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Flux measurements as indices of H^+ and metal effects on freshwater fish

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The gills are the principal site for uptake and losses of major body electrolytes, acid-base equivalents, ammonia, respiratory gases, and many waterborne contaminants in freshwater fish. Measurements of these fluxes offer a powerful tool for assessing the toxic effects of extreme water pH's and dissolved metals. Advantages of the flux approach over conventional methods based on blood or tissue sampling include: (i) 10–400-fold greater sensitivity; (ii) non-invasiveness; (iii) simplicity and applicability to field work; and (iv) measurements made at the site of toxic action (the branchial epithelium). Limitations of the approach include: (i) changes in water quality during the flux determination; (ii) extra-branchial routes of flux; and (iii) adsorption and precipitation phenomena leading to artifactual flux values. Current ideas on the mechanisms of O_2, CO_2, Na^+, Cl^-, Ca^{2+}, K^+, acid-base equivalent, and ammonia fluxes across the gills are reviewed, together with the actions of low pH, high pH, and metals (Al, Cu, Cd, La, Zn, Hg, Mn) on these fluxes. Recent kinetic analyses of flux relationships are described which provide new information on the mechanism(s) of toxicant action.

Key words: Metal; Low pH; High pH; Flux; Gill; Kinetics

I. INTRODUCTION

The gills of freshwater fish comprise over half the total body surface area, and the mean blood to water diffusion distance here is only a few microns (Hughes, 1984). The branchial epithelium is the point of most intimate contact between the internal environment of the animal and an external environment which is always osmotically hostile and which at times may be significantly polluted. It is not surprising that the gills represent the principal site for osmotic water gain, for diffusive losses of major body electrolytes, and for the entry of many waterborne contaminants. At the same time, the gills are also the most important site for the active uptake of major ions needed to counter these passive diffusive losses, for nitrogenous waste excretion, for

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acid-base regulation, and for respiratory gas exchange. Many environmental toxicants not only enter through the gills but also exert their primary toxic effects right on the branchial epithelium by interfering with one or more of these essential physiological processes.

This is especially true for the toxic agents on which the present review focusses –dissolved metals and water pH's significantly above or below neutrality. The traditional experimental approach for assessing their toxic effects has been to *sample the fish* for changes in whole body, tissue or blood levels of major electrolytes, ammonia, acid-base status, respiratory gases, and the toxicant itself. The purpose of the present article is to champion an alternative approach, that of *sampling changes of these parameters in the water*, rather than in the fish. In essence, the method involves simply monitoring changes in water composition over time either in a closed external environment, or in one which is slowly renewed at a known rate.

The *net fluxes* per unit weight (J_{net}) of substances between the fish and its environment are measured as losses or gains by the water compartment. Thus:

$$J^X_{net} = \frac{([X]_i - [X]_f)\ V_{ext}}{W_t} \tag{1}$$

where $[X]_i$ and $[X]_f$ refer to initial and final concentrations of the substance X in the water, V_{ext} is the volume of the system, t is the elapsed time, and W is the body mass. Thus losses from the water represent gains by the fish and have positive values, while gains by the water represent losses by the fish and have negative values. (If a flowthrough rather than a closed system is used, then inflowing and outflowing concentrations would be substituted for $[X]_i$ and $[X]_f$ respectively, and water flow rate for V_{ext}/t). For electrolytes such as Na^+ and Cl^-, determinations of J_{net} may be further factored into separate *unidirectional influx* (J_{in}) and *unidirectional outflux* (J_{out}) components (*e.g.* Fig. 1) by monitoring the disappearance of radioisotopes (^{22}Na, ^{36}Cl) from the water compartment as they enter the fish (only feasible in a closed system):

$$J^X_{in} = \frac{([R]_i - [R]_f)\ V_{ext}}{\frac{1}{2}\left[\frac{[R]_i}{[X]_i} + \frac{[R]_f}{[X]_f}\right] W_t} \tag{2}$$

