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Fish Gills: Mechanisms of Salt Transfer in Fresh Water and Sea Water

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Fish gills: mechanisms of salt transfer in fresh water and sea water

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CONTENTS

The teleostean gill is a multi-purpose organ, specialized for respiratory gas exchanges, clearance of waste products of nitrogenous metabolism and maintenance of acid-base and mineral balances.

Structural studies reveal a complex epithelium. The 'chloride-cells are almost certainly the site of ion exchange in relation to salt balance.

Functional studies show that the gill is responsible for the net absorption of Na^+ and Cl^- occurring in fresh water and extrusion of these ions in sea water. In fresh water, a coupling between endogenous NH_4^+ or H^+ and HCO_3^- excretion and Na^+ and Cl^- absorption is observed. In sea water active Na^+ excretion is linked with K^+ absorption from the external medium. In parallel, active Cl^- excretion occurs. The gill is also the site of Na^+/Na^+ and Cl^-/Cl^- exchanges which involve 25 to 75 % of the internal NaCl per hour. The relative importance of simple diffusion and exchange-diffusion in these exchanges is assessed.

Biochemical studies reveal two enzymes playing important roles in the ionic pumps: carbonic anhydrase and Na–K activated ATPase.

Studies involving transfer of euryhaline fishes from low to high salinity, show that the switch from fresh-water to seawater types of gill function is far from instantaneous. Synthesis or destruction of functional sites and renewal of specialized cells are involved. The role of external or internal NaCl concentration changes as stimuli for these 'inductive processes' and the endocrine control of these functional changes are briefly discussed.

Introduction

The present review will be limited to the teleosteans or bony fishes, which are characterized by the capacity to maintain a more or less constant internal osmotic pressure in aquatic environments ranging from fresh water to sea water.

The classic model proposed by H. W. Smith (1932) and completed by Krogh (1939) summarizes our knowledge of the contrasting ways by which several effector organs, the gill, the gut and the kidney permit the maintenance of mineral and water balance. Figure 1 illustrates this model. In fresh water, water enters through the gills, and the kidney has the task of removing excess water by excreting abundant and dilute urine. The renal Na^+ and Cl^- loss and the passive loss of these electrolytes along the concentration gradient across the external boundaries is compensated by an active uptake of ions by the gills. As the freshwater fish drinks very little and is able to maintain its salt balance during prolonged periods without food, the gut is believed to play a negligible role in osmoregulation. In sea water, water is lost across the gills, and the gut becomes the site of the compensatory mechanism permitting the water balance to be maintained. The fish swallows the external medium and water is absorbed by the gut together with salt. This electrolyte entry is balanced by extra-renal excretion of monovalent ions by active transport, probably by the gills. The kidney function is characterized by reduction of the free-water clearance and excretion of the bivalent ions absorbed by the gut.

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Teleosteans are not the only 'hetero-osmotic' regulators. Other groups of animals including invertebrates have developed branchial mechanisms similar to those of freshwater or seawater fish.

The purpose of this review is to emphasize the functional differences observed for the gill when the fish is living in fresh water or in sea water and the functional changes occurring when a euryhaline fish migrates from one medium to the other. The alternation of two pumping activities oriented either towards the outside or towards the inside of the organism makes the branchial epithelium one of the most fascinating biological epithelial membranes.

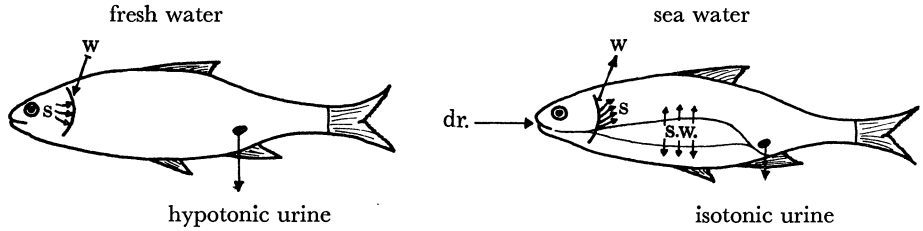

FIGURE 1. Schematic representation of the intervention of various effector organs in the maintenance of salt (s) and water (w) balance in freshwater and seawater teleosts.

1. GENERALITIES CONCERNING THE STUDY OF THE GILL AS A BIOLOGICAL MEMBRANE

(*a*) *Structure and function of the branchial epithelium*

The fish gill is a 'multi-purpose' organ, specialized for respiratory gas exchanges, the clearance of the waste products of nitrogenous metabolism and the maintenance of acid–base and mineral balances. It is also the site of passive transfer of water. We shall see below that most of these various and apparently unrelated functions may in fact be intimately linked.

As oxygen has a relatively low solubility in water, the fish has to maintain a high rate of water flow over the gills in order to meet its oxygen needs (Rahn 1966). The branchial epithelium is therefore in contact with an extremely well-stirred external medium (Hughes 1965). Maximal surface contact between internal and external media (up to 10 $cm^2\ g^{-1}$) in a minimal space has been evolved by the development of numerous lamellae along the double row of gill filaments supported by the gill arch (see figure 2), and there is also a counter flow of the internal and external media. According to the haemodynamic studies of Steen & Kruysse (1964), blood flows from afferent to efferent filamental vessels along two main pathways: the central compartment inside the filament and the lamellae. The branchial haemodynamics may be under either endocrine or nervous control (Maetz & Rankin 1969).

The epithelial cells bordering the two main paths of blood flow are different in type. The flat epithelial cells of the gill lamellae are obviously specialized in respiratory gas exchange, being no more than 3 to 5 μm thick. Their cytoplasm contains relatively few mitochondria, but surprisingly an abundant endoplasmic reticulum of the 'rough' variety and a conspicuous Golgi apparatus suggest more complex functions (Hughes & Grimestone 1965; Newstead 1967). The surface coating is generally more apparent in freshwater species. Mucous cells and mitochondria-rich cells are more abundant in the inter-lamellar region bordering the central compartment, and both types of cells are also found on the edges of the filament along the afferent and efferent vessels, the mitochondria-rich cells being more numerous on the afferent

side. These cells were first described by Keys & Willmer (1932) and because of their acidophily were considered to be analogous to the oxyntic cells responsible for hydrochloric acid secretion in the amphibian stomach. The authors coined the term 'chloride cell' for these cells, as Keys (1931) had demonstrated by means of his 'heart-gill' preparation that the gill is the site of the extra-renal active extrusion of chloride discovered a few years previously by H. W. Smith (1930). Since 1932, the literature concerning these cells has increased enormously and some authors have raised doubts as to their supposed excretory role (Parry 1966). Electron microscope studies revealed, in addition to the numerous mitochondria, an extraordinary development of cytoplasmic microtubules superficially ressembling a smooth endoplasmic reticulum. These tubules are finger-like projections of the lateral and serosal borders of the 'chloride cell' (Philpott & Copeland 1963; Philpott 1965). Such a cell is shown diagrammatically in figure 3.

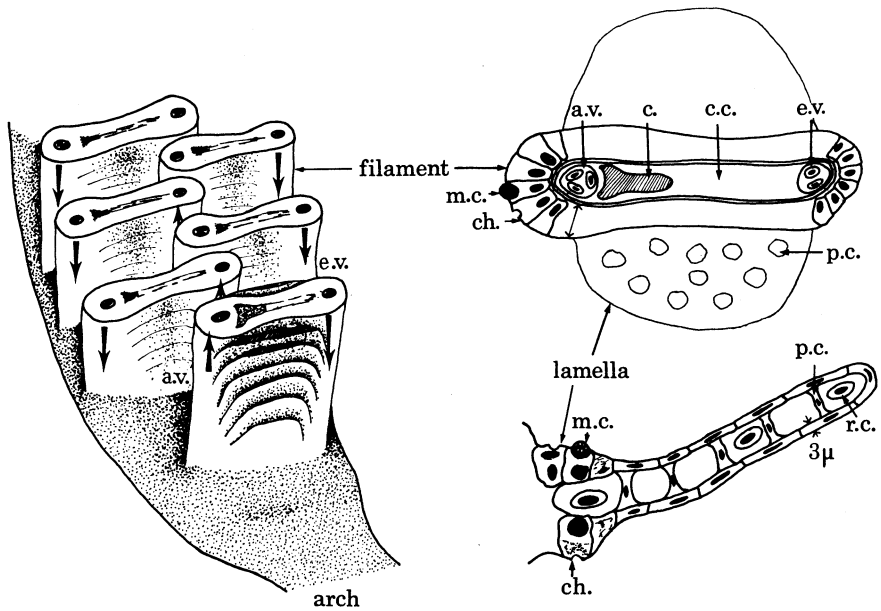

FIGURE 2. Simplified anatomical and histological features of the teleostean gill. The branchial apparatus consists of four pairs of gill arches with two rows of projecting filaments. The lamellae extend as flattened leaflets from each side of the filament (see Conte 1969). a.v. and e.v., afferent and efferent vessels with red cells (r.c.); c., cartilagenous spine supporting the filament; c.c., central vascular compartment; m.c., mucous cell; ch., 'chloride cell'; p.c., pillar cells.

The presence of chloride has been confirmed by histochemical techniques in the seawater cell and found to be located in the 'pit' characterizing the mucosal border or inside the microtubuli (Philpott 1965; Petrik 1968). After injection into the blood, heavy metal complexes such as lanthanum chloride, or fairly big organic molecules such as peroxidase have been located inside the tubular apparatus (Philpott 1967; Ritch & Philpott 1969). Experimental changes of external salinity are followed by conspicuous alterations in the diameter of the microtubuli (Lasker & Threadgold 1968) and disappearance of the 'pit' (Philpott & Copeland 1963). Long-term adaptation to sea water also leads to an increase in the number of the mitochondria-rich cells (Shirai & Utida 1970). The branchial epithelium is therefore complex in structure as well as in function. Concerning passive electrolyte transfer, it has been suggested that it occurs across the thin respiratory cells (Keys & Bateman, 1932; Kerstetter, Kirschner & Rafuse 1970). There is evidence that active handling of Na^+ or Cl^- occurs in the 'chloride cells' as

shown by the abundance of energy-providing mitochondria and the presence of rate limiting enzymes related to Na or Cl transport, namely carbonic anhydrase and Na–K activated ATPase. The activities of both enzymes increase during adaptation to sea water in parallel with the increase of 'chloride cell' number (Leiner 1938; Utida, Kamiya & Shirai 1971). Other enzymes, such as glutamic dehydrogenase, associated with nitrogenous metabolism and forming part of the enzymatic equipment of mitochondria are also found in high activities in the gill (see review by Forster & Goldstein 1969).

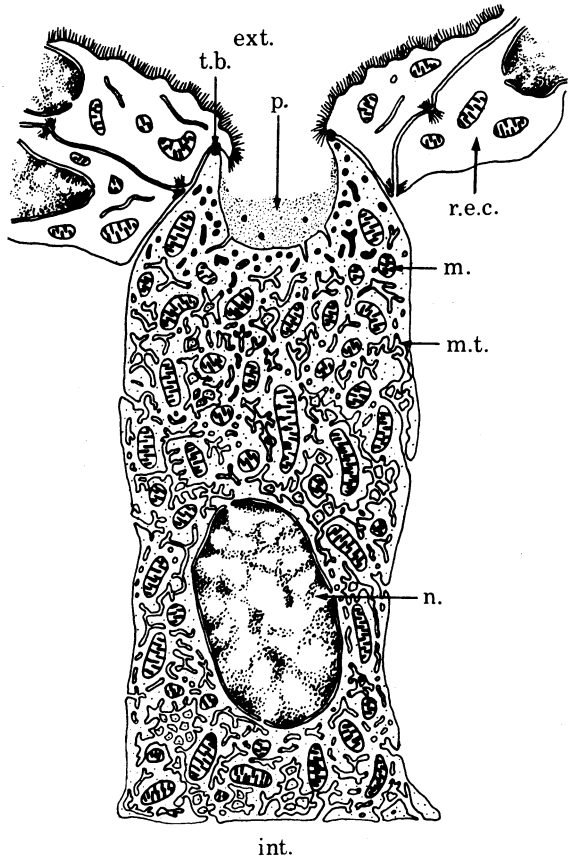

FIGURE 3. A typical 'chloride cell' redrawn from Ritch & Philpott (1969). The pit (p) characterizes the cell in seawater adapted fish. Numerous mitochondria (m) and microtubules (m.t.) are also typical. (Enlarged 6000×.) n., nucleus; t.b., terminal bar; r.e.c., flat respiratory epithelial cell.

(*b*) *The technical difficulties in studies of electrolyte transfer across gills* in vivo *and* in vitro

Most of the work presented below was performed on whole animals. Kirschner (1970) has recently summarized the difficulties in obtaining *in vivo* reliable thermodynamical data such as unidirectional fluxes and electrochemical potential differences in relation to salt transfer across the gill.

The use of tracers to measure unidirectional fluxes is sometimes severely limited by the complexities of compartmental analysis, although this is fortunately not the case for Na^+ and Cl^- exchanges (techniques in Maetz (1956*b*) and in Motais (1967)).

Account must also be taken of the effector organs other than the gills which play a role in ion exchange. Thus in freshwater fish, while gut ionic exchanges are negligible in unfed

specimens, renal loss of ions must be eliminated by catheterization of the urinary papilla. Recently Kerstetter *et al.* (1970) advocated an 'irrigated gill preparation' to obviate this complication. In seawater fish, catherization is unnecessary as the renal loss of NaCl is negligible, but gut ionic influx on the other hand is of importance.

The measurement of electric potentials also presents certain complications. Most of the authors cited below (see also Kirschner (1970) simply insert a catheter connected via agar KCl bridges and calomer electrodes to a high impedance millivoltmeter into the intraperitoneal cavity and record the potential difference with a similar electrode kept in the outside medium. Only two preliminary reports (Tosteson 1962; Maetz & Campanini 1966) are based on microelectrode impalement directly across the gill epithelium.

The determination of the chemical activities of the electrolytes in blood and in the outside medium does not present major difficulties. Very few fishes, however, lend themselves to vascular catheterization allowing for continuous blood sampling without perturbing the fish. Shock effects from handling upset the fish mineral balance. Experimental modifications of the electrochemical potential were attempted by means of continuous infusion of hyper or hypo saline Ringer solutions into the intraperitoneal cavity or vascular bed. In analysing the effects of such interventions it is difficult to assess the part played by the feed-back regulatory mechanisms intervening to restore mineral balance and the direct effects on the membrane under study. Attempts to modify the external Na^+ or Cl^- concentration by so-called 'transfer experiments' have yielded important information on the nature of the ionic transfer mechanisms thanks to improved electronic techniques permitting the study of the kinetics of the flux readjustments.

Various groups have recently reported ionic flux measurements across perfused gill preparations (Richards & Fromm 1970; J. C. Rankin & J. Maetz, in preparation). Three major problems have to be solved before an adequate preparation can be obtained. First, it is essential to provide adequate stirring of the outside medium to ensure homogenization of the fluid in direct contact with the epithelium and to allow it to reach the active site of ion transport between the lamellae. Secondly, it is necessary to master the complex problems of the haemodynamics of the perfusion fluid. The use of adequate hydrodynamic pressures at the perfusion inlet and outlet, the addition of catecholamines to obtain lamellar perfusion and the use of a filtered perfusion fluid are prerequisites for an adequate *in vitro* preparation. Thirdly, the catherization of the efferent blood vessel is difficult in some species: leakage of effluent fluid complicates flux and potential measurements. Despite all these problems, a few laboratories have devoted themselves to the study of the branchial mechanisms of electrolyte transfer.

The present report deals with observations collected for over 10 years by R. Motais at Monaco, by the Groupe de Biologie Marine at Saclay and Villefranche-sur-Mer and since 1967 by the Laboratoire de Physiologie Animale at Nice directed by R. Motais.

2. Electrolyte transfer mechanisms in fresh water

(*a*) *Sodium transport and its control*

Figure 4 summarizes the essential features of Na^+ transport in the freshwater eel. The Na^+ turnover resulting from the overall ionic exchanges (about 0.2 % per hour) represents a very small fraction of the internal (exchangeable) Na. The branchial net uptake compensating for the renal loss of Na^+ results from an influx which is about 20 to 50 % higher than the efflux. Contrary to earlier observations, the fish does drink to a certain extent (see review by Maetz

1970*a*), but as only a small quantity of salt is taken up in this way, the gut does not intervene in salt balance, provided the fish has not ingested food.

As can be seen in figure 5, the Na^+ influx (f_{in}) of the flounder increases as a function of external Na concentration ($[Na]_{ext}$). The curve obtained displays saturation kinetics and fits the well-known Michaelis–Menten equation:

$$f_{in} = \frac{f_{max}\,[Na]_{ext}}{K_m + [Na]_{ext}},$$

where f_{max} is the maximal rate of Na^+ influx (in μmol h^{-1} 100 g^{-1}) and K_m, the Michaelis constant corresponding to $[Na]_{ext}$ when $f_{in} = \frac{1}{2} f_{max}$.

