$, the extent to which bound $[\text{H}^3]\text{jouabain}$ was displaced by increasing concentrations of KCl and pH optima. 4. Electrophoresis on polyacrylamide gels in sodium dodecyl sulphate showed that enzyme preparations from both sources had an identical number of protein components. 5. The higher specific activity of $(\text{Na}^+ + \text{K}^+)$ -dependent adenosine triphosphatase from tissue of seawater eels was accompanied by increased amounts of two protein components. One of these proteins retained ^{32}P after treatment of the enzyme with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and had mol.wt. 97 000; the other component was a glycoprotein with mol.wt. approx. 46 000. 6. The results are discussed in terms of the nature of the transepithelial NaCl pumps in the gills of freshwater and seawater fish.

It is well established that the gills of a euryhaline fish, such as the common eel, are concerned with osmoregulation as well as with respiratory gas exchange. In fresh water, an eel pumps NaCl from the outside medium, across the gill epithelium and into the blood; on adaptation to sea water, NaCl derived from drinking is pumped from the blood across the gill and into the outside environment (Maetz, 1971; Motais & Garcia-Romeu, 1972). Apart from mucus cells, the gills of both freshwater- and seawater-adapted eels contain 'chloride cells' and respiratory epithelial cells. The chloride cell in the gill of a freshwater eel is large, acidophilic and contains numerous mitochondria together with a fairly well developed smooth endoplasmic reticulum (Shirai & Utida, 1970). On adaptation to sea water the number of chloride cells increases some fivefold, but the cells hypertrophy in the sense that they are enlarged and contain increased numbers of mitochondria together with a very extensive smooth endoplasmic reticulum and heavily infolded basal and lateral plasma membranes (Shirai & Utida, 1970). Some 5–10% of the total cell population in the gill of seawater eels consists of chloride cells, the remainder being largely respiratory epithelial cells.

Adaptation of freshwater eels to sea water also results in a 3–5-fold increase in the activity of

$(\text{Na}^+ + \text{K}^+)$ -dependent ATPase* in each gill (Epstein *et al.*, 1967; Kamiya & Utida, 1968; Utida *et al.*, 1971). Histochemical staining at the level of the electron microscope shows that Mg^{2+} -dependent ATPase is heavily concentrated in the smooth endoplasmic reticulum and plasma membranes of the chloride cells (Mizuhira *et al.*, 1970; Shirai, 1972). Direct isolation of enriched populations of chloride cells and respiratory epithelial cells from the gills of seawater- and freshwater-adapted eels shows that the specific activity of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase in respiratory epithelial cells is unchanged during adaptation; the specific activity of the enzyme in chloride cells from seawater eels, however, is nearly 40 times that in respiratory epithelial cells (Kamiya, 1972; Sargent *et al.*, 1974). Since the volume of a chloride cell is nearly ten times that of a respiratory cell it is clear that the bulk of the total $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity of gills of both seawater and freshwater eels is present in the chloride cells.

The present work was undertaken to compare the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPases of gills from fish adapted to fresh water and to sea water on the grounds that these activities are probably related to ion-pumping activities in the gills. Of particular concern was the possibility that the change in direction of the NaCl

* Abbreviation: ATPase, adenosine triphosphatase.

pump, brought about by changes in environmental salinity, might be accompanied by changes in the subcellular distributions, kinetic properties or subunit structures of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPases.

Experimental

Materials

Radioisotopes. [γ - ^{32}P]ATP (15.9 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [U - ^3H]Ouabain (11.63 Ci/mmol) was purchased from N.E.N. Chemicals G.m.b.H., Research Products, D6072 Dreieichenhain, Germany.

Chemicals. Heparin, the disodium salt of ATP, ouabain, trypsin, ovalbumin, phosphorylase α and lactate dehydrogenase were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Glutamate dehydrogenase was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Acrylamide, bis(NN' -methylene-bisacrylamide) and sodium dodecyl sulphate were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Sodium deoxycholate was purchased from E. Merck A.G., Darmstadt, Germany. All other chemicals were of AnalaR grade from BDH Chemicals Ltd., Poole, Dorset, U.K. All solutions were prepared in glass-distilled water.

Animals. Common eels (*Anguilla anguilla*) were netted in the estuaries of the Rivers Dee and North Esk, Scotland. The animals were maintained in either running tap water or running sea water at a temperature of 8–12°C. All experiments were carried out on fish caught during August and September and maintained in the aquaria from September to April. During this time fish were offered food. Animals were not used until they had been maintained in the aquaria for at least 1 month.