where $[R]_i$ and $[R]_f$ are initial and final concentrations of radioactivity in the water and the other symbols are as in eq. (1). Eq. (2) is most suitable for short measurement periods where non-steady state conditions apply, which is often the case in the presence of toxicants. A logarithmic equation for J_{in} (*cf.* Kirschner, 1970) may be more suitable for long measurement periods, but makes the important assumptions that the decline in external radioactivity follows a simple exponential function because J_{in} is constant, and that there is no change in $[X]$ because J_{net} is zero. These conditions are often not true in toxicological work. Finally, the conservation equation can be applied to estimate J_{out}:

$$J^X_{out} = J^X_{net} - J^X_{in} \quad (3)$$

This paper will first outline the advantages of the flux approach as a monitoring, screening, and diagnostic tool for use in the lab and field. The limitations of the flux approach will then be critically evaluated, and important methodological and interpretive considerations noted. Next, our present understanding of the transport processes on the gills of freshwater fish which are responsible for observed fluxes (Fig. 5) will be reviewed and integrated with current knowledge on the negative impacts of metals and extreme pH's on these fluxes. The focus will be mainly on cation fluxes; of the various anions which may cross the gills, to date only Cl^- fluxes have been well-studied. Finally, a relatively new development, *kinetic analysis* of fluxes, will be discussed as a tool for understanding the nature of toxicant effects at the gills.

II. ADVANTAGES OF THE FLUX APPROACH

(i) Sensitivity

This is perhaps the greatest advantage of the flux approach in comparison to the traditional method of measuring changes in blood plasma or whole body concentrations, as illustrated in Table 1. The higher sensitivity derives simply from the much lower concentrations of most electrolytes in freshwater than in fish plasma or tissues. With standard analytical techniques (atomic absorption, coulometric titration, colorimetric assays), one can reliably detect a change in concentration of about 2%. The toxic effects of most metals and pH extremes are typically investigated in the medium of environmental relevance, which is usually softwater. For Na^+, Cl^-, Ca^{2+}, and K^+,

TABLE 1

Plasma concentrations, exchangeable internal pools, and whole body pools of major electrolytes in freshwater rainbow trout (*Oncorhynchus mykiss*), and a comparison of the minimum change (2% of pool size) which can be detected by measuring concentration changes in the plasma (exchangeable pool), whole body, and external water. The latter represents the flux approach.

	Plasma concentration (mEq/l)	Exchangeable internal pool (μEq/kg)	Whole body pool (μEq/kg)	2% of internal pool (μEq/kg)	2% of whole body pool (μEq/kg)	2% of external pool* (μEq/kg)
Na^+	150[a]	42,050[a]	54,560[c]	841	1,091	10
Cl^-	130[a]	32,840[a]	45,920[c]	657	918	10
K^+	3.2[b]	4,930[b]	88,890[c]	99	1,778	10
Ca^{2+}	3.4[d]	6,010[d]	210,070[c]	120	4,201	10

*Note: The external medium is softwater with a concentration of 50 μEq/l for all four electrolytes and a volume to fish mass ratio of 10:1.

[a]Wood (1988); [b]Eddy (1985); [c]Wood and McDonald (1982); [d]Hobe et al. (1984a).