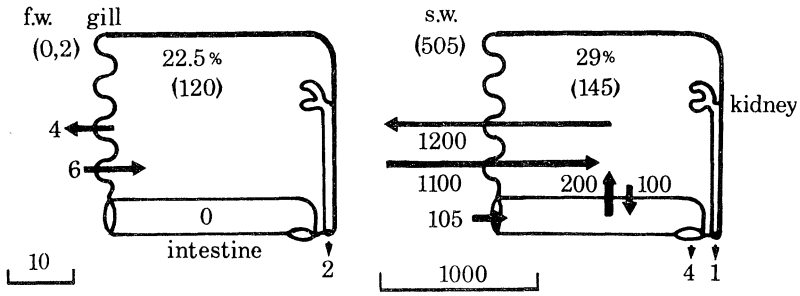

FIGURE 4. Comparative Na exchanges in freshwater (f.w.) and seawater (s.w.) eel. External and internal Na concentrations are given in brackets. Internal Na space, in %. Fluxes across gills and gut are represented in μmol h^{-1} 100 g^{-1}. Note different scales. Only the net Na loss by the kidney is given. For the exchange fluxes across the renal tubuli, see review by Lahlou (1970).

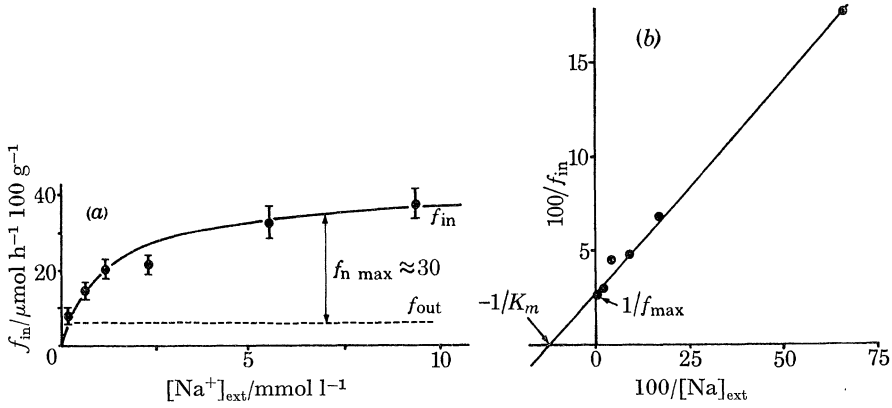

FIGURE 5 (*a*) Relationship between Na influx (f_{in}), efflux (f_{out}) and net flux (f_{net}) and external sodium concentration in the freshwater flounder. (*b*) the reciprocal plot permitting the evaluation of the parameters f_{max} and K_m (according to Maetz 1971).

Values for these various parameters were calculated from a reciprocal plot of the same data, also shown in figure 5. f_{max} is about 35 μmol, while K_m is about 0.8 mmol/l at 16°C. In the lower range of Na^+ concentrations $f_{in}/[Na]_{ext}$ attains about 30 to 40 μmol per mmol external concentration. It must be pointed out that these flux studies were done by using a large group of flounders adapted to waters containing 0.2 mmol/l Na. The fluxes in other salinities were measured during short periods of time (1 to 3 h) in order to avoid an adaptation to new environmental conditions resulting in changes of the pumping characteristics of the gill. A similar curve has recently been published by Kerstetter *et al.* (1970) for the trout.

The Na^+ branchial f_{out} also augments with increasing external Na^+ concentrations, but the

observed increase is negligible for the range of external salinity depicted in figure 5. Motais, Garcia-Romeu & Maetz (1966) have observed a fourfold augmentation of the Na^+ f_{out}, after more than a 500-fold increase of $[Na]_{ext}$.

The branchial Na^+ uptake is subject to feed-back control mechanisms. In 'unshocked' fish, at the external Na^+ concentration to which the fish has been adapted, mineral balance is achieved and f_{in} equals the total f_{out}: the sum of the branchial f_{out} and of the renal loss. At a lower $[Na]_{ext}$, there is a salt loss and at a higher $[Na]_{ext}$ a salt gain. The resulting changes of the internal Na^+ level trigger off regulatory mechanisms modifying the pattern of ionic exchanges and restoring salt balance. Sodium depletion results in a decrease of f_{out} and an

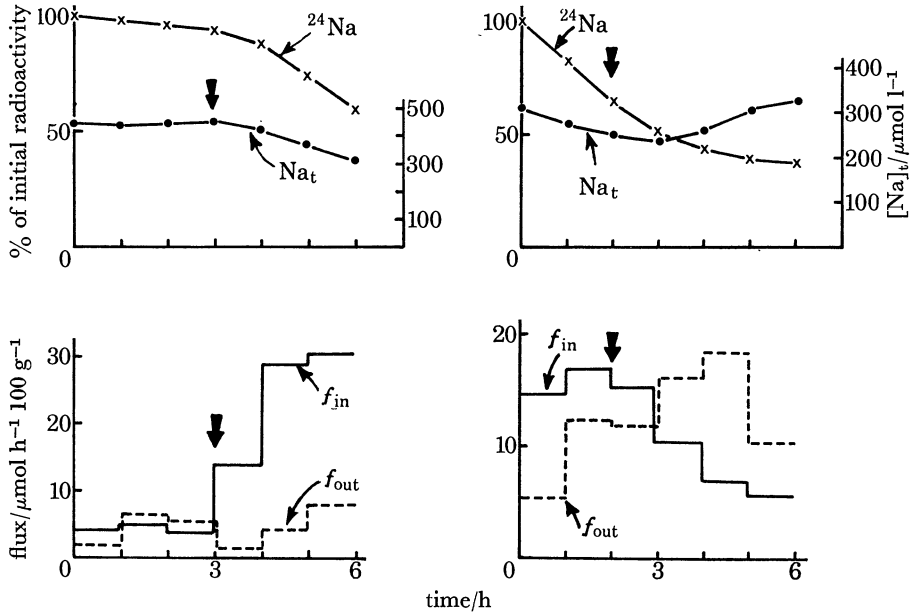

FIGURE 6. Effects of experimental decrease (left) or increase (right) of the internal Na level on the Na exchanges across the gill of the goldfish (according to Bourguet *et al.* 1964). Vertical arrows indicate time of decrease or increase. Upper graphs: evolution of the external total Na and Cl and ^{24}Na concentrations. Lower graphs: evolution of influx (f_{in}) and branchial efflux (f_{out}).

increase of f_{in} and net uptake by the gills. Such a response is obtained after keeping the fish in deionized water and then replacing it in its original adaptation medium (Krogh 1939; Maetz 1964) or after injection of hypotonic saline into the intraperitoneal cavity (Bourguet, Lahlou & Maetz 1964). Salt loading by keeping the fish in isotonic saline or by injection of hypersaline solutions produces opposite effects (see also Mayer & Nibelle 1970).

Figure 6 illustrates these contrasting changes in gill function as a result of experimental changes of $[Na]_{int}$ in the goldfish. Incidently this figure illustrates the simplest technique (technique A) proposed by our group for isotopic flux measurements in fresh water (Maetz, 1956*b*). The tracer is added to the outside closed-circuit bath. Influx is calculated by dividing the disappearance rate or radioactive influx (in counts min^{-1} h^{-1} 100 g^{-1}) by the mean external specific radioactivity of Na^+ (in counts min^{-1} μmol^{-1}). Efflux is given by the relation

$$f_{net} = f_{in} - f_{out}.$$

f_{net} is calculated from the rate of Na^+ appearance (f_{net} negative)or disappearance (f_{net} positive) in the external bath as measured by flame photometry. An $[Na]_{ext}$ of 10 mmol/l is the upper limit for technique A, for with an external volume of 300 to 500 ml per 100 g fish, internal and

external sodium compartments are equal and the variations of external radiosodium and Na^+ concentrations cannot be detected with precision. The technique advocated by Kerstetter *et al.* (1970) has the advantage of using a smaller external volume and avoiding catheterization. The fish has, however, to be anaesthetized. For higher external Na^+, Motais *et al.* (1966) used an alternative method (technique B) consisting of the evaluation of the radiosodium which appeared in the internal Na^+ pool, the fish being placed, as in the preceding technique, in 'hot' external media. Radioactive influx is the product of the tracer plasma concentration and the sodium space. The sodium space varies however as a function of time (Mayer & Nibelle 1969). To avoid this complication some authors (Potts & Evans 1967) use a 'total body counter' for small fishes. Unfortunately all these techniques can be criticized in view of recent evidence showing that fishes increase their drinking rate as soon as they are submitted to osmotic shock (R. Kirsch, personal communication). As the external medium thus swallowed contains sodium at very high specific activity, the sodium influx includes an important 'intestinal component'. Such are some of the difficulties encountered in flux studies *in vivo*. We now suspect that the influx curve published by us (Motais *et al.* 1966; Maetz 1970*a*) for freshwater flounders transferred to waters of higher salinities may not be a true branchial influx curve. The 'second component' of the influx curve as given by me in addition to the saturable component (Maetz 1970*a*) is probably almost entirely due to the intestinal component. The maximal rate of 400 μmol h^{-1} 100 g^{-1} given by Motais *et al.* (1966) agrees relatively well with the maximal rate of gut influx given for the freshwater eel by Skadhauge & Maetz (1967). Recent unpublished observations show that eels drink up to 2 ml sea water per 100 g per hour after sudden transfer from freshwater (R. Kirsch, in preparation). Drinking is therefore not a rate limiting step for intestinal absorption of radioactive Na.

(*b*) *Chloride transport and its control*

Very little work has been done on Cl transport in freshwater fish. In some teleosts, for example the eel, Cl^- is taken up mainly by the gut together with food, the gill being almost impermeable to Cl^- (Garcia-Romeu & Motais 1966). In one recent experiment, I reassessed this 'impermeability' and found f_{in} = 0.3 μmol for $[Cl]_{ext}$ = 1.47 mmol/l while f_{out} (total) was 1.05, the overall balance being thus negative. At a similar Na^+ concentration, the f_{in} for Na^+ would have been 100 times higher.

In other fishes, for example the goldfish, the gill is able to absorb Cl^- at rates similar to those measured for Na^+ (Krogh 1939; Meyer 1948; Garcia-Romeu & Maetz 1964; Maetz, Bourguet, Lahlouh & Hourdry 1964). We observed for a $[Cl]_{ext}$ of 1 mmol/l an f_{in} of 17.5 $\pm$ 3.0 μmol/l and for a $[Na]_{ext}$ of 0.45 mmol/l the Na^+ influx was 9.7 $\pm$ 1.0 μmol, the relative ratios of f_{in} to $[Na]_{ext}$ or $[Cl]_{ext}$ being 17.5 and 21.5 respectively.

No study relating Cl^- influx to $[Cl]_{ext}$ has been made. It may be assumed that for the goldfish as for the crayfish (Shaw 1960*c*) saturation kinetics prevail. In both the freshwater teleostean and the crustacean, Cl depletion produces an increase of the branchial pumping efficiency and a reduction of the Cl efflux resulting in a positive Cl^- balance. It must be noted, however, that in both organisms Cl^- balance is achieved by more complicated feed-back mechanisms than those prevailing for Na^+ according to preliminary observations in collaboration with Garcia-Romeu. Keeping goldfishes in deionized water for example results in an increased Na^+ net uptake when the animals are replaced in NaCl solutions, while the Cl^- balance remains negative. If the depleted fishes are first placed in a Na_2SO_4 solution to readjust the internal

Na^+ level, Cl^-uptake is then very rapid when the fish is placed in a NaCl solution. It seems therefore that the relative internal Na^+ and Cl^- levels also play a role in the regulation of the pumping rate of Cl^-.

(*c*) Na^+ *and* Cl^- *independent uptakes: evidence for separate exchanges with endogenous ions*

Table 1 summarizes selected fluxes measured in goldfish placed in dilute NaCl solutions (0.05 to 2 mmol/l), which clearly show the independence of Na^+ and Cl^- absorptions (Garcia-Romeu & Maetz 1964). Sodium and chloride exchanges are frequently of very different intensities, resulting in net transfers which may be of the same sign but of different values, or

TABLE 1. INDEPENDENCE OF SODIUM AND CHLORIDE ABSORPTION BY THE GOLDFISH IN SODIUM CHLORIDE SOLUTION

	Na^+ exchange			Cl^- exchange		
pretreatment solution	f_{in}†	f_{net}	f_{out}	f_{in}	f_{net}	f_{out}
Na_2SO_4	5	−8	13	36	+9	27
	18	+4	14	73	+29	44
choline chloride	57	+17	40	9	−35	44
	40	+23	17	12	−13	25
deionized water	79	+34	45	21	−44	65
	66	+48	18	.	−31	.
NaCl	25	+2	23	31	+14	17
	40	+1	39	25	+16	9
	1	−3	4	35	+18	17
	8	−2	10	15	−19	34
	27	+6	21	.	−23	.

† f_{in}, influx; f_{net}, net flux; f_{out}, efflux in μmol h^{-1} 100 g^{-1}.
Each horizontal line represents a separate experiment. According to Garcia-Romeu & Maetz (1964).

they may be of opposite signs, indicating a net absorption of one ion and a loss of the other. While this independence is occasional in animals kept in NaCl solutions or in running tap water, it is constant in animals preadapted in deionized water, Na_2SO_4 or choline chloride solutions in order to obtain selective depletions. The independence of the absorption mechanisms is also demonstrated by the vigorous uptake of sodium or chloride ions, notwithstanding the presence of a non-permeant counter-ion such as sulphate, choline or Mg^{2+}. Similar observations were recently made on the trout by Kerstetter *et al.* (1970).

These differences in the absorption of ions of different charges can only be explained in terms of the laws of electroneutrality of solutions, by exchange with endogenous ions of the same charge. The existence of such exchanges is confirmed by the results of conductivity measurements of the external medium. Figure 7 illustrates such experiments showing that the conductivity remains constant or even increases slightly even though Na^+ or Cl^- are absorbed rapidly. Figure 8 shows an experiment in which both ions are absorbed simultaneously and yet the conductivity increases. This figure again shows the effects of a sudden decrease of the internal salt concentration upon the rates of salt absorption for the goldfish.

As regards a counter-ion in exchange of Na^+, one can eliminate the possibility that metallic cations such as K^+ or Ca^{2+} play an important role for their normal rate of loss is much lower than the maximal rate of Na^+ uptake. In the case of Ca^{2+} a net absorption is sometimes observed. Similarly, monovalent or divalent anions such as Br^-, I^- or SO_4^{2-} can be eliminated as exchange

ions for Cl^- because they are present in relatively low concentration in the internal medium and the gill is impermeable to these ions (see Garcia-Romeu & Maetz 1964). Finally, the only endogenous ions which could fulfill the role of exchange ions are either NH_4^+ or H^+ for Na^+, and HCO_3^- or possibly OH^- for Cl^-.

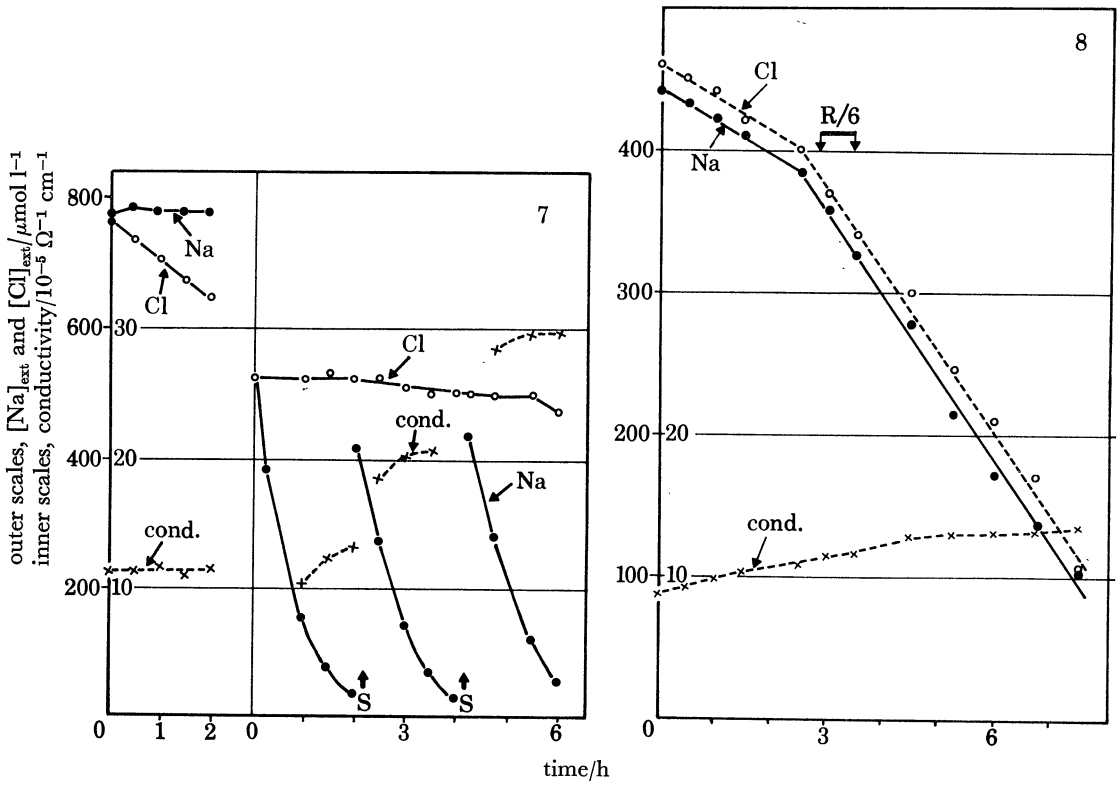

FIGURE 7. Independence of Na and Cl uptakes from an NaCl solution by the goldfish and evolution of the conductivity of the external bath (according to Garcia-Romeu & Maetz 1964). On the left, control fish (preadapted in NaCl solution). On the right, fish preadapted in choline chloride solution for 6 weeks. Arrows (S) indicate successive additions of sodium sulphate made to raise the external Na concentration without altering that of Cl.