Methods

Fractionation of gill tissue. Throughout the entire procedure fish adapted to sea water and fresh water were treated identically. Single fish (100–150 g wet wt.) were killed by transection posterior to the heart. The gills were perfused *in situ* by slowly injecting 20 ml of chilled 0.9% (w/v) NaCl containing 1 mg of heparin/ml into the sinus venosus of the heart. Individual gill arches were immediately excised and placed in 0.9% (w/v) NaCl on ice.

All operations were subsequently carried out at 0–2°C. Gill arches were blotted dry and the mucosal layers scraped from the underlying cartilage bars with a scalpel at right angles to the gill filaments. The scrapings were pooled and 1 g wet wt. was homogenized with 10 ml of 0.32 M-sucrose containing 1 mM-EDTA (disodium salt) and 0.1% (w/v) sodium deoxycholate, the final pH being adjusted to 8.0 with 1 M-

NaOH. A Teflon-in-glass homogenizer (0.125–0.175 mm clearance) operated at about 500 rev./min (15 complete strokes) was used. The homogenate was filtered through a nylon gauze of rectangular mesh area 106 μm^2 . A small portion of the filtrate was retained for enzyme and protein assays and the remainder fractionated as follows.

Fractionations were carried out in an MSE 75 centrifuge (MSE Ltd., Crawley, Sussex, U.K.) in an angle rotor (type 59112) operated at 2°C. The homogenate was centrifuged at 1000 g_{av} for 10 min to yield a pellet (1K pellet) and a supernatant. The latter was centrifuged at 15000 g_{av} for 15 min to yield a pellet (15K pellet) and a supernatant. The latter was finally centrifuged at 100000 g_{av} for 60 min to yield a pellet (100K pellet) and the final supernatant. All pellets were suspended in the original homogenizing medium at an approximate protein concentration of 0.5–1.0 mg/ml.

ATPase assays. The ($\text{Na}^+ + \text{K}^+$)-dependent ATPase was assayed in a conventional system containing 30 mM-imidazole-HCl buffer, pH 7.0 at 30°C, 100 mM-NaCl, 20 mM-KCl, 6 mM-MgCl₂ and 4 mM-ATP (added as the disodium salt). Enzyme protein was added in the range 20–100 μg and assays were carried out at 30°C for 15 min in a total volume of 1.0 ml in a shaking water bath. The ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity was calculated from the difference in phosphate liberated when the above assay was carried out in the presence and absence of 0.1 mM-ouabain. The Mg^{2+} -dependent ATPase activity was derived from the value obtained in the presence of 0.1 mM-ouabain. The reaction was stopped by adding 4.0 ml of 5% (w/v) trichloroacetic acid and the liberated phosphate measured in the AutoAnalyser (Technicon Instrument Co., Basingstoke, Hants., U.K.) by the method of Young (1966). Reaction rates under the above conditions were proportional to time and protein concentration. ATPase activities measured in the presence of 0.1 mM-ouabain were identical with those obtained when either Na^+ or K^+ was omitted from the assay. Units of ATPase activity are expressed as μmol of phosphate released/h.

Binding of [^3H]ouabain. A modification of the method of Hansen *et al.* (1971) was used. The assay was identical with that described above for ($\text{Na}^+ + \text{K}^+$)-dependent ATPase except that 20 mM-KCl was omitted, the enzyme fraction (100K pellet) was present at a concentration of 50–100 μg /ml and the 0.1 mM-ouabain was replaced with 1 μCi of [U - ^3H]ouabain (11.63 mCi/mmol). Tubes were incubated at 30°C for 60 min in a shaking incubator and 0.5 ml samples rapidly filtered through a Millipore filter of pore size 0.22 μm . The filters were air-dried and counted for radioactivity in 5 ml of Instagel (Packard Instrument Co. Ltd., Caversham, Berks., U.K.) in a model 3385 Packard Tri-Carb liquid-scintillation spectrometer operated at a gain of 60%.

The efficiency of counting ³H radioactivity under these conditions was 50%. All values were corrected by subtracting the value obtained when assays were carried out in the presence of 20mM-KCl, substituted for 100mM-NaCl.