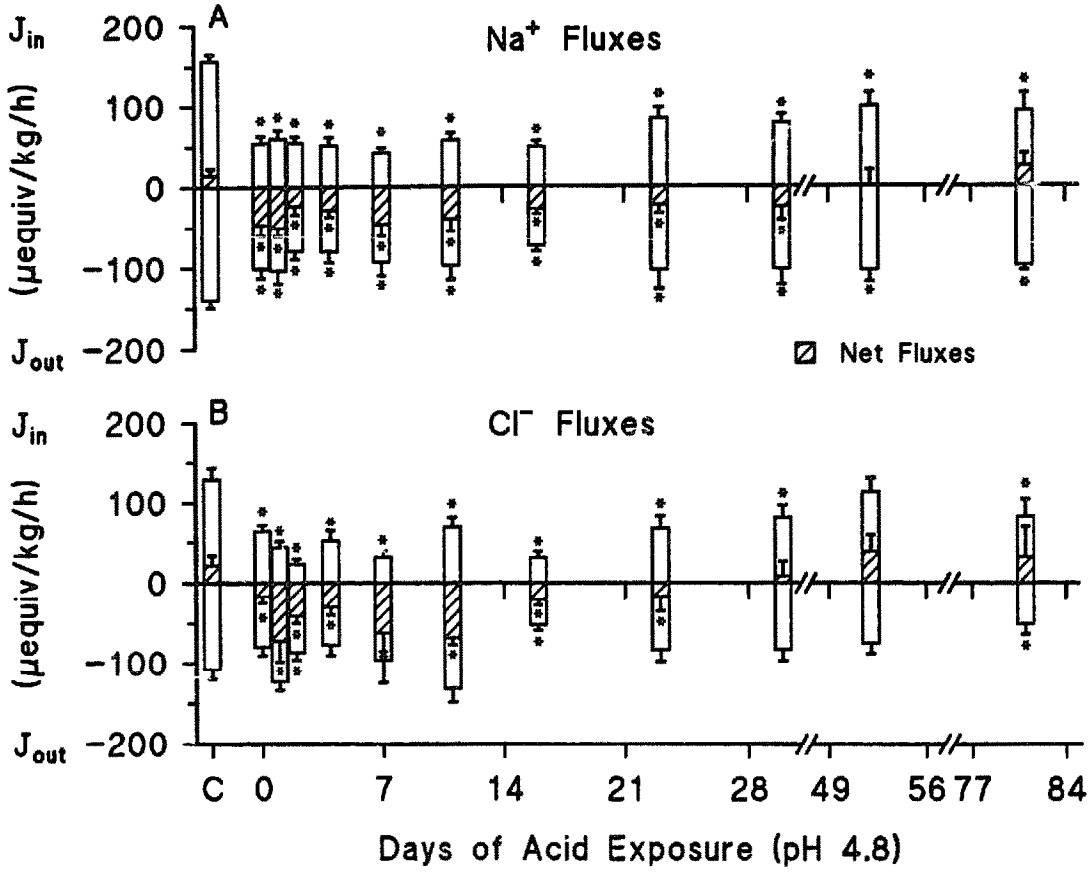

Fig. 1. Unidirectional (J_{in}, J_{out}) and net flux rates (J_{net}) of Na^+ and Cl (means ± 1 SEM) in the same 12 rainbow trout during long-term acid exposure (pH 4.8) in synthetic softwater. The *N* number is lower (7–8) in the last two flux periods due to mortalities after day 30. Asterisks indicate means significantly different ($p<0.05$) from the pre-acid control value. Redrawn from Audet et al. (1988).

concentrations in softwater are typically 50 μEq/l or lower. Therefore a change of about 1 μEq/l can be detected. Assume a standard experimental situation where the control net flux rate (J_{net}) is zero (i.e. the fish is in ion balance prior to exposure to the toxicant), the water volume (V_{ext}) to body mass (W) ratio is 10:1 and the duration (t) is 1 h (eq. 1). One can reliably detect a net flux (J_{net}) of 10 μEq/kg. (Even greater sensitivity is obtained if J_{net} is factored into J_{in} and J_{out} components via radiotracers.) On the other hand, if one samples the blood plasma of the fish, the concentration is representative of the exchangeable internal pool which is very large. The minimum detectable change (2%) in blood plasma concentration represents a 10-fold (K^+) to 84-fold (Na^+) larger disturbance of electrolyte balance (Table 1). A 2% change in whole body concentration represents a 92-fold (Cl^-) to 420-fold (Ca^{2+}) larger disturbance of electrolyte balance (Table 1).