FIGURE 8. Simultaneous absorptions of Na and Cl by the goldfish and variations of the external conductivity (figures 8 to 13 according to Garcia-Romeu & Maetz 1964). Experiment on a control fish. The arrow R/6 indicates intraperitoneal injection of hypotonic saline (20 mmol/l NaCl). Observe feed-back stimulation of the Na and Cl uptake rates.

(*d*) NH_4^+ *as the counter-ion exchanged against* Na^+

I have recently reviewed and discussed the arguments in favour of the interaction of salt and ammonia transports in aquatic organisms (Maetz 1971). As pointed out by H. W. Smith (1953) ammonia, which is the principal waste products of aquatic ammonotelic animals, is mainly excreted by way of the gill, this organ being only the site for cation absorption, at least in freshwater animals. He suggests that in the gill as in the mammalian kidney, both mechanisms are linked. Krogh (1939) was actually the first to present experimental observations in favour of such an interaction. The problems remaining to be solved are whether this interaction corresponds to a tight specific coupling or if other ions such as H^+ may also be exchanged and whether it is the Na^+ *net uptake* or *influx* which is to be exchanged.

The measured rates of ammonia excretion (15 to 100 $\mu mol\ h^{-1}\ 100\ g^{-1}$) across the gills of various fishes appear to be sufficient to account for the rate of Na uptake or influx *normally*

observed (up to 100 μmol). Only in one species, the trout, have both rates been compared simultaneously. Kerstetter *et al.* (1970) found that the Na^+ *influx* equals the ammonia excretion when the Na uptake takes place in the adaptation medium and sodium balance is achieved.

Two types of experiments have been designed to study further whether ammonia excretion and Na^+ absorption go hand in hand. One type consists of altering the ammonia excretion while keeping the fish in the same external medium, and to follow the induced modifications of the sodium uptake rate. A second type consists of placing the fish in various external salinities, including distilled water or solutions of impermeant electrolytes, and to correlate the changes in the Na uptake with the corresponding rates of ammonia excretion.

(i) *Indirect evidence in favour of* NH_4^+/Na^+ *exchange*

In collaboration with Garcia-Romeu (Maetz & Garcia-Romeu 1964). I attempted to demonstrate an obligatory Na^+/NH_4^+ exchange in the goldfish in a series of experiments designed to alter ammonia excretion either by adding ammonium salts to the outside closed-circuit medium or by injecting ammonium salts into the intra-peritoneal cavity. The first procedure is known to depress ammonia excretion while the second hastens ammonia clearance via the gills (Fromm & Gillette 1968; Wolbach, Heinemann & Fishman 1959; Pequin 1967). Figures 9 and 10 illustrate the effects on Na exchange of ammonia addition and ammonia injection respectively. A depression of the Na^+ uptake is observed and the sodium balance becomes negative when ammonia is added to the external bath. After rinsing and elimination of the added ammonia, sodium uptake is resumed. Intraperitoneal injection of ammonium sulphate is followed by a prompt increase of Na^+ uptake and net absorption. Both experimental interventions are specific insofar as the Cl^- uptake remains more or less unchanged. Similar observations were made on the eel by Garcia-Romeu & Motais (1966), on the flounder by Motais (1967) and more recently on the trout by Kerstetter *et al.* (1970). Unfortunately in all these experiments no attempt was made to correlate the variations in the rate of ammonia excretion with those of sodium absorption. In a recent series of preliminary experiments on the goldfish, I found that intraperitoneal injection of 500 to 1000 μmol of NH_4Cl per 100 g is accompanied by increased rates of branchial ammonia excretion attaining 150 μmol h^{-1}, a maximal rate which appeared to be in the range of that measured for Na influx. More work is necessary to verify in individual fish whether a satisfactory correlation can be obtained between the two variables.

(ii) *Evidence against an obligatory* NH_4^+/Na^+ *exchange*

The results from the second type of experiment clearly indicate that an obligatory exchange of Na^+ against NH_4^+ does not exist. De Vooys (1968) showed in the carp that when Na absorption is rendered impossible by keeping the fish in deionized water, ammonia excretion continues and is even increased after 24–48 h. Returning the fish to tap water which presumably 'turns on' the Na pump does not induce extra ammonia excretion. Similar observations have been made in the crayfish by Shaw (1960*a*) who also showed that there was no stoichiometric relation between ammonia excretion and Na^+ absorption.

More recently Kerstetter *et al.* (1970) confirmed this absence of correlation in the trout. Sodium exchanges and ammonia excretion were measured in Na^+ phosphate buffer solution of three different sodium concentrations, the pH being kept at 7.0 to 7.5 in order to prevent NH_3 escape from the solution. The three different solutions were tested in consecutive 1 h periods on the same fish. When tested in the external Na concentration (1 mmol/l) at which

the fishes were adapted, $Na^+ f_{in}$ was equal to the rate of ammonia excretion (about 20 μmol h^{-1} 100 g^{-1}) while $Na\, f_{net}$ was nil. Ammonia excretion remained constant however when the sodium influx varied experimentally by a factor of 7 to 8 and the $Na\, f_{net}$ was either positive or negative at the higher or lower range of external Na concentrations. Ammonia excretion also remained within the normal range for fishes kept in sodium-free solutions ($MgCl_2$).

Thus any obligatory exchange between Na^+ and NH_4^+ as suggested by the model given by us

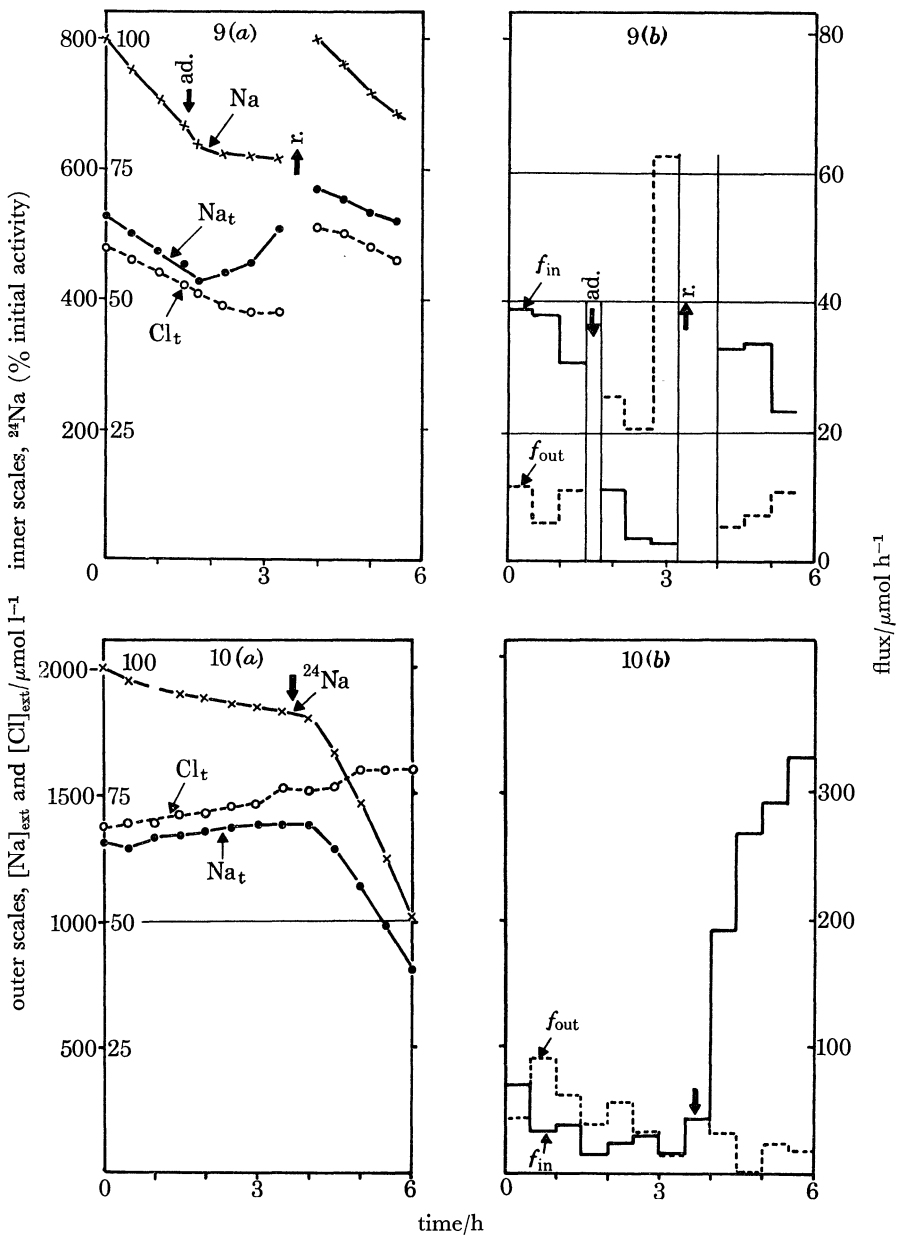

FIGURE 9. Effect of addition of NH_4 ions to the external medium on the sodium exchanges of the goldfish. (*a*) Evolution of external total Na and Cl and ^{24}Na concentrations as a function of time. (*b*) Evolution of the Na unidirectional fluxes (per fish; body weight 85 g). Arrow ad., addition of a solution of ammonium sulphate giving 9 mmol/l concentration in the bath; r., rinsing and renewal of external bath.

FIGURE 10. Effect of intraperitoneal injection of NH_4 ions on the Na exchanges of the goldfish. Arrow, injection of 0.6 mmol ammonium sulphate per 100 g fish. Body weight 312 g.

(Maetz 1971) for the mechanisms of NaCl uptake by the 'freshwater gill' must be ruled out. A decade ago, Shaw (1960*a* to *c*) had already arrived at the same conclusion for the crayfish. He suggested that Na^+/NH_4^+ exchange constitutes the 'normal' mechanism, but adds that 'there is *a priori* no reason for supposing that the exchange process involves only a single ion species'. Obviously a H^+/Na^+ exchange must be considered as an alternative possibility.

(*e*) H^+ *as the counter-ion exchanged against* Na^+

(i) Na^+/H^+ *exchange in amphibian skin*

Garcia-Romeu, Salibian & Pezzani-Hernandez (1969) recently demonstrated the existence of a Na^+/H^+ exchange as part of the mechanism of Na uptake through the skin of the Chilean frog *Calyptocephallela gayi*, *in vivo*. Adult amphibians, in relation to their invasion of dry-land and restriction of the availability of water, have changed from ammonotelism to ureotelism. Hence, the quantities of ammonia excreted by the skin (ranging from 1 to 7 μmol) are far too small to account for the maximal rates of Na uptake (Garcia-Romeu & Salibian 1968). In frogs, as in fishes however, independence of Na^+ and Cl^- uptake prevails. Garcia-Romeu and his colleagues observed that Na^+ transport in the presence of impermeant sulphate is accompanied by a downward shift of pH of 0.30 ± 0.04 units per hour in the external medium. In all these experiments, the measured increase of H^+ activity however, does not represent the actual H^+ addition to the bath, because of the progressive buffering of the external medium. They showed by measuring with NaOH the increase of titrable acidity, that an excellent correlation occurs between H^+ excretion and Na^+ absorption, suggesting a one to one relationship.

That Na^+/H^+ exchange is the prevailing mechanism is further confirmed by the fact that acidification of the external bath is followed by a specific inhibition of Na uptake, in both *in vitro* (Schoffeniels 1955) and *in vivo* skins (Garcia-Romeu *et al.* 1969). In the crayfish also, Shaw (1960*c*) observed that H^+ ions are 10 times more effective than NH_4^+ in depressing Na^+ uptake. A similar effect of external acidity has also been demonstrated in the goldfish (Maetz 1971) and the trout (Packer & Dunson 1970). In both species Na^+ influx was totally inhibited at an external pH below 4.0, while Na efflux increased markedly and as a result a loss of body sodium occurred, suggesting that such a H^+/Na^+ exchange also prevails in fish.

(ii) Na^+/H^+ *exchange in fishes*

Kersetter *et al.* (1970) investigated the possibility of an Na^+/H^+ exchange in the trout. Flux determinations were made in a chloride-free phosphate buffer to which Na_2SO_4 was added to give three different sodium concentrations. Chloride-free solutions were used in order to eliminate a production of bicarbonate from a Cl^-/HCO_3^- exchange (see below). The pH of the external solutions before and after hourly test periods was measured. The results are given in table 2. First, it can be seen that, as discussed above, there is no correlation between Na^+ influx and NH_4^+ output. Secondly, at the highest external Na^+ concentration, the pH decreases significantly as the Na^+ influx greatly exceeds the ammonia output. The corresponding H^+ output calculated from the pH shift of the buffer is about 10 μmol as against 33.4 μmol h^{-1} Na uptake. The difference corresponds to the rate of ammonia excretion (about 23 μmol). At 1 mmol external Na^+ concentration, the Na uptake is more or less compensated by the ammonium output and a slight, barely significant downward pH shift is observed. At 0.1 mmol external Na^+, the Na^+ balance is negative and a slight but not significant upward shift of pH is observed. When unbuffered external media were used, pH shifts were erratic and usually

upward at all rates of f_{in}. The authors recorded copious mucous secretion which may have masked the addition of H^+ ion, but nevertheless feel justified in concluding that an obligatory H^+/Na^+ exchange occurs in fish as in amphibians.

TABLE 2. EFFECTS OF DIFFERENT Na^+ CONCENTRATIONS ON Na^+ FLUXES, NH_4^+ OUTPUT, AND pH CHANGES

$[Na^+]$/mmol l^{-1}	f_{in}†	f_{out}†	NH_4^+†	ΔpH $\pm$ S.E.
7.0	33.4 $\pm$ 6.3	29.8 $\pm$ 5.7	15.4 $\pm$ 2.4	$-0.31 - 0.08$
1.0	17.9 $\pm$ 1.1	16.0 $\pm$ 2.9	17.4 $\pm$ 0.6	-0.07 ± 0.04
0.1	6.2 $\pm$ 1.5	11.6 $\pm$ 2.8	12.4 $\pm$ 1.8	$+0.07 \pm 0.08$

† μmol h^{-1} 100 g^{-1} $\pm$ S.E.
According to Kerstetter *et al.* (1970).

(iii) *Branchial permeability to free ammonia or ammonium ion*

Kerstetter *et al.* (1970) suggest that all the ammonia excreted is in the molecular form, but with a pK_a of 9.3, this molecule would inevitably act as a H^+ trap in neutral solutions, accepting H^+ not only from a Na^+/H^+ exchange but also from the carbonic acid formed by respiratory CO_2 excreted in the bath, and this would prevent an excessive decrease of the pH in the closed-circuit bath. Thus, at the higher rates of H^+ production in relation to Na^+ absorption, the pH would decrease despite the trapping of H^+ by ammonia, since insufficient ammonia would be available. At very low rates of Na^+ absorption, however, the excreted ammonia would occur as a free base and an upward pH shift would be expected, especially if the CO_2 production were not too high.

Shaw (1960*b*) and Maetz (1971) favour a different model. They suggest that ionized NH_4^+ is normally exchanged against Na^+. At the higher rates of Na^+ absorption H^+ ion excretion supplements the ammonium excretion to account for the Na^+ exchanged. At rates of Na^+ uptake lower than ammonia output, we agree with Kerstetter *et al.* that some of the ammonia must then be excreted in molecular form. Our model suggests that alternative H^+ or NH_4^+ exchanges may occur through the same channel. According to Shaw 'sodium may be exchanged for either, depending on the relative rates of excretion and the type of metabolic activity displayed by the animal at the time'. Maetz (1971) stresses that an important question is whether the branchial epithelium is able to transfer both forms of ammonia or only free ammonia. Recent evidence strongly suggests active transport of NH_4^+ across some epithelial or cellular membranes (Mossberg 1967). Indications that the gill may be the site of active NH_4^+ transport based on data which cannot be explained in terms of simple diffusion of free ammonia are found in a preliminary report by Wolbach *et al.* (1959). In their experiments concerning the fate of intraperitoneally injected ammonium solutions in catfish kept in a closed-circuit aquarium, the final concentration of ammonia excreted in the bath was observed to be two to three times the concentration in the blood. This occurred even when the blood was more acid than the bath: excretion of ammonia was only reduced by one-third even against a 2 mmol/l gradient. Such experiments, if confirmed, would invalidate the part of the model suggested by Kerstetter and his colleagues in which free ammonia was considered to be excreted under all experimental conditions.