Phosphorylation with [γ -³²P]ATP. The method used was based on that of Hokin *et al.* (1973). The assay was exactly as detailed for ATPase except that 20mM-KCl was omitted and the [γ -³²P]ATP concentration was 25 μ M (2 μ Ci/ml). Assays were set up in iced water and the reaction was started by rapidly adding enzyme and mixing. After 30s at 0°C the reaction was stopped by adding 10ml of 5% (w/v) trichloroacetic acid containing 0.6mM-ATP and 0.6mM-P_i, and the suspension filtered through a glass-fibre disc. The disc was washed with four separate lots of 20ml of 5% (w/v) trichloroacetic acid containing 0.6mM-ATP and 0.6mM-P_i. The discs were air-dried and counted for radioactivity in 5ml of Instagel in the Tri-Carb instrument set at 1.5% gain. Under these conditions the efficiency of counting ³²P radioactivity was 98%. All values were corrected by subtracting control values determined in assays containing 20mM-KCl instead of 100mM-NaCl.

When the enzyme fraction was subjected to electrophoresis after phosphorylation, the reaction was stopped by adding 1 vol. of the medium used to solubilize the sample (see below).

Polyacrylamide-gel electrophoresis. Electrophoresis was carried out in tubes 8.6cm long and 7mm diam. exactly as described by Hokin *et al.* (1973). The separating gel, 8.75% (w/v) acrylamide, was prepared in 0.375M-Tris-HCl, pH8.8, containing 0.1% (w/v) sodium dodecyl sulphate, and the stacking gel, 3.0% (w/v) acrylamide, was prepared in 0.12M-Tris-HCl, pH6.8, containing 0.1% (w/v) sodium dodecyl sulphate. The sample (10–60 μ g of protein) was applied after solution in 2 vol. of 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, 3% (w/v) sodium dodecyl sulphate and 0.0625M-Tris-HCl, pH6.8. The electrophoresis buffer was 0.19M-glycine, 2mM-Tris, pH8.3, containing 0.1% (w/v) sodium dodecyl sulphate. Electrophoresis was carried out at 2°C for 4h, starting with 1mA/gel for the first hour and continued at 4mA/gel. Gels were stained for total protein by immersion in 0.1% (w/v) Coomassie Blue R 250 and destained in 7% (v/v) acetic acid. Glycoprotein components were detected by staining by the method of Zacharius *et al.* (1959). In one series of experiments, gels containing ³²P were cut into slices 1mm long with a Mickle slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). The slices were placed in scintillation vials and incubated at 40°C for 16h with 0.1ml of 30% (v/v) H₂O₂. After adding 5ml of Instagel the samples were counted for radioactivity in the Tri-Carb instrument at a gain of 1.5%.

Densitometry. A Vitatron TLD 100 densitometer (Vitatron, Dieren, Holland) was used with a slit

width of 0.25mm, a span setting of 850, a scan speed of 0.5cm/min and a chart speed of 2cm/min. For scanning gels stained with Coomassie Blue a red filter, transmitting light of more than 600nm wavelength, was used. No filter was used for scanning gels stained for glycoprotein.

Protein determinations. Protein was measured in the AutoAnalyser by using the method of Lowry *et al.* (1951) as modified by Schuel & Schuel (1967).

Results

The initial objective of the present work was to obtain a relatively enriched preparation of (Na⁺+K⁺)-dependent ATPase in good yield from gill tissue. Mucosal scrapings of gill tissue were initially disrupted in 0.32M-sucrose and a simple subcellular fractionation scheme was applied. At best 50% of the total (Na⁺+K⁺)-dependent ATPase activity recovered was in a fraction sedimenting between 10000g_{av}-min and 225000g_{av}-min. The specific activities of the enzymes in this fraction, after activation with deoxycholate, were 7 and 22 μ mol of phosphate/h per mg of protein for tissue from freshwater and seawater eels respectively. The remainder of the (Na⁺+K⁺)-dependent ATPase was equally distributed in fractions sedimenting at 10000g_{av}-min and between 225000g_{av}-min and 6000000g_{av}-min, but in both cases the specific activities of the enzymes were less than that in the fraction sedimenting between 10000g_{av}-min and 225000g_{av}-min.

Table 1 shows the subcellular distributions of (Na⁺+K⁺)-dependent ATPases in gill tissue from freshwater and seawater eels disrupted in 0.32M-sucrose containing 0.1% (w/v) deoxycholate. Nearly 70% of the total activity recovered is in the fraction sedimenting between 225000g_{av}-min and 6000000g_{av}-min and the specific activities of the enzymes from freshwater and seawater eels are 10 and 37 μ mol of phosphate/h per mg of protein respectively. Thus tissue disruption in the presence of deoxycholate has increased both the yield and specific activity of the enzyme in a single subcellular fraction. Consequently this fraction was used as a source of (Na⁺+K⁺)-dependent ATPase in all subsequent experiments.