In practical terms, this means that the flux approach can detect toxic effects long before they are reflected in internal concentration changes, and long before they cause irreversible damage to the fish. For example in the chronic acid exposure (pH 4.8)

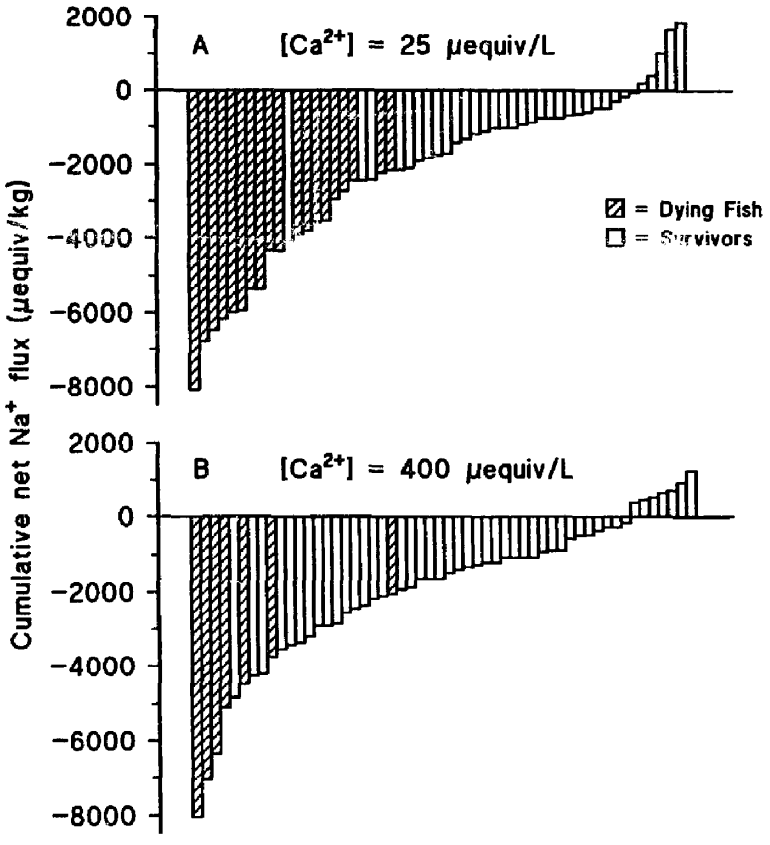

Fig. 2. Cumulative net fluxes of Na^+ in brook char during the first 24 h of exposure to various combinations of low pH and Al (after a first 24 h of exposure to low pH alone) in synthetic softwater at two Ca^{2+} concentrations. The fish are ranked according to the magnitude of the fluxes. Open bars indicate fish that survived for the full 11 days of the experiment, and stippled bars indicate fish that died subsequent to this first 24 h of Al exposure. Redrawn from Booth et al. (1988).

experiment illustrated in Fig. 1 (Audet et al. 1988), marked changes in J_{in}, J_{out}, and J_{net} were seen within the first 5 h of exposure, whereas the first significant changes in plasma electrolytes were not seen until 72 h. Furthermore, one can resolve changes (e.g. increasing damage or recovery) as they develop over time (Fig. 1), rather than just the cumulative loss or gain reflected in internal concentrations. Several other recent studies directly comparing the two methods for metal (Al, Cu, Cd) and low pH effects (Booth et al., 1988; Wood et al., 1988a,b; Reid and McDonald, 1988) have noted this much greater sensitivity.