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(iv) *The origin of* H^+ *excreted*

With regard to the origin of H^+, which directly or indirectly in combination with ammonia is exchanged against Na^+, hydration of CO_2 producing carbonic acid, a reaction catalysed by carbonic anhydrase, is the obvious source, as suggested by Maetz & Garcia-Romeu (1964). Maetz (1956*b*) showed that inhibition of this enzyme in the goldfish is followed by a depression of Na uptake, an effect confirmed by Kerstetter and his colleagues (1970) on the trout. The latter group also made the important observation that acetazolamide injection is not followed by an alteration of the total ammonia excretion rate. Table 3 summarizes the results obtained by Kerstetter *et al.* Instead of the significant downward shift of the external pH, observed during

TABLE 3. EFFECTS OF ACETAZOLAMIDE ON Na^+ FLUXES, NH_4^+ OUTPUT, AND pH CHANGES IN THE TROUT

$[Na^+]$/mmol l^{-1}	f_{in}†	f_{out}†	NH_4^+†	pH ± S.E.
1.0 before Diamox	18.0 ± 1.6	15.7 ± 3.6	15.4 ± 1.6	−0.15 ± 0.03
1.0 after Diamox	2.6 + 0.9	10.2 ± 2.6	12.6 ± 1.2	± 0.06 ± 0.05

† μmol h^{-1} 100 g^{-1} ± S.E.
According to Kerstetter *et al.* (1970).

the control period, an upward shift is recorded after acetazolamide injection, which suggests release of free-ammonia into the bath. I suggest that when H^+ is not readily available, the ratio of unionized to ionized forms of ammonia crossing the membrane is increased, and Na^+ exchange is hindered.

(*f*) *Evidence for a* Cl^-/HCO_3^- *exchange system*

(i) *Indirect evidence*

The hypothesis that HCO_3^- ions are the endogenous ions exchanged against Cl^-, as originally suggested by Krogh (1939), stems from the fact that the gill is the major route of CO_2 excretion and the amount of CO_2 excreted is more than sufficient (up to 500 μmol h^{-1} 100 g^{-1}) to cover the needs of the postulated HCO_3^-/Cl^- exchange. Furthermore, the presence of carbonic anhydrase in the gill tissue also suggests such an exchange. This hypothesis was submitted to experimental analysis by Maetz & Garcia-Romeu (1964). The indirect method, consisting of an analysis of the effects on sodium and chloride absorption rates of experimental changes of bicarbonate concentrations in external or internal media, was preferred to the direct method involving physicochemical analysis of the external medium because of the difficulty of determining to ratios of ionized and unionized forms of CO_2 crossing the membrane. Figures 11 and 12 illustrate typical experiments which are exact replicas of the experiments depicted in figures 9 and 10. The addition of bicarbonate (in the form of the K^+ salt) to the external medium produces a specific inhibition of Cl^- uptake and removal of the bicarbonate by rinsing fully restores the initial pumping activity. HCO_3^- added to the external medium probably acts by competing for the chloride pump.

(ii) *The role of carbonic anhydrase*

With regards to the importance of carbonic anhydrase as an accelerator of the exchange process, Maetz & Garcia-Romeu demonstrated that inhibition of the enzyme by injection with

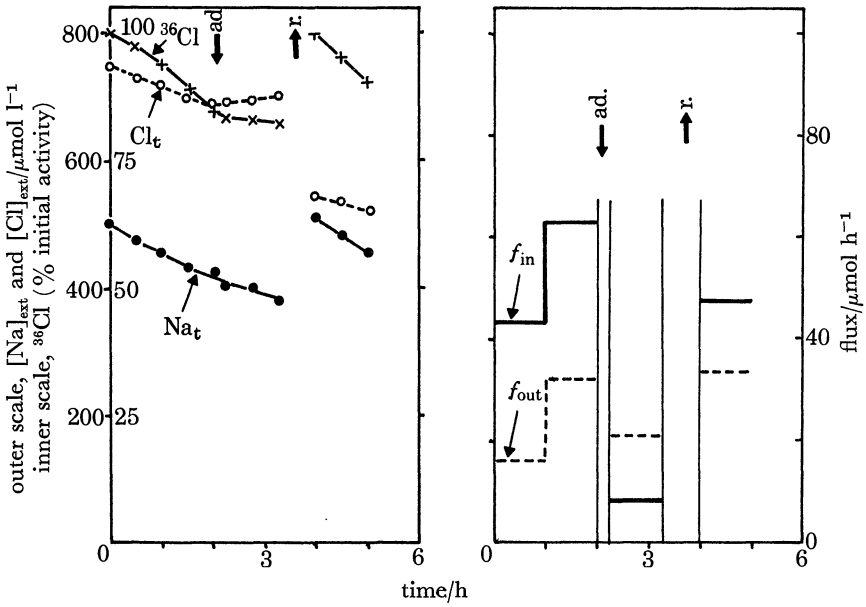

FIGURE 11. Effect of addition of HCO_3^- ions on the Cl exchanges of the goldfish. Ad., addition of K bicarbonate giving an 18 mmol/l concentration in the bath. r, rinsing and renewal of external bath. Weight of fish 273 g.

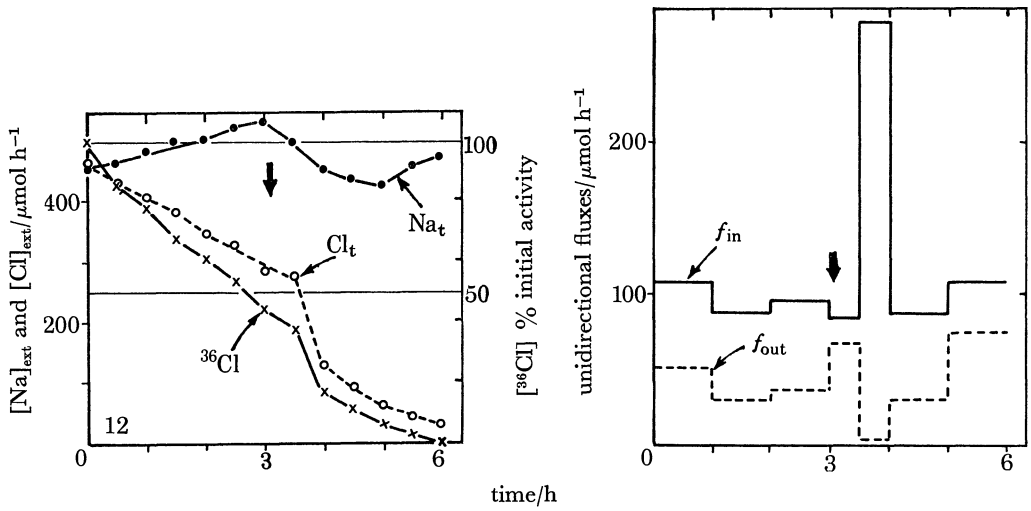

FIGURE 12. Effect of intraperitoneal injection of HCO_3^- ions on the Cl exchanges of the goldfish. Arrow, 0.6 mmol per 100 g fish of $KHCO_3$ solution. Weight of fish 307 g.

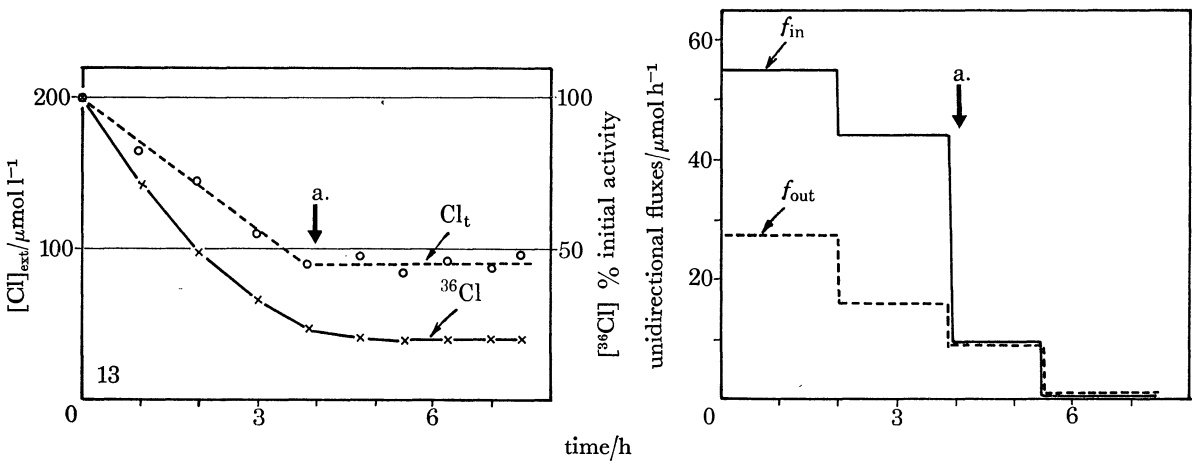

FIGURE 13. Effect of acetazolamide injection (*a*) on the Cl exchanges of the goldfish. Left hand-graph: evolution of external total Cl and ^{36}Cl concentrations. Right-hand graph: Evolution of the unidirectional fluxes. Dose of inhibitor: 2 mg/100 g fish (weight of fish 160 g).

acetazolamide is followed by a 75% decrease of the Cl^- uptake (see figure 13). Conversion of respiratory CO_2 into bicarbonate must be considered to be a slow process which is rate limiting in the proposed exchange.

(iii) *Stoichiometry of the* Cl^-/HCO_3^- *exchange*

For the Cl^-/HCO_3^- exchange as for that of NH_4^+/Na^+, it remains to be verified whether a stoichiometric relation exists between the excretion rate of endogenous HCO_3^- and the Cl^- absorption flux. Such a relation was observed by Garcia Romeu *et al.* (1969) for the Chilean frog during Cl^- absorption from a choline chloride solution in the absence of Na^+/H^+ exchange. If both exchanges occur simultaneously in NaCl solutions, the counter-ions H^+ and HCO_3^- combine to produce H_2O and CO_2 in the external bath making the titration of the endogenous ions appearing in the outside bath impossible. Such a combination explains previous observations by Salibian, Pezzani-Hernandez & Garcia-Romeu (1968) on the related *Leptodactylus* of a decrease of the conductivity of the external solution during simultaneous Na^+ and Cl^- absorption.

(iv) *Evidence for an obligatory exchange*

There is further compelling evidence for the goldfish that HCO_3^-/Cl^- exchanges may be obligatory. Dejours (1969) observed that when the ionic composition of the external medium is suddenly changed by transfer from a NaCl to a Na_2SO_4 solution, a sharp reduction of the output of respiratory CO_2 is observed within 30 min. In some cases a reversal of CO_2 excretion, that is an absorption of CO_2 is seen, sometimes for as long as 24 h. When the animal is then replaced in NaCl solution CO_2 excretion is resumed and considerably increased. The above-mentioned variations of the CO_2 transfer across the gill do not reflect variations of the metabolic CO_2 production, as O_2 consumption remains essentially steady during the experiment.

According to Dejours (1969), the dependence of the CO_2 transfer upon the ionic composition of the water is best explained in terms of an obligatory Cl^-/HCO_3^- exchange: HCO_3^- entry against Cl^- loss after the suppression of external Cl^-, massive HCO_3^- exit upon return to the NaCl solution probably as a result of compensatory Cl^- uptake induced by either internal Cl^- depletion or elevated plasma HCO_3^- concentration. This interpretation suggests that most of the CO_2 released is not in molecular but in the ionized form.

The occurrence of a H^+ or NH_4^+/Na^+ exchange together with that of a HCO_3^-/Cl^- exchange in the gill, with each ion uptake mechanism operating independently, exemplifies the role of the gill in the maintenance of acid-base balance, a function similar to that of the mammalian distal kidney tubule (Pitts 1964).

(*g*) *Inclusion of the proposed exchanges into carrier-mediated active transports*

(i) *Evidence for active transport of* Na^+ *and* Cl^-

When ionic exchanges linked with ionic uptake (across membranes) from a medium of low sodium and chloride concentration are considered, the question arises whether the mechanisms involve endothermic processes of either the 'facilited diffusion' type or of that of 'active transport'. All present evidence points to the active transport of both Na^+ and Cl^- ions. As shown by Garcia-Romeu & Maetz (1964) in salt-depleted goldfish, the ratio of influx to efflux (f_{in}/f_{out}) may be as high as 2.5 for Cl^- and 5.5 for Na^+ for a ratio of internal to external concentrations and presumably activities (C_{in}/C_{ext}) averaging 300 for Na^+ and 650 for Cl^-.

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Applying Ussing's criterion to calculate the difference of potential that would explain these ratios in term of passive transport, a difference of 180 to 190 mV positive inside for chloride and negative inside for sodium should occur. Such a high bioelectrical potential has never been encountered.

The few reports of the potential differences occurring across the gills of fish in fresh water or in dilute sea water are contradictory. On the one hand and despite differences in the techniques used, House (1963), Evans (1969) and Maetz & Campanini (1966) report potentials negative with respect to the blood in three euryhaline species: *Blennius pholis*, *Pholis gunnellus* and *Anguilla anguilla*. For the eel (see figure 14) potentials as high as 20 mV have been observed by us. On

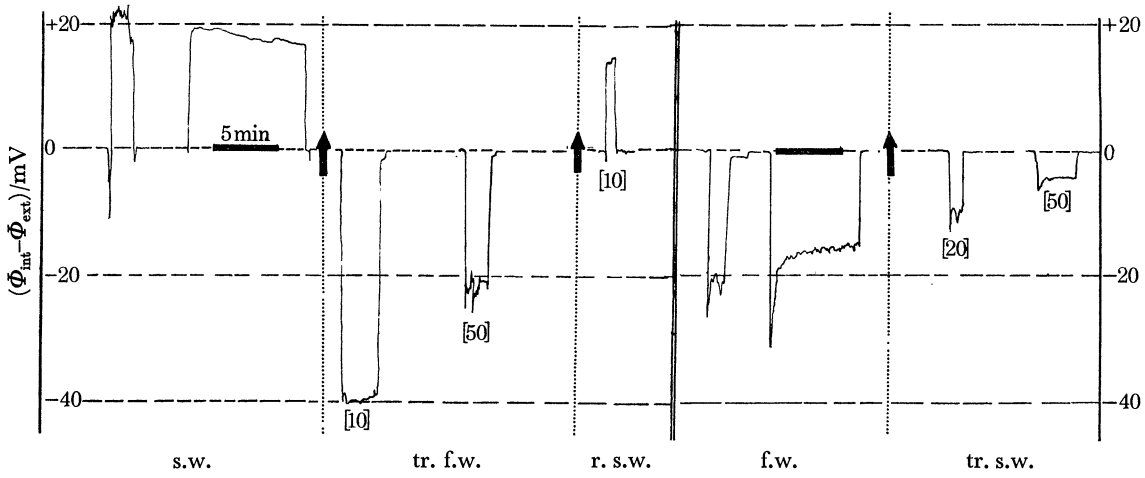

FIGURE 14. Recordings of potential measurements across the branchial epithelium of the eel *in vivo*. Successive impalements (according to Maetz & Campanini 1966). Left-hand side: seawater adapted eel in sea water (s.w.), after transfer to fresh water (tr. f.w.) for 10 or 50 min, or 10 min after return to s.w. (r. s.w.) following a 20 min period in f.w. (Time lags are indicated in brackets under recordings.)

the other hand, potentials up to 8 mV positive inside were recently reported for the trout by Kerstetter *et al.* (1970). In this respect the trout seems to resemble other aquatic vertebrates such as the frog (Brown 1962) or salamanders (Dietz, Kirschner & Porter 1967). According to Kerstetter and his colleagues, however, a negative potential inside as high as 15 mV is observed for the trout kept in 1 mmol/l NaCl without Ca^{2+}. The eels studied by Campanini and me were kept in running fresh water containing about 2 mmol/l Ca^{2+}.

(ii) *Partitioning of the exchange fluxes*

Let us assume for the goldfish a potential difference negative inside by 20 mV and the ratios of fluxes and concentrations given above, and determine from these data the 'partitioning' of the fluxes into various active and passive components. If, in addition, we assume for the goldfish as for the flounder that the exchange-diffusion component is negligible, we find that practically all the influx corresponds to the active component for both electrolytes, while the totality of the outflux is diffusive. According to Kerstetter *et al.* (1970), the respiratory epithelium may well be a pure leak path for both electrolytes. The authors report a personal communication by D. J. Randall that Na^+ efflux may rise with physical activity in the trout, while Na^+ influx remains unchanged. Physical activity is known to increase lamellar circulation. We have already discussed the arguments in favour of the 'chloride cells' as the sites of active electrolyte

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transport. If the leak pathway is located in the respiratory epithelium, the simplest assumption is that most of the passive Na^+ and Cl^- effluxes are linked. Hence for the 'active cell', we must assume that it is Na *influx* or Cl *influx* which must be related to the extrusion rates of NH_4^+ and H^+ or HCO_3^-.