It may be noted that natural populations of eels were used in the present work, so that selection to eliminate natural variation caused by previous dietary history, sex, stage of development and seasonal factors was not possible as a routine. Eels were selected on the basis of size alone. Throughout the work considerable variation in the specific activity of (Na⁺+K⁺)-dependent ATPase was found. Thus the minimum and maximum values observed for the enzyme preparation from tissues of seawater eels were 32 and 96 μ mol of phosphate/h per mg of protein respectively. The mean ($\pm$ S.E.M.) specific activities of enzyme

Table 1. *Subcellular distribution of (Na⁺+K⁺)-dependent and Mg²⁺-dependent ATPases in gill tissue from freshwater and seawater eels*

Experimental details are described under 'Methods'. Results refer to tissue disrupted in 0.32M-sucrose containing 1 mM-EDTA and 0.1% (w/v) sodium deoxycholate, pH 8.0. The homogenate contained 100mg of protein. Units are expressed as μmol of phosphate/h and specific activities are expressed as units/mg of protein. The 1K pellet refers to the fraction sedimenting at 10000 $g_{av.}$ -min, whereas the 15K and 100K pellets refer to fractions sedimenting at 10000–225000 $g_{av.}$ -min and 225000–6000000 $g_{av.}$ -min respectively. Results are the means of duplicate determinations. FW, fresh water; SW, sea water.

Fraction	Source	...	Mg ²⁺ -dependent ATPase				(Na ⁺ +K ⁺)-dependent ATPase			
			Total activity (units)		Specific activity (units/mg of protein)		Total activity (units)		Specific activity (units/mg of protein)	
			FW	SW	FW	SW	FW	SW	FW	SW
Homogenate			300	430	3.0	4.3	310	790	3.1	7.9
1K pellet			11	16	1.7	1.6	5	45	0.9	4.1
15K pellet			45	68	3.9	6.5	61	156	5.9	13.8
100K pellet			74	84	5.0	6.8	138	459	10.1	37.3
100K supernatant			57	107	2.2	2.8	22	42	0.3	0.8

Table 2. *Comparison of specific enzymic activity, specific binding of [³H]ouabain and specific phosphorylation with [γ -³²P]ATP for (Na⁺+K⁺)-dependent ATPase preparations from the gills of freshwater and seawater eels*

The enzyme fractions used were the 100K pellets described in Table 1. Experimental details are given under 'Methods'. Results are expressed as means $\pm$ S.E.M., with the number of determinations given in parentheses. Values for seawater eels differ from those for freshwater eels by 0.02 < P < 0.05, P < 0.01 and 0.01 < P < 0.02 for parameters A, B and C respectively.

		Freshwater	Seawater	Seawater/freshwater ratio
A	Enzymic activity (μmol of phosphate/h per mg of protein)	16 $\pm$ 4 (4)	51 $\pm$ 12 (5)	3.1
B	Binding of [³ H]ouabain (pmol of ouabain bound/mg of protein)	48 $\pm$ 7 (4)	154 $\pm$ 28 (3)	3.2
C	Phosphorylation with [γ - ³² P]ATP (pmol of phosphate bound/30s per mg of protein)	37 $\pm$ 0 (3)	126 $\pm$ 25 (3)	3.4

preparations from freshwater and seawater eels throughout the work were 16 $\pm$ 4 (n = 4) and 51 $\pm$ 12 (n = 5) μmol of phosphate/h per mg of protein respectively (Table 2).

Table 2 presents a summary of some properties of the enzymes from freshwater and seawater eels. The specific activities of the enzymes are compared with their abilities to bind [³H]ouabain and the degrees to which they are phosphorylated with [γ -³²P]ATP. Values quoted for specific activity related to the ouabain-sensitive component of total (Mg²⁺+Na⁺+K⁺)-dependent ATPase, whereas values for the other two parameters relate to differences between measurements carried out in the presence of NaCl and in the presence of KCl. Considerable variation exists between individual determinations, but differences between preparations from freshwater and seawater eels are highly significant. The 'seawater/freshwater' ratios of enzyme activity are essentially the same in all cases.