(ii) Non-invasiveness

The fish are subjected to no disturbance apart from the mild stress of confinement in the flux chamber. Repetitive measurements can be made without trauma. In Fig. 1, fluxes were monitored periodically in the same 12 rainbow trout (*Oncorhynchus mykiss*) over a period of almost 3 months during chronic low pH exposure. In con-

trast, for blood or tissue sampling, the fish is often chased, netted, anaesthetized or stunned, and finally air-exposed prior to venipuncture or biopsy, a process which makes repeated sampling of questionable value. The accompanying hemoconcentration tends to raise most plasma electrolytes, and measurements of blood respiratory gases, ammonia, and acid-base status are grossly disturbed. Many of these problems can be overcome by the use of chronic arterial catheterization to allow blood sampling without physical disturbance (Soivio et al., 1972). Some studies have combined the flux and cannulation approaches to allow simultaneous monitoring of internal and external pools (e.g. McDonald and Wood, 1981; McDonald, 1983b; Wilkie and Wood, 1991). However, it must be appreciated that even after long recovery periods, cannulated fish are invariably more sensitive to toxicant stresses than non-cannulated fish (e.g. Wood et al., 1988a; Wilkie and Wood, 1991).

A related benefit of the non-invasive flux approach is its predictive value. The measurement procedures themselves have no influence on the fish's survival or death from the toxicant, but provide physiological information which may be predictive of eventual fate. For example, Fig. 2 demonstrates that for brook char (*Salvelinus fontinalis*) in low [Ca^{2+}] softwater, cumulative J_{net}^{Na} over the first 24 h of exposure to various low pH/Al combinations was an excellent predictor of survival or mortality over the following 10 days of continued exposure (Booth et al., 1988). A cumulative Na^+ loss of about 2000 μEq/kg in the first day was the threshold for mortality, even though some of the deaths did not occur until many days later. Reference to Table 1 demonstrates that this would cause less than a 5% drop in plasma [Na^+]. In higher [Ca^{2+}] water, net Na^+ losses were reduced, the threshold loss for eventual mortality was higher (Fig. 2B), and the frequency of mortality was lowered.

(iii) Ease and applicability to field work

Fluxes are one of the very few non-terminal physiological measurements that can be made in a field situation. The only equipment required is a suitable chamber, a source of compressed air (e.g. battery air pump) for aeration and mixing in the chamber, and pipettors and vials for sampling. However, it must be appreciated that the water samples must be brought back to the lab for later analysis using non-portable and expensive equipment such as atomic absorption units, spectrophotometers, scintillation counters, gamma counters etc. With the exception of titration alkalinity measurements (required for the calculation of acidic equivalent fluxes – McDonald and Wood, 1981, e.g. Fig. 6) which must be performed soon after collection, all other parameters can be preserved by acidification for these later analyses. We have measured fluxes in the field on fish freshly collected from a softwater lake in northern Canada (Hobe et al., 1984b) and an alkaline lake in Kenya (Wood et al., 1989). Indeed, the data of Fig. 3 (Spry and Wood, 1985) showing the action of Zn on various branchial fluxes in rainbow trout in natural softwater were obtained under rather primitive conditions in a small hut at lakeside. Despite their humble origin,