(iii) *A simple model of the chloride cell*

Let us now consider the simplest model for the 'chloride cell' across which ion movements may be considered in terms of two barriers, the outer cell membrane and the inner (blood) border. Kerstetter *et al.* (1970) suggest that the Na–K activated ATPase which has been reported in the teleostean gill (see above), plays a key role in the sodium pump and is located at the inner border in a manner analogous to that in the frog skin model proposed Ussing (1960). This location is suggested by the fact that K^+, when added to the outside bath, even at concentrations up to 50 times that of the Na^+ concentration, has no effect on the Na pumping mechanism (Maetz 1970*a*). Kerstetter *et al* (1970) found that the K_m for the ATPase *in vitro* was 5–10 mmol/l for Na^+, the maximal rate being observed at 100 mmol/l, in sharp contrast with the K_m observed for Na^+ transport (0.5 mmol/l) discussed above. If this discrepancy between the values of K_m is real, two models are suggested by the authors to account for it. In the first, the Na^+ movements through the outer border are supposed to be diffusive and mediated by fixed charges located inside the membrane. To obtain a Michaelis–Menten type curve for transport resulting from the ATPase activity but 'reflected an order of magnitude lower to the external concentration' it is necessary to assume impossibly high intracellular Na^+ concentrations, up to 100 mmol/l. In addition, an electrochemical gradient for passive H^+ movement outward at neutral pH outside would require an intracellular pH of 6.0 or lower.

The second model proposed suggests that the observed curve of concentration dependence of Na^+ influx must be generated by the outer membrane, which implies that the movement of Na^+ would not be diffusive but would involve binding at a finite number of sites mediating an obligatory exchange between H^+ and Na^+. Na^+ transport would be completed by the Na–K dependent ATPase activity of the inner border and H^+ ions would result from the action of carbonic anhydrase, which would also supply HCO_3^-. This second model seems to us to be the more probable, with the additional hypothesis that NH_3 originating from the clearance of plasma ammonia or from the metabolic activity of the deaminating or deamidating enzymes located in the branchial cells (see Forster & Goldstein 1969) would combine with H^+, the outer binding site accepting indifferently NH_4^+ or H^+, as suggested by Shaw (1960*b*). As regards the role of the ATPase in a Michaelis–Menten type of kinetics, it must be added that Motais (1970*a*), in a recent study of the branchial ATPase of the eel, found that its interaction with the Na–K substrate cannot be interpreted in terms of classical theories of enzyme action but that it is probably mediated by allosteric transition. Enzyme kinetics may therefore not reflect Na transport kinetics at all.

With regards to the chloride transport coupled with HCO_3^-, I favour the model recently proposed by Sachs (1970) for the active chloride transport of the oxyntic cells. This author has extracted and purified from this tissue the HCO_3^- activated ATPase discovered by Kasbekar & Durbin (1965) which is probably responsible for the HCO_3^-/Cl^- exchange in a manner comparable with that suggested for the Na^+/K^+ exchange mediated by the Na–K dependent ATPase. Furthermore, carbonic anhydrase plays an important role in both transport mechanisms. To complete the model depicted in figure 15, many more assumptions have been made.

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I suggest that the K^+ entering the chloride cells by way of the Na–K pump replaces the K^+ lost by passive diffusion through the inner border. In addition, I suggest that the chloride entering by way of the exchange pump located at the outer border is taken up by a second pump presumably electrogenic, permitting Cl^- to pass the inner border. The observed potential difference would thus be explained.

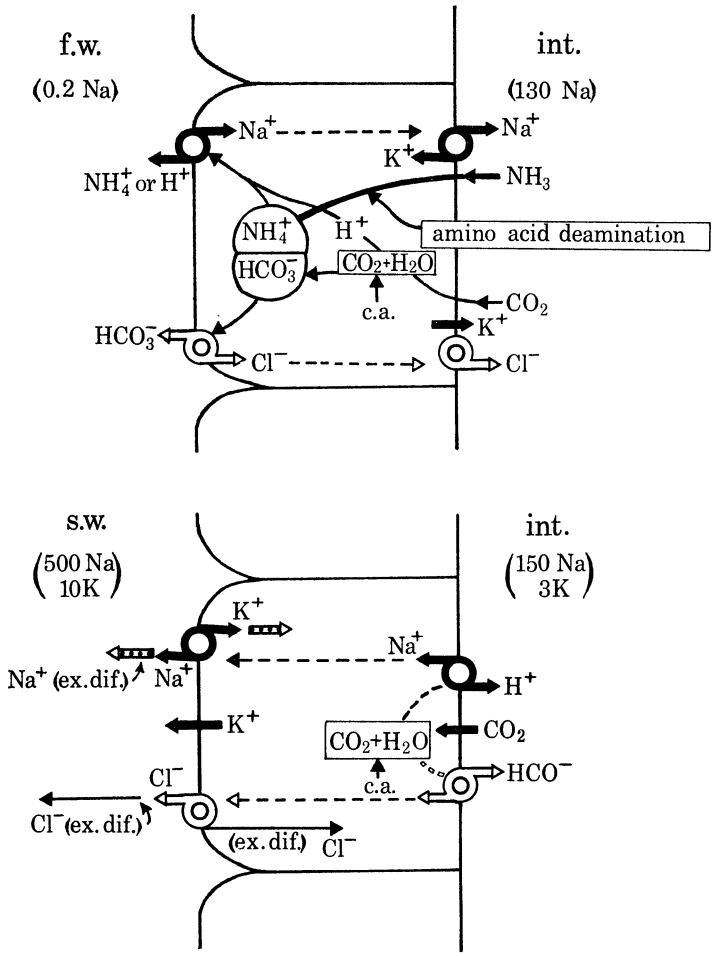

FIGURE 15. Functional model of the 'chloride cell' in fresh water and sea water. In fresh water, independent Na^+/NH_4^+ or H^+ and Cl^-/HCO_3^- exchanges are located on the mucosal border. The role of carbonic anhydrase (c.a.) and of deaminating enzymes in the production of HCO_3^-, H^+ and NH_3 is also shown. Most of the NH_3 is extracted by blood clearance. On the inner border, a Na^+/K^+ exchange and a Cl pump are depicted. In sea water, independent Na^+/K^+ exchanges associated with Na^+/Na^+ exchange and a chloride pump associated with Cl^-/Cl^- exchanges are located on the outer border of the cell. On the inner border, Na^+/H^+ and Cl^-/HCO_3^- exchanges are depicted. The central role of carbonic anhydrase (c.a.) in the production of H^+ and HCO_3^- is also shown.

3. ELECTROLYTE TRANSFER MECHANISMS IN SALT WATER

(*a*) *Relative role of the gill and other effector organs of osmoregulation in the overall turnover of sodium and chloride*

Figure 1 indicates that the Na^+ exchange across the gill in the seawater eel is very fast, representing about 25 to 30 % of the exchangeable Na^+ per hour. The chloride turnover was found by me (in unpublished experiments) to be as rapid as the Na turnover rate. In some fishes

such as *Fundulus heteroclitus* (Potts & Evans 1967) or the salmon smolt (Potts, Foster & Stather 1970) and also in the brine shrimp, *Artemia salina*, studied by our group (Thuet, Motais & Maetz 1968), the chloride exchange is faster than the Na^+ exchange. In other fishes, for example, *Platichthys flesus* (Motais 1967), *Xiphister atropurpureus* and *Pholis gunellus*, (Evans 1967, 1969) the contrary is observed. Figure 16 illustrates the pattern of the appearance of ^{24}Na and ^{36}Cl injected into two fish in the external closed-circuit bath. An exponential rate is observed corresponding to an exchange between two compartments in steady-state. For the teleost *Serranus*, the turnover rates are fast (50 % per hour) and similar for both electrolytes. For the

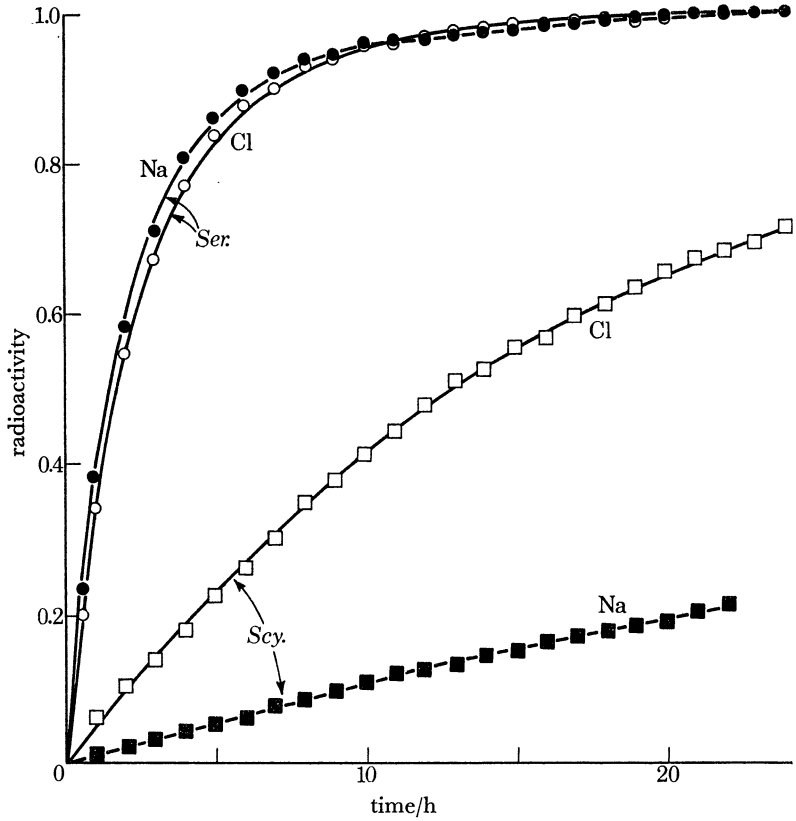

FIGURE 16. ^{24}Na and ^{36}Cl appearance curves from a seawater teleost *Serranus* and a seawater elasmobranch *Scyliorhinus* (according to Payan & Maetz 1970). Note the slow turnovers in the elasmobranch. *Scy.*, *Scyliorhinus*; *Ser.*, *Serranus*.

elasmobranch *Scyliorhinus* the rates of exchange are very slow: 0.5 and 5 % h^{-1} 100 g^{-1} respectively for Na^+ and Cl^-. As pointed out by Payan & Maetz (1970) such a difference emphasizes the contrasting ways in which teleosts and elasmobranchs achieve their osmotic balance.

Figure 1 also shows the relative roles of the various boundaries or effector-organs of osmoregulation. The skin has been considered as impermeable to ions, in view of its impermeability to water as demonstrated by Motais, Isaia, Rankin & Maetz (1969). The kidney plays a minor role in NaCl excretion, although the exchange fluxes across the kidney tubule are very fast (Lahlou 1970). The gut plays a major role, permitting the absorption of water compensating for the osmotic water loss by the gills. The intestinal water absorption is secondary to salt uptake (Skadhauge 1969; Maetz 1970*a*, *b*) but the mineral balance is maintained as a result of the active NaCl excretion by the gill. Our latest determinations of water uptake for the eel obtained with the help of colloidal gold ^{198}Au, yield a value of 167 ± 28 μl h^{-1} 100 g^{-1} (Maetz

1970*a*). Assuming complete absorption of the water swallowed, the rate of Na and Cl net absorption by the gut attains 90 to 100 μmol h^{-1} 100 g^{-1}. We assume that a similar quantity is excreted by the gill. This net excretion rate is at least ten times lower than the isotopically measured efflux, about 1200 μmol for Na^+ and 1050 μmol for Cl^-. Consequently, most of the sodium or chloride influx must occur by way of the gills. Motais & Maetz (1965) were first to point this out for the flounder. They showed in addition that for influx studies concerning sea water adapted fish in sea water or dilute sea water, the gut influx component is negligible as the gut already contains 'cold' sea water which 'dilutes' the specific radioactivity of the Na swallowed during the experiment. Motais *et al.* (1966) report for the flounder a branchial sodium influx of 2250 μmol against an efflux of 2600 μmol. The netflux thus found -350 μmol is somewhat greater than that of the -100 μmol calculated from the drinking rate (192 μl, see Motais *et al.* 1969). The difference is probably explained by the difficulties of estimating the internal sodium space which was given as 19% body weight for the half hourly influx measurement.

The brine shrimp, *Artemia salina*, displays mechanisms of salt and water balance very similar to those described for teleosteans in sea water. Croghan (1958*a*, *b*, *c*) was the first to point out this interesting physiological convergence. Drinking rate amounts to 2 to 3% of the body weight, relatively more than in teleosteans. Very high Na^+ and Cl^- turnover rates have been found, the exchange occurring mainly by way of the gills (Thuet *et al* 1968; P. G. Smith 1969*a*, *b*).

(*b*) Na^+ *and* Cl^- *exchanges in relation to internal* NaCl *level*

Mayer & Nibelle (1970) in our laboratory have demonstrated that in the seawater eel experimental changes of the internal sodium level triggers off changes in the branchial Na^+ excretion

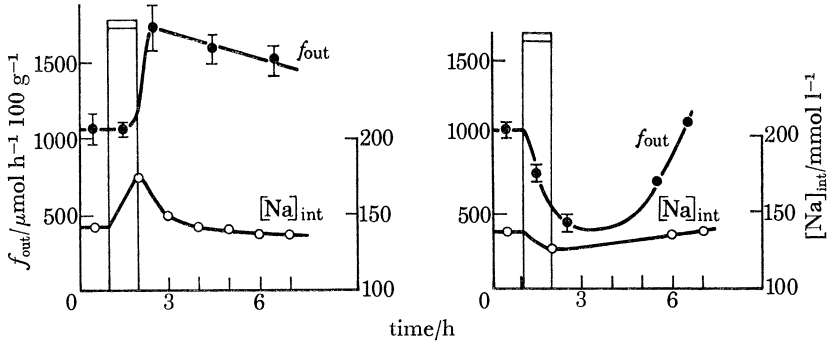

FIGURE 17. Evolution of the Na efflux and internal Na in eels subjected to hypersaline (left) or hyposaline (right) intravenous infusions (according to Mayer & Nibelle 1970). Compare with figure 6.

rate. A salt load induces an increase, while water loading is followed by a decrease of f_{out} (see figure 17), the influx remaining unchanged. The branchial net flux is thus either increased or reversed and mineral is restored within a few hours. Thus, feed-back mechanisms permitting the maintainance of the internal Na^+ level are of importance in seawater as well as in freshwater fish. Similar studies concerning chloride balance have recently been made by R. Kirsch (personal communication).

Shock in seawater fish induces increased Na turnover (Maetz, Sawyer, Pickford & Mayer (1967*c*) in the killifish; Mayer & Maetz (1967), in the eel). In shocked fish, the drinking rate is much higher. Motais & Maetz (1965) found 1000 μl for handled flounders in which the anal papilla had been ligatured according to the technique of H. W. Smith (1930). As Maetz &

Skadhauge (1968) pointed out, salt swallowed by drinking does not tax the salt absorbing capacity of the gut except in media hypersaline to sea water. I suggest that increased drinking following shock imposes an additional salt load on the fish, which in turn induces an increased Na^+ and Cl^- efflux.

(*c*) *Electrical potentials across the gill in sea water*

Few measurements of potentials have been made but these agree that the blood is electropositive to sea water by about 20 mV (*Blennius* + 23 mV, House (1963); *Anguilla* + 18 mV, Maetz & Campinini (1966); *Pholis* + 18 mV, Evans (1969)). Similarly, in the brine shrimp P. G. Smith (1969*a*) found + 23 mV. Figure 14 illustrates typical measurements obtained on the eel in our laboratory. Applying Ussing's passive flux ratio criterion for the eel, with external concentrations of Cl^- and Na of 610 and 510 mmol/l respectively and internal concentrations 135 and 145 mmol/l, and with a potential of + 18 mV, the expected ratio of fluxes (f_{in}/f_{out}) across the gill should be about 1.75 for Na^+ and 9.1 for Cl^-. In fact the observed ratios are 0.9 for both Na^+ and Cl^-. There is therefore no doubt that Cl^- is excreted actively while for Na^+ most *but not all* movements are diffusional. In the brine shrimp, P. G. Smith (1969*b*) also concludes that there is active transport of Cl^-. As regards Na^+, I disagree with P. G. Smith's conclusion that the totality of the Na exchange in *Artemia* is passive in nature, because he discusses the flux–force relation in terms of the Nernst equation, assuming $f_{in} = f_{out}$, and ignoring that the efflux is greater than the influx by 1000 to 1500 μmol h^{-1} 100 g^{-1} to compensate for the salt absorbed by the gut. The actual flux ratio is 0.6 according to Thuet *et al.* (1968). For *Artemia*, as for teleosts, an active sodium transport outwards occurs against the chemical gradient and despite the electrical gradient which favours passive efflux of Na^+. My conclusion therefore contradicts that recently expressed by Kirschner (1970) and agrees with that given by Potts and his colleagues (1970); who also suggest active transport of Na^+ for salmon smolts in sea water. It is also supported by the recent discovery of increased Na–K dependent ATPase activity in the gills of seawater fish and *Artemia* (Epstein, Katz & Pickford 1967; Kamiya & Utida 1968; Augenfeld 1969; Jampol & Epstein 1970; Motais 1970*a*, *b*). This enzyme has been linked in many cells and epithelia to the presence of an active Na pump. Further considerations concerning the nature of the branchial Na^+ pump as a Na^+/K^+ exchange pump give additional support to this conclusion.