The [³H]ouabain bound as shown in Table 2 was

displaced by increasing concentrations of KCl. The curves for the amount of [³H]ouabain displaced with increasing concentrations of KCl were essentially identical for enzymes from both sources. In both cases, [³H]ouabain was maximally displaced at a minimum KCl concentration of 20mM.

The sea water and fresh water (tap water) used in the present work had pH values of approx. 8.0 and 6.8 respectively. Despite this difference, the enzymes from freshwater and seawater eels were both optimally active at pH 7.0, and the curves for pH versus activity were essentially identical.

Preparations of (Na⁺+K⁺)-dependent ATPase from freshwater and seawater eels were next subjected to disruptive polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. Fig. 1 depicts the densitometric profiles recorded from gels stained for total protein. The profiles in Figs. 1(a) and 1(b), where the total amount of protein applied to the gels is the same in both cases, show an identical number of protein components. About 20 zones could be

Fig. 1. Location of ^{32}P on polyacrylamide gels after treating preparations of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ from gills of freshwater and seawater eels with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$

The enzyme preparations used were those in Table 2. Conditions of reaction with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and electrophoresis are described under 'Methods'. Continuous curves represent densitometric tracings of gels stained for protein with Coomassie Blue. Solid horizontal bars represent areas that stained positively for glycoprotein. Preparations were treated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of NaCl ($\circ$, $\bullet$) or KCl ($\square$, $\blacksquare$) before gel electrophoresis. (a) Freshwater-eel preparation ($55\text{ }\mu\text{g}$ of protein; 1.05 enzyme units); (b) seawater-eel preparation ($55\text{ }\mu\text{g}$ of protein; 4.85 enzyme units).

detected by visual examination of the gels. However, the profiles differ quantitatively in that two zones, A and B, are present in greater amounts in the seawater-eel preparation. Staining the gels for glycoproteins revealed a single, diffuse component whose mid-point coincided with zone B (Fig. 1).

When enzymes from both freshwater and seawater eels were treated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of NaCl before electrophoresis, single peaks of radio-

activity which coincided with zone A were found for both enzymes (Fig. 1). The peak of radioactivity was greater for the seawater-eel enzyme, and peaks for both enzymes were abolished when treatment with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was carried out in the presence of KCl (Fig. 1). It may be concluded that peak A is an active unit of the $(\text{Na}^+ + \text{K}^+)\text{-dependent ATPase}$ of both freshwater and seawater eels. Comparison of the mobilities of zones A and B with the mobilities of

marker proteins of known molecular weights indicates that the molecular weights of zones A and B are 97000 and approx. 46000 respectively.

Discussion

In the present work most of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase was recovered from homogenates of gill tissue in 0.32M-sucrose in the conventional mitochondrial fraction. Neither the specific activity of the enzyme nor its yield in the latter fraction were notably high. Since it is known that the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase of gill tissue is heavily concentrated in the smooth endoplasmic reticulum and plasma membranes of the chloride cell (Mizuhira *et al.*, 1970; Shirai, 1972; Kamiya, 1972; Sargent *et al.*, 1974), it may be inferred that the distribution of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase in subcellular fractions reflects the distribution of smooth vesicles derived from chloride cells. It may be further inferred that homogenization in 0.32M-sucrose causes formation of relatively large vesicles that sediment mainly with the mitochondria. This finding may be generally applicable to cells of salt-secreting epithelia, since the bulk of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase from both *Squalus* rectal gland and the pseudobranch of a marine teleost is recovered in the 'heavy microsomal' or 'light mitochondrial' fractions (Hokin *et al.*, 1973; Dendy *et al.*, 1973).

Homogenization in 0.32M-sucrose containing 0.1% (w/v) deoxycholate, however, resulted in the appearance of nearly 70% of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase in the conventional microsomal fraction with enhanced specific activity (Table 1). Subcellular fractionation in deoxycholate has been used to isolate a microsomal fraction from the gills of seawater-adapted salmon that contains a specific ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity of 90 μmol of phosphate/h per mg of protein (Zaugg & McLain, 1970). It is noteworthy that, in the present work, fractionation in the presence of deoxycholate caused the appearance of substantial amounts of Mg^{2+} -dependent ATPase activity in the high-speed supernatant. Clearly at least part of the latter enzyme is distinct from ($\text{Na}^+ + \text{K}^+$)-dependent ATPase and may well be derived from damaged mitochondria. At the same time it was found (J. R. Sargent & A. J. Thomson, unpublished work) that the bulk of the succinate dehydrogenase activity in the homogenate was recovered in the conventional mitochondrial fraction so that the mitochondria were not entirely disrupted. Fractionation in deoxycholate proved to be a convenient and simple method to isolate enriched preparations of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase from gill tissue. The average specific activity of the preparations here compared well with the maximum value reported so far of 45 μmol of phosphate/h per mg of protein obtained by treating a combined mitochondrial and

microsomal fraction from gills of seawater-adapted eels with NaI (Kamiya & Utida, 1968).