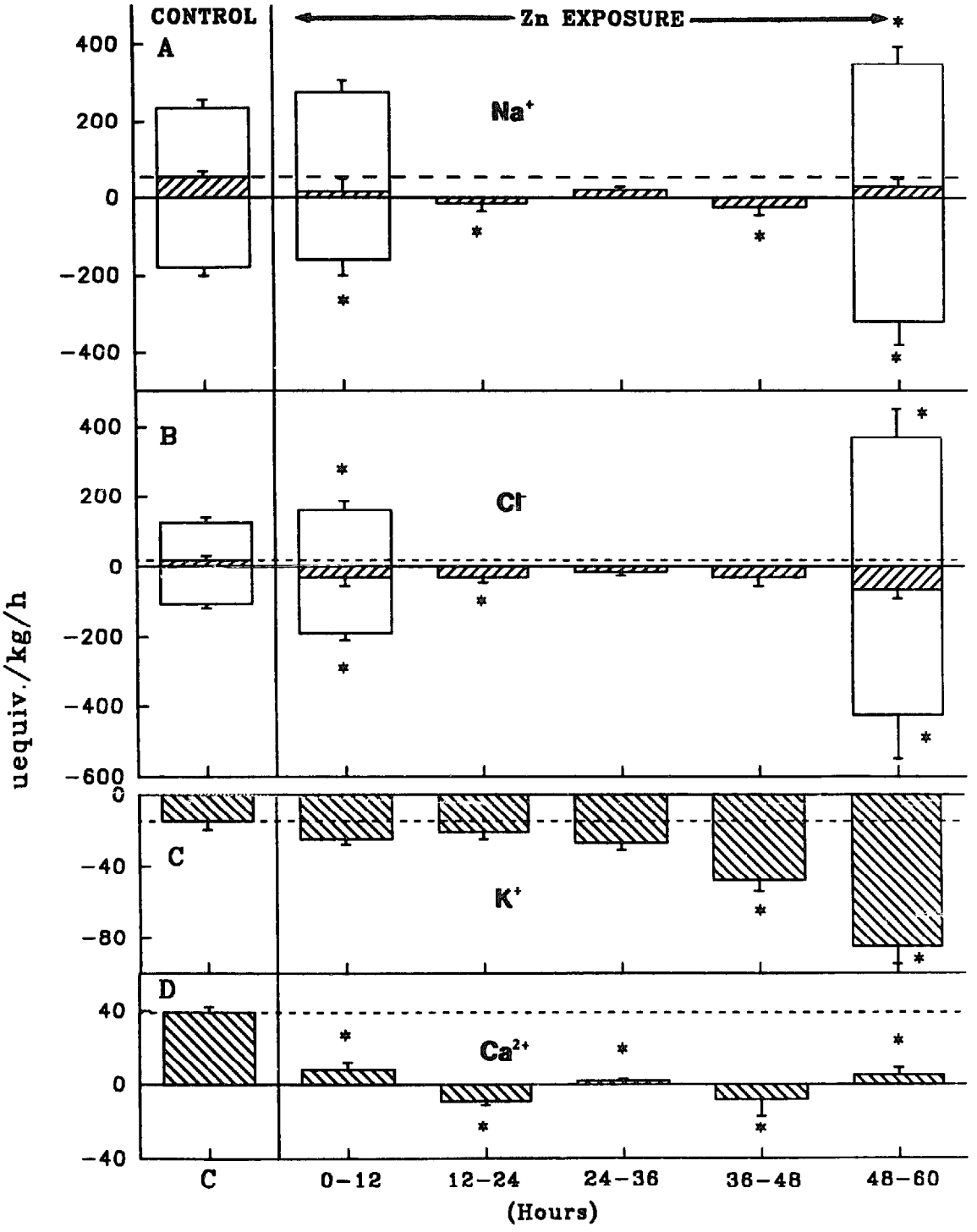

Fig. 3. Net branchial flux rates (J_{net}; cross-hatched bars) of Na^+, Cl^-, K^+, and Ca^{2+} (means ± 1 SEM, N = 12–13) in rainbow trout during 60 h exposure to Zn (0.8 mg/l) in natural softwater. Unidirectional flux rates (J_{in}, J_{out}; open bars) were measured only for Na^+ (N = 9–13) and Cl^- (N = 3–9) at 0–12 h and 48–60 h. Asterisks indicate means significantly different ($p<0.05$) from the pre-exposure control value. Redrawn from Spry and Wood (1985).