(*d*) *Branchial* K^+ *exchange in seawater adapted fishes*

In sea water, the concentration of K^+ is 10.5 mmol/l, while the concentration found in fish plasma ranges from 3 to 10 mmol/l (see review by Holmes & Donaldson 1969). I have recently measured the rates of K influx and efflux in two euryhaline teleosts adapted to sea water. For the flounder (Maetz 1969) I found: $f_{in} = 120 \pm 24$ μmol h^{-1} 100 g^- and $f_{out} = 145 \pm 46$ μmol h^{-1} 100 g^{-1}. This balanced exchange concerns, in contrast to the Na^+ and Cl^- exchanges, only a small fraction of the internal K^+ per hour. For the eel, very similar values were found (J. Maetz, unpublished). As regards the site of the exchanges, it is certain that the gill plays the exclusive role, as the renal loss and gut absorption do not account for more than 1 or 2 μmol h^{-1} (Lahlou 1970; Maetz & Skadhauge 1968).

Taking into account a concentration ratio $[K]_{ext}/[K]_{int}$ of 2.95 for the eel, the equilibrium potential should be 28 mV (inside positive) to account for passive exchanges. In conclusion part of the K^+ transported by the gill must be brought about by active transport. P. G. Smith (1969*b*) arrived at a similar conclusion for *Artemia*.

(*e*) Na^+ *and* Cl^- *exchanges in relation to external salinity*

In considering the variations of the exchange fluxes with external salinity, it is essential to distinguish between results from 'rapid-transfer' experiments where no acclimation to the new salt concentration has occurred and observations in which the fish is allowed to acclimatize to the new environment. Most of the experiments were carried out with euryhaline fishes.

(i) *Studies concerning adapted or 'steady-state' fish*

Potts & Evans (1967) on *Fundulus heteroclitus* and Maetz & Skadhauge (1968) on *Anguilla anguilla* observed that adaptation to media of increasing or decreasing salinity is accompanied by variations in the drinking rate. Increased drinking when the fish is living in higher salinities

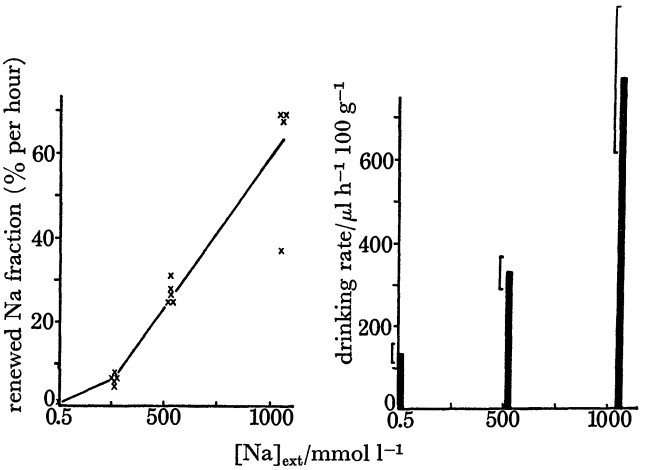

FIGURE 18. Drinking rate and branchial Na turnover as a function of external Na concentration in the eel (according to Maetz & Skadhauge 1968).

permits the maintenance of water balance by compensating for increased osmotic water loss through the gill. Skadhauge & Maetz (1967) and Oide (1967) discovered that the gut adapts to the increased salinity by increased efficiency of water and salt uptake. As the internal Na^+ and Cl^- levels remain more or less constant in euryhaline fish, we must assume that increased intestinal salt uptake is compensated by increased branchial NaCl excretion. The existence of such compensating mechanisms is also suggested by the above-mentioned studies by Mayer & Nibelle (1970) on the feed-back regulations intervening in mineral balance. Adaptation to increased external salinity is accompanied by an increased salt turnover, as first shown in the flounder by Motais *et al.* (1966), and as confirmed for the killifish by Potts & Evans (1967), and the eel by Maetz & Skadhauge (1968). Figure 18 illustrates our observations concerning drinking and Na^+ turnover rate on the eel.

Stenohaline fishes, e.g. *Serranus*, normally found in sea water may also adapt to dilute ($\frac{1}{4}$ strength) sea water and the Na^+ turnover rate is then reduced, although less so than in euryhaline fishes.

(ii) *Studies concerning rapid-transfer experiments: 'instantaneous' and 'secondary' efflux readjustments*

The kinetics of the unidirectional flux readjustments during the adaptation of euryhaline fishes to a new environmental salinity have given a wealth of information concerning the

mechanisms of ionic transfer. Such studies would have been impossible without the help of isotopic tracers. Pioneer studies were made by Motais (1961 *a*, *b*) on the flounder and by Croghan (1958*c*) on the brine shrimp. An interpretation of the observed readjustments was later given by Motais *et al.* (1966) and Motais (1967). The reader is also referred to reviews by Maetz (1968, 1970*a*, *b*) and by Potts (1968).

When seawater adapted fishes are suddenly transferred to fresh water, the Na^+ or Cl^- influx is instantaneously reduced to very low levels. If the salt efflux which represents 30 to 50% of the exchangeable salt content of the fish, continued unchanged, the fish would die of

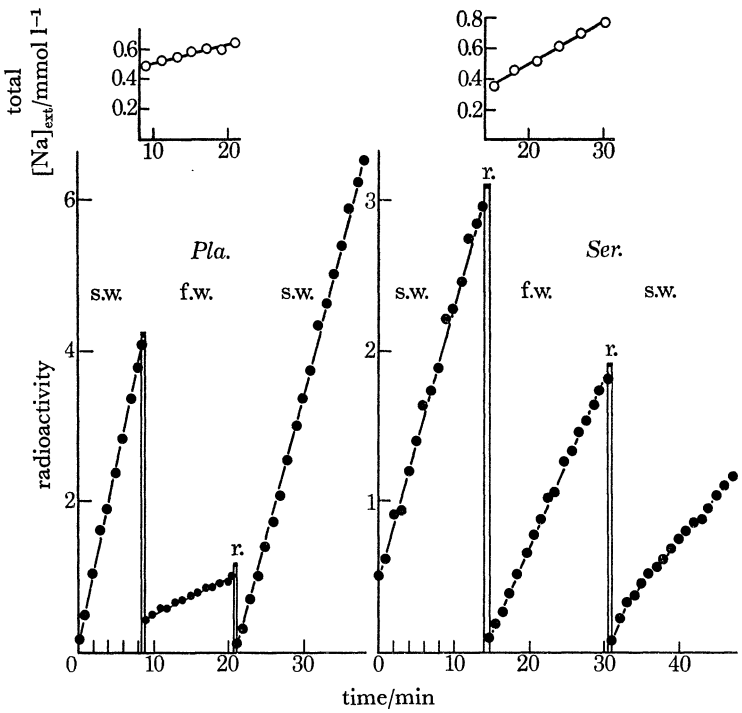

FIGURE 19. Relative ^{24}Na appearance rates of seawater adapted *Platichthys* (*Pla.*) and *Serranus* (*Ser.*) transferred from sea water (s.w.) to fresh water (f.w.) and back to sea water. External ^{24}Na was measured every 30 s but only plotted every minute. Inset graphs above show evolution of total external Na concentration during periods in f.w. (according to Motais *et al.* 1966)

demineralization. Figure 19 compares the efflux readjustment patterns of the euryhaline flounder and of the stenohaline *Serranus* as studied with radiosodium by Motais *et al.* (1966). An instantaneous eightfold reduction of the efflux occurs immediately after transfer, while scarcely any reduction is observed in *Serranus*. By following the external concentration changes of the sodium during the freshwater period, we confirmed the huge differences in Na loss: -315 ± 22 μmol for the flounder against -1770 ± 150 μmol for *Serranus*, incurred during this period. Figure 19 shows that upon return to sea water, a readjustment of the Na^+ efflux promptly occurs Motais and his colleagues showed that such a reversibility prevails in flounders kept less than 30 min in fresh water. In the eel, this period is even shorter. Figure 20 illustrates for the eel, the effects on the efflux readjustment of a period of 1 h in fresh water with subsequent return to sea water. It may be seen that during a prolonged period in fresh water, the 'instantaneous' efflux reduction is completed by a slow delayed 'secondary' diminution of the efflux. Such a delayed

regulation is confirmed by simultaneous measurements by flame photometry of the sodium net loss in the external closed-circuit bath (see Motais *et al.* 1966, for the flounder and the killifish, and Maetz, Mayer & Chartier-Baraduc, 1967*a* for the eel). This secondary regulation obviously corresponds to a progressive impermeabilization of the gill. Motais & Maetz (1964) showed that the longer the fish remains in fresh water, the slower is the readjustment pattern when the fish is challenged again with sea water. The completion of the impermeabilization of the fish during adaptation to fresh water may take several days (Maetz *et al.* 1967*a*, *c*).

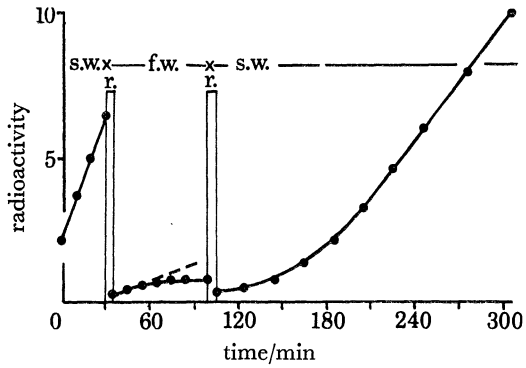

FIGURE 20. Occurrence of a 'delayed' or 'secondary' regulation in the efflux readjustment pattern of the eel during a prolonged period in fresh water and upon subsequent return into sea water (Maetz 1970*a*).

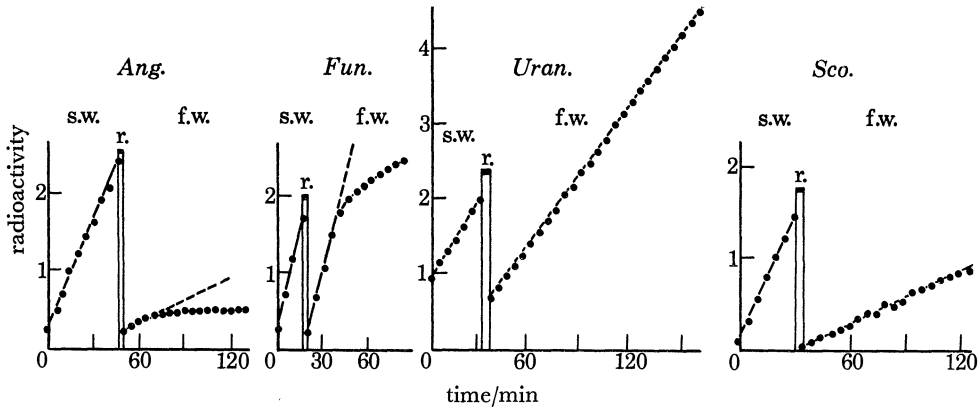

FIGURE 21. Relative ^{24}Na appearance rates upon transfer from sea water to fresh water in various teleosts (according to Motais *et al.* (1966) and Motais (1967)). *Ang.*, *Anguilla*; *Fun.*, *Fundulus heteroclitus*; *Uran.*, *Uranoscopus*; *Sco.*, *Scorpaena*.

Figure 21 illustrates the efflux readjustment pattern for various fishes according to Motais *et al.* (1966). It shows that only the euryhaline fishes (*Anguilla*, *Fundulus*) possess this delayed regulation when subjected to fresh water. The extent of the instantaneous efflux reduction may vary from species to species irrespective of their 'euryhalinity'. *Fundulus heteroclitus* (euryhaline), for example, exhibits only a limited reduction of the efflux upon transfer to fresh water and heavy loss of NaCl is observed during the first 10 min, while the stenohaline *Scorpaena* reduces the sodium efflux by a factor of 3. But only the former fish later shows the delayed regulation, permitting it to survive and to recover its mineral balance. The latter ultimately dies.

(iii) *Mechanism of efflux readjustments: relative importance of an 'osmotic component'*

Motais *et al.* (1966) report a series of experiments designed to assess whether the instantaneous efflux reduction is due to interactions of salt and water flow inside the branchial epithelium. The relative importance of the Na^+ efflux of various fishes was compared during two short periods one in fresh water and the other in fresh water made isotonic to sea water by addition of mannitol, the flux observed in sea water serving as a control. Table 4 summarizes the results which show that in some species such as *Platichthys* there is no osmotic component to the efflux reduction. In others, such as *Serranus* or *Anguilla*, such a component is of importance. In a later

TABLE 4. RELATIVE IMPORTANCE OF THE 'OSMOTIC COMPONENT' IN THE Na EFFLUX READJUSTMENTS OF VARIOUS TELEOSTS DURING SEA WATER–FRESH WATER TRANSFER

	relative Na f_{out} in	
fish	f.w. + mannitol	f.w.
Uranoscopus†	95.3 ± 4.3	52.1 ± 7.6
Serranus†	97.0 ± 8.2	58.0 ± 5.3
Gobius†	72.8 ± 8.3	31.8 ± 7.9
Sargus†	47.5 ± 2.2	37.3 ± 6.6
Chromis†	52.3 ± 5.9	25.9 ± 3.9
Scorpaena†	33.9 ± 3.4	29.9 ± 5.2
Anguilla‡	38.0 ± 6.7	19.2 ± 1.9
Platichthys‡	16.4 ± 2.2	15.9 ± 2.0
Mugil‡	38.8 ± 11.8	12.9 ± 0.7
Fundulus‡	60.0 ± 10.8	68.8 ± 6.0

† Stenohaline. ‡ Euryhaline.
According to Motais *et al.* (1966).

series of experiments on *Serranus*, Motais (1967) showed that the efflux reduction was directly proportional to the osmotic gradient between external and internal media. The importance of this osmotic component varies from species to species. In the eel, only 19 % of the total efflux is 'osmotic dependent', while in *Gobius*, *Uranoscopus* and *Serranus*, up to 40 % of the efflux reduction upon transfer to fresh water is accounted for by this mechanism. The absence of this phenomenon in the flounder made this fish the ideal subject for further studies on the instantaneous flux reduction.

(iv) *Mechanism of efflux readjustments: independence of* 'Na-*free*' *and* 'Cl-*free*' *effects*

Table 5 summarizes a series of experiments by Motais *et al.* (1966) showing that the relative efflux reduction upon transfer to fresh water or to fresh water made isotonic to sea water was the same for Na^+ and Cl^-. Table 5 also shows that transfers to artificial sea waters containing Na^+ or Cl^- with an impermeant co-ion are accompanied by an independent switching-off of Na^+ or Cl^- effluxes. Mechanisms of Na^+ and Cl^- transfer seem therefore to be independent in seawater as well as in freshwater fish. In any case this series of experiments rules out any explanation of the reduction of effluxes in terms of changes of the pattern of gill haemodynamics with blood flow alternatively in contact with epithelia of high or low ionic permeability.

It is therefore apparent that it is the external Na^+ or Cl^- concentration changes which intervene in the efflux changes. Similar observations concerning 'Na-free' or 'Cl-free' effects have been made on other cellular (muscle, red cell) or epithelial (gastric or intestinal mucosa)

tissues. The term 'exchange diffusion' was coined by Ussing (1960) for such a type of flux pattern in which an ionic flux across a membrane appears to depend on the concentration of the ionic species on the *trans*-side of the membrane. The simplest hypothesis suggested by Ussing is that the membrane contains a mobile carrier which is responsible for a coupling between the 'uphill' and 'downhill' transport of ions of the same species, irrespective of the electric potential gradient. Such an exchange, which can only be observed when using isotopic tracers would necessitate a minimum expenditure of energy, but it could also result in no net transport of ions (see also the recent review by Stein 1967).

TABLE 5. RELATIVE SODIUM AND CHLORIDE EFFLUX OF *PLATICHTHYS* AND *SERRANUS* TRANSFERRED FROM SEA WATER TO VARIOUS MEDIA (IN PER CENT OF CONTROL VALUES OBSERVED IN S.W.)

	Platichthys. Media in which fluxes are measured				*Serranus*. Media in which fluxes are measured	
electrolyte	f.w.	f.w. + mannitol	$CaCl_2$	Na_2SO_4	f.w.	f.w. + mannitol
Na	15.9 ± 2.0	16.4 ± 2.2	11.7 ± 2.5	106.0 ± 12.1	58 ± 5.3	97 ± 8.2
Cl	16.0 ± 3.0	14.5 ± 2.5	72.6 ± 7.5	26.4 ± 3.1	53	—

f.w. + mannitol: f.w. with mannitol solution isosomotic to s.w.
$CaCl_2$: calcium chloride solution with mannitol isosmotic to s.w.
Na_2SO_4: sodium sulphate solution with mannitol isosmotic to s.w.
Mean relative values ± S.E.
According to Motais *et al.* (1966).