The major objective of the present work was to compare enriched preparations of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase from gills of freshwater- and seawater-adapted eels. The present data show that the subcellular distribution of the enzymes from the two sources is the same (Table 1). The extent to which the enzymes are phosphorylated with [γ - ^{32}P]ATP and the extent to which they bind [^3H]ouabain show a 1:1 relationship in each case (Table 2), in agreement with the findings of Hansen *et al.* (1971). The latter two properties are related to the specific activities of the enzymes in the same manner, irrespective of their source (Table 2). The competitive relationship between ouabain and KCl for the two enzymes is found to be the same. Both enzymes are optimally active at pH 7.0, as is the case for ($\text{Na}^+ + \text{K}^+$)-dependent ATPase from the gills of freshwater- and seawater-adapted trout (Pfeiler & Kirschner, 1972). Although the latter authors reported that the enzyme from freshwater-adapted trout gills had a somewhat broader pH optimum than the corresponding enzyme from seawater-adapted trout, the work here shows that the plots of pH versus activity were at least very similar. It may be concluded that the enzymes are indistinguishable on the basis of all these properties.

Very pure preparations of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase with a specific activity of 1500 μmol of phosphate/h per mg of protein have been isolated from *Squalus* rectal gland and canine kidney (Hokin *et al.*, 1973; Lane *et al.*, 1973). These preparations contain only two protein components as judged by electrophoresis on polyacrylamide gels in sodium dodecyl sulphate. However, it is not known with certainty whether one of the components, a glycoprotein with mol.wt. approx. 50000, is a component part of the enzyme, or a component part of the vesicles that contain the enzyme, or merely a contaminant. Both these protein components are present in crude preparations of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase from gills of freshwater and seawater eels (Fig. 1), and the only detectable difference between the crude preparations is that both components are present in increased amounts in the seawater fish. It has been found that the two components are associated with the chloride cells of gills rather than with the respiratory epithelial cells (J. R. Sargent & A. J. Thomson, unpublished work).

Thus adaptation to sea water has resulted in the appearance in gills of increased amounts of a ($\text{Na}^+ + \text{K}^+$)-dependent ATPase that is indistinguishable from the enzyme in gills from freshwater fish on the basis of subunit structure, as well as subcellular distribution and kinetic properties. The conclusion is in line with the increased content of membranes present in the greater number of chloride cells in the seawater fish (Utida *et al.*, 1971). It is perhaps surprising that only

the two protein components associated with the (Na⁺+K⁺)-dependent ATPase were found to be increased in amount in the present work. Either these two components are the only major proteins present in the membranes in the chloride cells of seawater fish, or else other proteins have been removed during the isolation of the crude membrane fractions studied here.

Although the above findings account for the enhanced load of NaCl pumping in gills of seawater fish, they do not explain the change in direction (Motaïs & Garcia-Romeu, 1972). Electron-microscopic data strongly indicate that, in the chloride cell of seawater fish, the (Na⁺+K⁺)-dependent ATPase is present on the heavily infolded basal and lateral plasma membrane, that is on the serosal side of the cell (Shirai, 1972). Less evidence is available for the chloride cell of freshwater fish, but it is probable, by analogy with amphibian skin (Farquhar & Palade, 1966), that the enzyme is also present on basal and lateral plasma membranes. The latter probability is supported by the present findings that the two enzymes are indistinguishable kinetically, since such results imply that the enzymes operate in essentially identical micro-environments. The hypothesis presented by Shirai (1972), that NaCl is transported across the chloride cell of seawater fish while sequestered within vesicles that are derived from the infolded plasma membrane, is in accord with the present findings and remains the most plausible explanation at present for the reversal in direction of the NaCl pump during adaptation to sea water. The molecular basis for such a process remains to be elucidated. However, the present work strongly implies that the (Na⁺+K⁺)-dependent ATPase itself is not responsible for the directional change in NaCl pumping during adaptation to sea water.

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