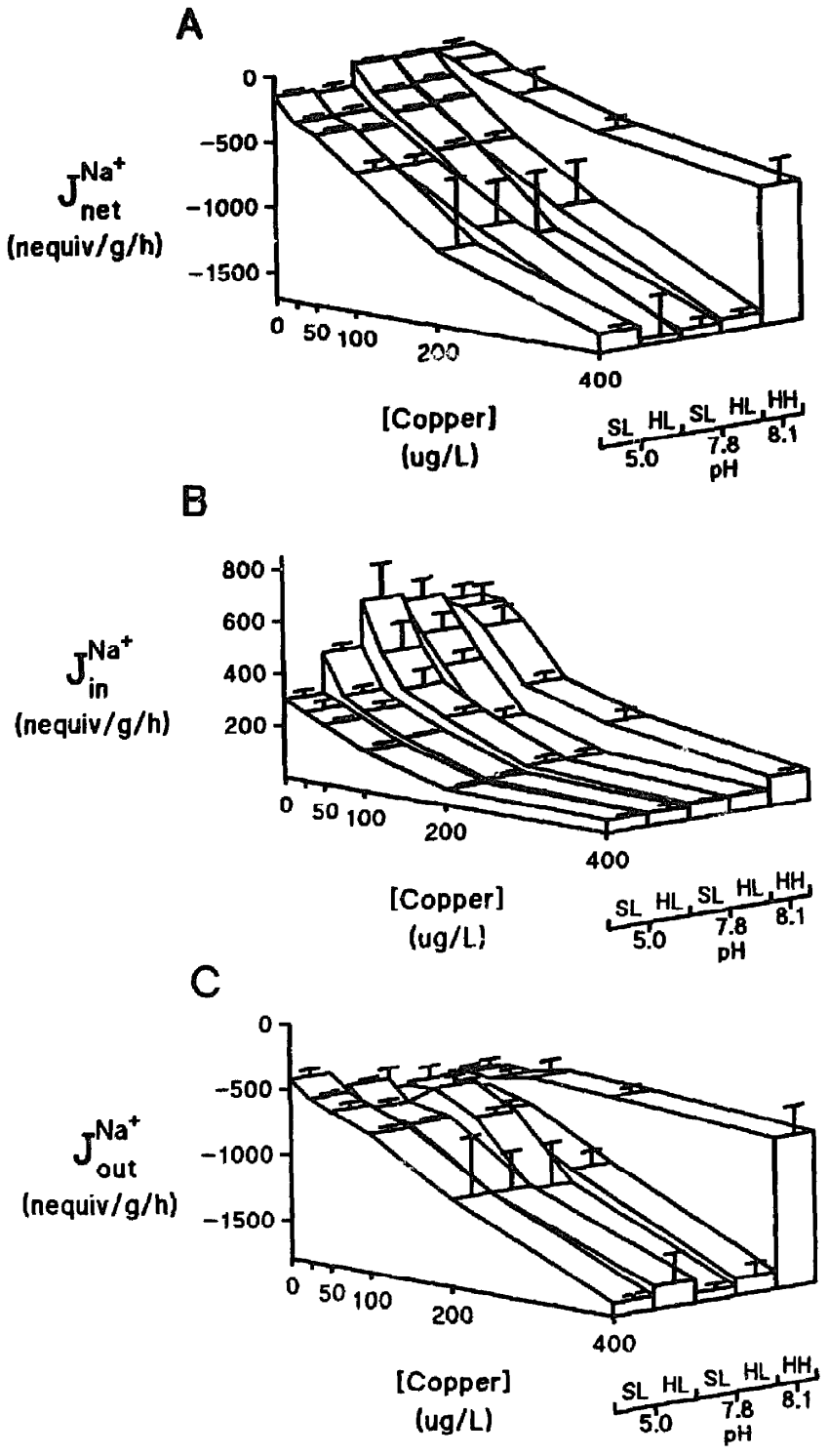

Fig. 4. Rates of Na^+ net flux (J_{net}), influx (J_{in}), and outflux (J_{out}) in juvenile rainbow trout exposed for 24 h to various levels of Cu in 5 different water qualities: SL 5.0 = softwater, low alkalinity, pH 5.0; HL 7.8 = hardwater, low alkalinity, pH 7.8; SL 7.8 = softwater, low alkalinity, pH 7.8; HL 8.1 = hardwater, low alkalinity, pH 8.1; HH 8.1 = hardwater, high alkalinity, pH 8.1. Means ± 1 SEM (N = 10–20). Redrawn from Lauren and McDonald (1986).