(v) *Mechanism of efflux readjustments: arguments in favour of 'exchange-diffusion'*

To investigate the possible occurrence of exchange-diffusion Motais *et al.* (1966) compare the Na^+ efflux readjustment pattern in flounders adapted to three different salinities (s.w., $\frac{1}{2}$ s.w. and $\frac{1}{4}$ s.w.) within 10 min of transfer into media of various salinities ranging from fresh-water to hypersaline seawater ('rapid-transfer' experiments). For all fishes, the efflux may be divided into an 'external Na concentration dependent' component (Δf_{out}) and an 'independent' component which is the efflux measured after transfer to deionized or fresh water. Similarly, the 'instantaneous' influx was measured within 10 min of transfer into the various media in a parallel series of experiments in order to correlate both 'external-concentration dependent' fluxes. Figure 22 compares f_{in} and Δf_{out} as a function of external Na concentration. It can be seen that the two coincide within experimental error and that they follow the same pattern, showing a plateau for high Na_{ext} suggesting some kind of saturation process. In point of fact, all three curves fit Michaelis–Menten equations. When the two parameters f_{max} and K_m are compared, it appears that these three 'non-steady-state' curves obtained from fishes adapted to the three chosen media differ by the maximal rate at saturation but not by the K_m, which is very high (400 mmol/l). Very similar curves were obtained by Thuet *et al.* (1968) for *Artemia* adapted to $\frac{1}{2}$ s.w., s.w. or 2 s.w. (double strength sea water), with a K_m = 65 mmol/l. Recently J. C. Rankin in my laboratory (see Maetz 1970*a*) also showed that the influx measured as a function of $[Na]_{ext}$ across isolated eel gill fitted a Michaelis—Menten curve with K_m = 285 mol/l.

Motais *et al.* (1966) and Motais (1967) interpreted these curves in terms of the exchange-diffusion carrier hypothesis with a poor apparent affinity of the carrier for Na in contrast to the high affinity found for the active transport carrier in fresh water (see above). They suggested, furthermore, that the number of available carrier-sites varies during adaptation to a new

salinity either by a process of synthesis or by the combination with some unknown 'non-specific inhibitor' which would not change the affinity of the sites for Na. During 'instantaneous' readjustment the fluxes would 'move' along the three curves drawn on figure 22. During delayed or secondary adaptation, the fluxes would slowly move from one curve to the other along an 'iso-concentration' line. This process may need several days, as discussed above. The rapid adjustment pattern is dependent on the characteristics of the carrier (number of sites and affinity), while the delayed regulation expresses changes in the number of sites.

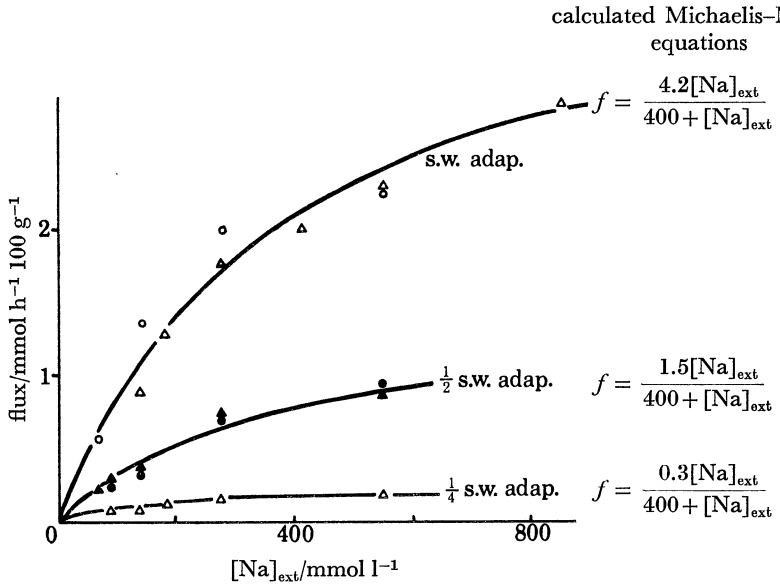

FIGURE 22. Influx and external Na dependent component of the efflux as a function of external Na concentration in the flounder (according to Motais *et al.* 1966). ● ○, influx, f_{in}; ▲ △, Na dependent component of f_{out}.

In most of the recent publications from both the Nice and Villefranche laboratories (see reviews by Maetz 1969; Maetz, Motais & Mayer 1969; Maetz 1970*b*) we have adopted the exchange-diffusion carrier hypothesis and we made the supplementary assumption that somehow the active transport of Na^+ occurring in the gill in sea water was in some way related to the exchange carrier. We considered the whole system: exchange and active carrier as being a kind of leaky imperfect pump. Experimental evidence concerning the effects of hypophysectomy or interrenalectomy or the effects of antibiotics showed that the two capacities of the pump: exchange and active transfer were altered simultaneously.

In a recent series of experiments (R. Maetz & D. H. Evans, in preparation) we tried to assess indirectly the 'minimum-energy expenditure' characteristics of the exchange-diffusion component of the efflux in flounders transferred rapidly from 17 to 7 °C. We found a Q_{10} of 1.7 for the Δf_{out} parameter, which suggests an unusually low activation energy for this process. The same Q_{10} ratio was found for this parameter when flounders adapted at 17 °C were compared with flounders kept for a week at 7 °C. Simultaneously, the drinking rates were measured at the two temperatures and found to have a Q_{10} of 3. As the drinking rate is a measure of the net salt uptake by the gut, compensated by the net extrusion rate by the gill, we suggest that this higher Q_{10} reflects the much higher activation energy related to active transport in the gill. These series of experiments suggest therefore that exchange-diffusion and active transport are not necessarily coupled, since they have different Q_{10} characteristics.

(vi) *Mechanism of efflux readjustments: arguments against the 'exchange-diffusion' concept for* Na^+

Several lines of evidence confute the exchange-diffusion concept for Na^+. The observations of Mayer & Nibelle (1970) show that experimental changes of the internal Na^+ level are followed by huge changes ($\pm 50\%$) of the Na^+ efflux (see figure 17), while the Na^+ influx remains unchanged. A similar independence of influx and efflux was observed by J. C. Rankin in our laboratory in perfused eel gill preparations. Decreasing the Na concentration of the perfusion fluid or even suppressing internal Na^+ does not alter Na^+ influx as would be expected from a coupling of influx and efflux.

In a recent report Britton (1970) critizes the abuse of the term 'exchange-diffusion' applied indiscriminately to solute flux changes monitored by alterations of the concentration of the solute on the *trans* side of complex membranes, the magnitude of the flux change upon removal of the solute being taken as equal to the exchange-diffusion flux. He insists that this 'solute-free test' requires that the membrane potential should remain unchanged if the solute is electrically charged. Two independent reports, concerning the effects of salinity changes on the potential observed across the gills of the eel (Maetz & Campanini 1966) and of the brine shrimp, (P. G. Smith 1969*a*, *b*) have definitely shown that potential reversal occurs during 'rapid-transfer' experiments from high to low salinity. Our 1966 paper was only a short note as there seemed no clear explanation of the observed changes (illustrated in figure 14) at the time. The excellent paper by P. G. Smith, followed by subsequent discussions with the author, led me to revise our views on exchange diffusion.

Figure 14 (left-hand side) illustrates the huge potential changes observed by us during s.w.–f.w. transition. While in sea water a positive potential inside is observed, within 10 min of the salinity change, a time lag which is imposed by the necessity for technical manipulation after change of medium, a reversal of the potential accompanied by a transient hyperpolarization is observed. The external medium thus immediately becomes electropositive to the blood by about 40 to 55 mV. If the fish is retransferred to its original medium within 10 min the blood again immediately becomes electropositive (r. s.w. in figure 14). If the fish is maintained in f.w., a slow readjustment of the potential is observed and within 24 h, the fish being replaced in a f.w. tank and re-used the following day, the potential observed becomes characteristic of the freshwater gill.

The right-hand side of figure 14 shows that during f.w.–s.w. transition, no immediate reversal of potential is observed. The potential, originally blood-negative, readjusts slowly, shifting towards 0 within a few hours and slowly becoming electropositive.

In *Artemia* during transfer from sea water to 10% sea water, P. G. Smith (1969*a*, *b*) found very similar potential changes to those observed in the flounder, the potential reversing from $+23$ to -23 mV. In addition, he studied the relative roles of Na^+ and Cl^- in the observed changes. Replacement of Cl^- by benzenesulphonate results in a barely significant potential change, while substitution of Na^+ by choline is followed by reversal of the potential. Substituting K^+ for Na^+ is only followed by a slight change ($+23$ to $+12$ mV). It is clear therefore that Na plays a major role in the potential changes observed. In another series of experiments P. G. Smith measured the electrical conductance of the boundaries of the brine shrimp which more or less agrees with that calculated from the sodium fluxes across the boundaries on the assumption of *passive*, *independent* (i.e. not linked) movements of Na^+. Furthermore, the large 'transport number' of Na^+, that is, the ratio between the observed potential changes and the

theoretical changes derived from the Nernst equation for a given external electrolyte concentration change, leaves little doubt that the movement of Na is largely diffusive in character. P. G. Smith challenges the conclusion drawn by Thuet *et al.* (1968) that most of the Na moves by way of exchange-diffusion. He rightly argues that a flux-concentration curve of the Michaelis—Menten type does not constitute evidence for a carrier-mediated process. Moreover, P. G. Smith proceeds to demonstrate that such curves may be explained purely in terms of diffusion. By assuming that the constant field theory holds true across the whole epithelium and taking into account that Na permeability is about 10 times that of Cl^-, he derived flux equations which agree with the dependence of the efflux upon external Na^+ (and Cl^-) concentration. Such equations are hyperbolic functions for which an f_{max} and a K_m can be calculated. Although reservations may be made of the use of the constant field theory and of the calculation of transport numbers across a whole epithelium in which potential 'jumps' most probably occur at least at the outer and inner boundaries (see Tosteson 1962) and in which the cellular concentration of electrolytes certainly does not remain constant when the concentration of the external electrolytes is changed, I agree with P. G. Smith that in the gills of the flounder as well as in the gills of *Artemia* most of the Na transfer is diffusional in nature. I disagree with P. G. Smith on one point already discussed, and maintain that a small part of the efflux is in fact active. This active component is mediated by a Na^+–K^+ exchange.

(vii) *Mechanisms of efflux readjustments: evidence for* Na^+–K^+ *exchange*

The presence of increased amounts of Na–K activated ATPase during seawater adaptation as well as the probable existence of an active K^+ transport (see above) suggest the possibility of active exchange of internal Na^+ against sea water K^+ (concentration 10.5 mmol/l).

Kamiya & Utida (1968) briefly reported experiments on the variations of the Na^+ content of excised non-perfused eel gills kept alternatively in K^+- and K^+-free sea water. The Na content was observed to increase in the absence of external K^+. This suggests that Na excretion is effected by a Na^+–K^+ exchange. I confirmed that Na extrusion is dependent on the presence of external K^+ in flounders studied *in vivo* (Maetz 1969). Rapid transfer experiments were performed on flounders alternatively placed in sea water and K-free sea water for comparison of the Na^+ effluxes. A small but consistent reduction of Na efflux was observed in K-free sea water, amounting to 80 ± 12 μmol h^{-1} 100 g^{-1}, a value similar to the net extrusion rate of Na^+ measured in sea water. If the animals were kept for 24 h in renewed K-free sea water, a progressive increase of the internal sodium (Na^+ plasma level and Na^+ space) was observed, amounting to 110 μmol/h, a value which would be expected if the Na^+ transporting mechanism had failed. After return to sea water for 48 to 72 h, the internal Na^+ level declined significantly as if the Na^+ pump had been 'turned-on' upon addition of external K^+. While the fish was kept in K-free sea water the Na turnover rate was measured and found to be almost as high as in ordinary sea water. It is evident that in the absence of external K^+, the high Na^+–Na^+ exchange (exchange diffusion or simple diffusion) is still operative, but it obviously is not the vital mechanism permitting the maintenance of the Na^+ balance of the fish.

The existence of a Na^+–K^+ exchange mechanism is further demonstrated by rapid transfer experiments permitting the comparison of the Na effluxes in sea water, deionized water and various NaCl or KCl solutions at concentrations ranging from 5 to 50 mmol/l. Figure 23 illustrates two such typical experiments. In deionized water, there was the expected 'instantaneous' Na^+ efflux reduction. It may be seen in the left-hand part of the figure, that transfer to 50 mmol/l NaCl

produces an increase of this 'basal' efflux which is also to be expected from the dependence of the efflux on the external Na^+ concentration (see upper curve of figure 22). Transfer to 50 mmol/l KCl however is also accompanied by an increase of the Na^+ efflux. In fact K^+ seems at least as efficient as Na for 'driving Na^+ out' of the fish. Similar observations were made on the brine shrimp which can be subjected to transfers into 500 mmol/l KCl. Thuet *et al.* (1968) report that in such a medium, Na^+ efflux is as fast as in 500 mmol/l NaCl, and Croghan (1958*c*) by studying the relative changes of the internal Na^+ and K^+ levels, observed that after such a transfer the Na^+ loss was equivalent to the K^+ gain. All these observations strongly suggest Na^+/K^+ exchanges.

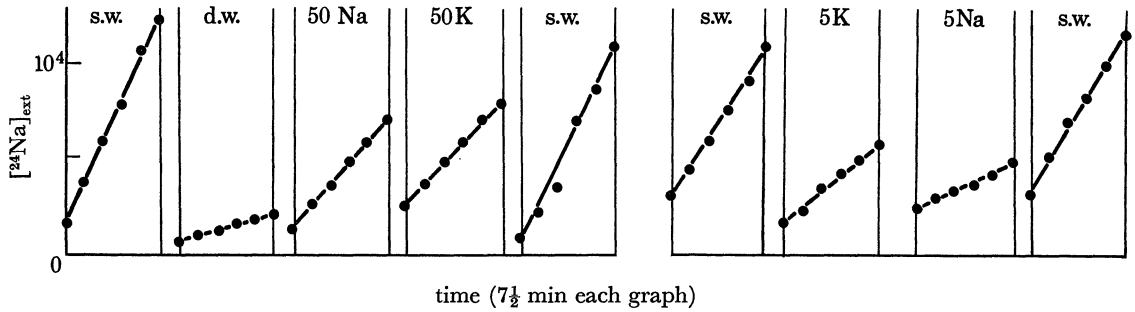

FIGURE 23. Comparison of the ^{24}Na appearance rates for the seawater flounder transferred into various media containing NaCl or KCl (according to Maetz 1969). d.w., deionized water; 5 Na or 5 K, 5 mmol/l Na or KCl solutions; 50 Na or 50 K, 50 mmol/l solutions. Note the relative effectiveness of K and Na in increasing the Na efflux above the d.w. level.

The right-hand part of figure 23 shows that at 5 mmol/l concentrations, K^+ was in fact much more efficient than Na^+ in driving Na^+ out of the fish. Figure 24 summarizes all the results obtained with such Na–K tests and illustrates that in the lower range of concentrations, K^+ is definitely much more efficient than Na^+ in increasing $Na^+ f_{out}$. At 5 and 10 mmol/l in particular, the extra-Na^+ efflux (above the 'basal' level in deionized water) is 200 and 300 μmol h^{-1} 100 g^{-1} with external sodium and 980 and 1105 μmol with external K^+, respectively. It was not feasible during the short transfer periods to measure the corresponding Na^+ or K^+ influx, but if the assumption is made that the K^+ influx equals the extra-sodium efflux, admitting a one-for-one exchange as for Na (see figure 22), it follows that the two curves depicted in figure 24 after deduction of the base-line efflux found in deionized water, correspond to the K^+ and Na^+ influxes as a function of their respective external concentrations. In comparing the two curves, it can be seen that the K^+ influx is almost maximal at the lowest K^+ concentration tested (5 mM). The Na^+ curve is the same as that depicted in figure 22 but the latter corresponds to a much higher range of Na^+ concentrations. For K^+ as for Na^+ we are therefore faced with curves which apparently fit Michaelis–Menten kinetics and the problem is to decide whether for K^+ as for Na^+ the hypothesis of passive diffusive fluxes proposed by P. G. Smith also holds true. The question is whether substitution of say 5 mmol/l KCl for NaCl is able by way of potential changes to explain the huge change in Na efflux observed experimentally. In the absence of potential measurements under such experimental conditions, we can only speculate. According to P. G. Smith (1969*a*), the potential changes are fairly well predicted by the equation for membrane p.d. derived by Hodgkin & Katz (1949) from the theory of Goldman (1943):

$$\Phi_{ext} - \Phi_{int} = \frac{RT}{F} \ln \frac{Na_{int} + \alpha K_{int} + \beta Cl_{ext}}{Na_{ext} + \alpha K_{ext} + \beta Cl_{int}}$$

α and β being the ratios of K^+ to Na^+ and of Cl^- to Na^+ permeabilities. For *Artemia* these ratios are 0.6 and 0.1 respectively according to P. G. Smith (1969*b*), and comparable values may to be expected for the flounder. It may be seen that when 5 mmol/l KCl is substituted for 5 mmol/l NaCl, the denominator of the above equation is smaller by about 10 % while the numerator

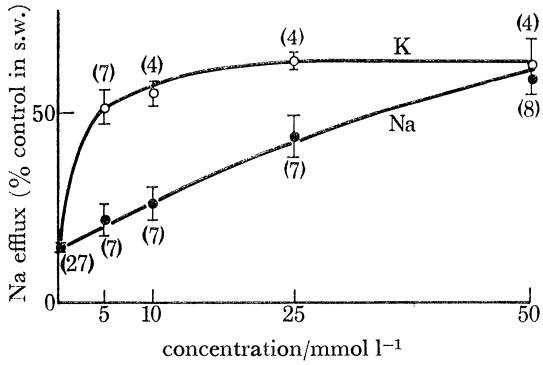

FIGURE 24. Na effluxes of the flounder as a function of external Na or K concentrations of the transfer media (according to Maetz, 1969).

remains unchanged. We can therefore predict an increase of the p.d. by about 3 mV for such a substitution, from which it can be concluded that not only is the predicted potential change too small but that it is also in the wrong direction to explain the huge change in Na^+ efflux induced by such a substitution. It seems to me that the curve depicted in figure 24 for K^+ is best explained in terms of a carrier with a high affinity for K^+ (apparent K_m about 2 mmol/l). For Na^+, we must agree with P. G. Smith that the apparent low affinity of the hypothetical carrier may solely reflect effects of diffusive potential changes, as discussed above.

There is, however, compelling evidence that at least part of the Na^+ fluxes result from a competition between external K^+ and Na^+ for the high affinity K carrier. This is suggested by the effects on the Na^+ efflux of simultaneous addition of Na^+ and K^+ to the external medium. For example, when a solution containing both KCl and NaCl at 5 mmol/l is used, the extra-sodium efflux is only 365 μmol as against 980 μmol for K^+ alone. For 10 mmol/l KCl the extra-efflux is 1105 μmol against 730 μmol only, for 10 mmol/l NaCl + KCl, and for sea water containing 500 mmol/l Na^+ and 10 mmol/l K^+, the extra-efflux due to K^+ is only 80 μmol. Maetz (1969, 1970*a*) suggested that this Na^+ efflux reduction reflects a decrease in the K^+ influx as a result of competition between Na^+ and K^+ for a common carrier. Such a competition has already been described and analysed by Armstrong & Rothstein (1967) in the yeast cell membrane.

A direct demonstration of the inhibition of K^+ influx across the gill by external Na^+ has been obtained in a preliminary experiment in collaboration with J. C. Rankin. Figure 25 illustrates our results on a perfused eel gill preparation. The K^+ influx with sea water as an external medium (10.5 mmol/l K) was found to be in the range of 15 μmol. When, however, the external medium was replaced by deionized water containing 10.5 mmol/l KCl, the influx showed a threefold increase. After return to sea water, the K^+ influx decreased again by a factor 5. Confirmation of these observations must await improvements in the perfused gill technique.

In conclusion, it is probable that an Na^+–K^+ exchange situated on the outer border of the 'chloride cell' is an integral part of the Na extrusion mechanism. Furthermore, part of Na^+–Na^+

exchange may result from a process in which external K^+ and Na^+ compete for a common carrier. It is at the moment impossible to decide whether this exchange-diffusion represents an important part of the sodium exchanges though this is unlikely in view of the work of P. G Smith. More work is necessary in particular with specific inhibitors of Na^+–K^+ exchanges such as cardiac glucosides, to determine the relative importance of the three components, namely

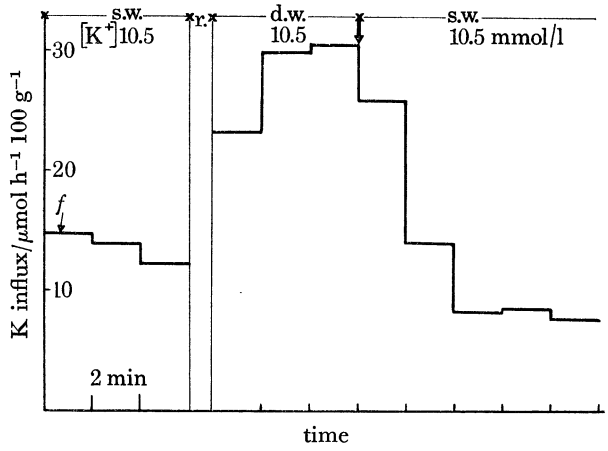

FIGURE 25. K influx of a perfused seawater eel gill preparation (according to Rankin & Maetz (in preparation) and Maetz (1970*a*)). $[K]_{ext}$ remained the same (10.5 mmol/l throughout the experiment Na_{ext} was 505 mmol/l in s.w. and about 0 in d.w. (deionized water). r, rinsing period of 6 min, to remove external Na.

active transfer, exchange-diffusion related to Na^+–K^+ exchange in a manner similar to that described by Garrahan & Glynn (1967*a*, *b*, *c*) for the red cell, and passive diffusional transfer. No conclusions can as yet be advanced as to the stoichiometric relation between active Na^+ extrusion and active K^+ entry into the epithelial cell. The fact that the Na^+ net extrusion rate in both flounder and eel is more or less equal to the K^+ influx, does not necessarily imply a one to one coupling.

In any case, if we admit that Na^+ extrusion is effected by a Na^+–K^+ pump, we must revise our earlier opinion (see Motais *et al.* 1966) that the Na^+ efflux observed immediately after transfer to fresh water is due to an active Na^+ pump operating for a short time until delayed regulation intervenes. As fresh water contains very little K^+, the pump would necessarily stop upon transfer.

(viii) *Mechanism of* Cl^- *efflux readjustments*

P. G. Smith agrees with Motais *et al.* (1966) about the interpretation of the 'chloride-free effect'. In the case of Cl^-, the potential changes following transfer from s.w. to dilute s.w. cannot explain the instantaneous reduction of the efflux, and Cl transfer is therefore mediated by simultaneous active transport and exchange-diffusion. A small passive component has also to be taken into account.

Two different views have been expressed concerning the origin of the potential observed in sea water. P. G. Smith (1969*a*) considers that it is solely an Na^+ ionic diffusion potential. Others (Potts *et al.* 1970; Maetz 1970*a*) suggest that the Cl^- pump is electrogenic. More work on improved isolated gill preparations permitting potential measurements in relation to internal

Cl^- concentration changes of the perfusion medium will decide between the alternative hypotheses.

It may be noted that some euryhaline fishes show no evidence of a 'chloride-free' effect while the 'Na-free effect' is clearly observed. This is the case for *Xiphister* and *Pholis* (Evans 1967, 1969). In these fishes the active chloride transport is accompanied by exclusively passive diffusional Cl^- fluxes. In view of the fact that some fishes such as the euryhaline *Fundulus heteroclitus* and the stenohaline *Serranus* and *Scopaena* exhibits a very small 'Na-free effect', it would be of interest to study the pattern of potential changes in rapid-transfer experiments.

(*f*) *The role of* Na–K *activated ATPase in* Na *extrusion*

Our observations concerning the Na^+–K^+ exchange in relation to active Na^+ extrusion give a renewed interest to the biochemical studies on the extra ATPase Na–K dependent activity

TABLE 6. EFFECT OF HYPOPHYSECTOMY AND CORTISOL TREATMENT ON THE VARIOUS COMPONENTS OF Na TRANSFER ACROSS THE GILL OF S.W. EEL AND ON THE Na–K ACTIVATED ATPASE ACTIVITY

	n	$[Na_{int}]$/mmol l^{-1}	f_{out}†	Δf_{out}	K test	ATPase Na–K
control	7	163 ± 1.7	1173 ± 199†	929 ± 174†	371 ± 40†	77.5 ± 4.5† (10)
hypx (2–5 wks in s.w.)	9	163 ± 3.6§	352 ± 24	216 ± 34	117 ± 43	44.8 ± 3.7 (8)
hypx + cortisol	9	148 ± 2.0‡	835 ± 102‡	581 ± 84‡	517 ± 70‡	107.8 ± 7.6‡ (8)

† f_{out}, Δf_{out} and K test in μmol h^{-1} 100 g^{-1}; ATPase activity in μmol P_i h^{-1}mg protein.
‡ $P < 0.01$. Comparison between hypx and either control or cortisol-treated animals.
§ Hypophysectomy is also followed by an impairment of the gut absorption mechanism (Hirano, Kamiya, Saishu & Utida 1967), for NaCl and water. The Na_{int} level remains the same after hypophysectomy presumably because both Na entry (by the gut) and Na extrusion (by the gill) are reduced to the same extent (see also Pickford *et al.* 1970). Cortisol acts on both effector-organs, but obviously it must act faster on the gill as Na_{int} level decreases significantly. According to Langford, Motais & Maetz (in preparation).

exhibited by the gill in relation to life in salt water. As originally pointed out by Epstein *et al.* (1967) the role of this enzyme in the sodium extrusion mechanism is strongly indicated by the parallel effects of hypophysectomy and cortisol treatment on the Na turnover rate (cf. Maetz *et al.* 1967*c*; Maetz, Mayer, Forster & Chan 1967*b*) and on ATPase Na–K dependent activity of the gill of seawater adapted *Fundulus heteroclitus*. That the overall Na turnover rate closely parallels the Na net extrusion rate remains in doubt. Studying freshwater to seawater adaptation in the eel, R. Langford, R. Motais & I found no parallel changes of the Na^+ turnover rate, of the extent of the Na-free effect, of the efficiency of external K^+ to drive out Na^+ (the K test) and of Na–K dependent ATPase. In other series of experiments with hypophysectomized eels adapted to sea water for about 3 to 5 weeks compared with control animals and cortisol-treated operated animals a very satisfactory correlation between all these parameters was found (see table 6).

(*g*) *The role of carbonic anhydrase in* Cl^- *extrusion*

With regard to the Cl^- pump, two observations suggest that carbonic anhydrase is involved in the Cl^- extrusion mechanism in seawater fish. Comparing the enzyme activities in freshwater and seawater perch (*Perca fluviatilis* and *Serranus* sp.) Maetz (1956*a*) observed a significantly higher activity (by about 100 %) in the sea water species. Recently, in collaboration with M. Istin and J. P. Girard, we found a similar difference between freshwater and seawater eels studying gills perfused with Ringer solution in order to eliminate the presence of carbonic

anhydrase activity from the red cells. Maetz (1956*a*) injected acetazolamide into the seawater perch and observed a transient significant increase of the internal Cl^- level which was interpreted as resulting from the inhibition of the branchial chloride extrusion pump. Control injection with sulphathiazole remained without effect. More work with the help of isotopic kinetic techniques is necessary to verify these results.

In any case the observed effect may be interpreted in terms of a Cl^-/HCO_3^- exchange mechanism forming part of the active Cl^- extrusion pump. As the source of bicarbonate ions can only be the 'chloride cell' containing carbonic anhydrase, the proposed exchange must link Cl^- efflux from blood to active cell with HCO_3^- influx from cell to blood, an exchange which therefore takes place at the inner face of the cell. Cl^- extrusion must therefore be completed by a second pump situated at the external face of the cell in contact with the external Cl^- where the exchange-diffusion process must also be located.

The model depicted in figure 15 illustrates my suggestions concerning the functioning of the chloride cell in sea water. I have assumed that the K efflux across the external boundary follows passively the active chloride extrusion. The K^+ balance in the cell is achieved by the Na–K exchange mechanism. On the inner face of the cell parallel exchanges of HCO_3^- with Cl^- and of H^+ with Na^+ are depicted. It would be of interest to verify whether carbonic anhydrase inhibition is also accompanied by a reduction of the Na extrusion rate as the model would suggest. Indeed in freshwater carbonic anhydrase inhibition simultaneously impedes both Na^+ and Cl^- absorption. Comparison of the two models shows that the inner border of the active cell in fresh water and the outer border of the active cell in sea water perform similar tasks in terms of electrolyte translocation. The same observation holds for the two other borders.

As to the passive components of the Na^+ and Cl^- exchange, it is suggested that these cross the respiratory epithelium. Keys & Bateman (1932) after adding adrenalin to the Ringer perfusing their heart-gill preparation, observed a progressive decrease of the chloride extrusion rate followed by chloride loss. Adrenalin is known to increase the blood flow in the respiratory lamellae, the blood being diverted from the central compartment in contact with the active cells (Maetz & Rankin 1969).

4. Concluding remarks about the mechanisms of euryhalinity

The present review is concerned mainly with the mechanisms of ion transfer in teleosts adapted to either fresh water or to sea water.

In freshwater fishes the present evidence strongly suggests the existence of two independent active pumps probably located on the mucosal side of the epithelial cells concerned with transport. We have reassessed the arguments for and against the presence of tightly linked NH_4^+–Na^+ and HCO_3^-–Cl^- exchanges as part of the active pumps. It is probable that H^+ ions may supplement NH_4^+ ions in the exchange, when the fish is faced with an abrupt increase of external Na concentration. It is obvious that ammonia excretion is not stopped when no Na^+ is present in the external medium. Ammonia is most probably excreted in both molecular and ionic forms according to the physiological needs of the organism. An obligatory HCO_3^-–Cl^- exchange is observed, at least in the goldfish. One puzzling aspect of the branchial pumping mechanisms in fish is that although in many respects their functioning is similar to that of the *in vivo* frog skin, the electric potential is reversed, blood being electro-negative whereas in the frog it is electropositive.

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In seawater fishes, two independent Na^+ and Cl^- pumps are again observed, but their activity results in salt extrusion. The Na^+ pump is operated by a Na^+–K^+ exchange located on the mucosal barrier of the 'chloride cell' and probably mediated by the Na–K activated ATPase, although the inhibitory effect of cardiac glucosides remains to be demonstrated. Na crosses the gill epithelium not only by way of the pump but also by diffusive flow along the electrochemical gradient and by exchange-diffusion. I have reassessed the relative importance of these two mechanisms. Because of the potential readjustments which are observed in parallel to the efflux readjustments during transfer from sea water to fresh water, the diffusive flow may be much more important than we had previously suggested.

The Cl^- pump is probably electrogenic, which would explain the electronegativity of the outside medium with respect to the blood. It is probable that Cl^- entry from the blood into the 'chloride cell' is mediated by a Cl^-–HCO_3^- exchange. In fresh water as in sea water such an exchange necessitates the rapid production of HCO_3^- by hydration of CO_2. Carbonic anhydrase is essential for this step. Cl^- crosses the branchial epithelium in sea water, not only by way of the pump but also by diffusive flow and by exchange diffusion. In at least the eel and the flounder, most of the chloride is exhanged by exchange-diffusion.

The models described in the present paper, are to a large extent speculative. Their purpose is to stimulate further investigation of the branchial epithelium.

The problems of euryhalinity will be discussed in greater detail later (review in preparation). Instantaneous and delayed flux adjustments after external salinity changes have been described in the present paper since their analysis permits a better understanding of the transport mechanisms. After transfer from fresh water to sea water, new pumps become located at different sites in the 'chloride cell' and passive permeability, especially of Na, increases. The question of how external salinity induces such functional changes is of considerable interest. Two suggestions may be advanced: salt may act directly on the target-cell externally or indirectly via the drinking reflex and the gut, in which case the stimulus would be an internal NaCl concentration change acting either directly or by some integrated control mechanism, such as endocrine secretion. The results obtained in our laboratory (Maetz 1968, 1970*c*; Mayer 1970) stress the importance of endocrine control. The important question remaining open is the origin of the rise of internal NaCl following transfer to a higher salinity. The relative importance of NaCl entry by the branchial route or by the intestinal route has to be determined. R. Kirsch (personal communication) maintains that in view of his recent work on the eel only the intestinal route need be considered.

In relation to the functional changes occurring in the gill duration adaptation to waters of higher salinity, two types of modification have been suggested (Maetz 1970*c*): first, molecular processes involving for example increases in the activity of two key-enzymes related to the ionic transfer mechanisms, and secondly, cellular processes including the shedding of old cells and the differentiation of new cellular types in relation to the new task of salt extrusion rather than salt uptake. The increase of DNA turnover observed in sea water adapted salmon by Conte & Lin (1967) and the disappearance of the B type 'chloride cell' simultaneous to the increase of the A type of cell in the eel during adaptation to sea water (Shirai & Utida 1970) are suggestive in this respect. Two major hormones intervene in these processes: cortisol and paralactin. Their relative importance and the nature of their effects will be discussed elsewhere.

In conclusion, my experience concerning the study of gill transport phenomena is dominated by frustration because of the difficulty of obtaining clear thermodynamic data. This is

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compensated by the wealth of information concerning feed-back responses of the gill epithelium and its endocrine control which it has been possible to amass. The question remains whether progress in membrane biology will come only from studies at the molecular level or whether it will also be advanced by studying the membrane as an integrated part of the whole organism in relation to its environment.

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