these observations were the first evidence that Zn blocks branchial Ca^{2+} uptake while stimulating unidirectional Na^+ and Cl^- fluxes (see Sections V, VII). This applicability to field use offers the potential for flux measurements on fish freshly collected from polluted environments, or on test fish exposed continuously to 'naturally' polluted water.

(iv) Measurements at the site of toxic action

The key toxic mechanisms of action for low pH, high pH, and many metals are interference with normal branchial uptake and/or excretion processes, and stimulation of passive diffusive losses of scarce electrolytes across the gills. These mechanisms are discussed in subsequent sections. Flux measurements quantify the toxic events as they occur (Figs. 1, 3), rather than their sequelae which are eventually reflected in internal composition. Furthermore, because of their speed and relative ease, they facilitate quick investigation of the mechanism(s) of toxicity by allowing the researcher to vary factors which might influence the toxicant's effect on fluxes. These include the concentration of the toxicant itself, the water quality, and the concentration of 'normal' substrate in the water which the toxicant is thought to antagonize. Experimental variation of the latter is a particularly powerful approach (*kinetic determination*), which will be dealt with in Section IX.

However, manipulation of the other two factors alone can prove very informative, as illustrated in Fig. 4. Here by varying water hardness ($[Ca^{2+}]$), pH, and alkalinity, Laurén and McDonald (1986) demonstrated: (i) Cu inhibits J_{in}^{Na} at a much lower threshold than its effect in stimulating J_{out}^{Na}; (ii) both effects of Cu are more or less insensitive to pH and to hardness; and (iii) elevated alkalinity protects against both effects of Cu, but with a greater protective action against the stimulation of J_{out}^{Na}. The entire data set of Fig. 4 were obtained by a single investigator in experimental periods totalling less than 1 week (D.G. McDonald, personal communication)!

III. LIMITATIONS OF THE FLUX APPROACH

(i) Changes in water quality during the flux period

A particular problem for the inexperienced flux investigator are the remarkable changes in water quality that can occur over time due to the presence of the fish in a closed system. For example, under the 'standard' flux conditions outlined in Section II(i) and Table 1, the water ammonia concentration would increase by 30–100 μmol/l in 1 h, an effect which in itself can significantly lower the rate of further ammonia excretion (e.g. Wright and Wood, 1985). The ammonia build-up (which is not affected by aeration) will in turn 'trap' HCO_3^- in the water resulting from the fish's CO_2 excretion (the remainder of the CO_2 is volatile and therefore removed by aeration at pH's below about 8.0). This will increase alkalinity, which may affect the toxicity of

certain metals such as Cu (Fig. 4; Laurén and McDonald, 1986), as well as raise water pH. If the test water is very soft, acidic, and poorly buffered, as is often the case in experiments with metals, pH could increase by 1–3 units within 1 h. This is a serious complication for other metals such as Al where the toxic effect is critically dependent on pH (e.g. Booth et al., 1988; Playle and Wood, 1989; Playle et al., 1989). The opposite problem occurs during exposures to highly alkaline pH; here a significant portion of respiratory CO_2 production is trapped as HCO_3^- directly, thereby lowering water pH (Wilkie and Wood, 1991). Furthermore the net uptake or loss of Na^+, Cl^-, and K^+ by the fish could raise or lower water concentrations of these ions by up to 50%, which in turn could alter their subsequent J_{in} values (Goss and Wood, 1991). The solution(s) to these problems is to shorten the flux periods, to increase the volume to fish mass ratio, or to use continuous flow systems (see Section I), though these all tend to lower measurement sensitivity, and the latter precludes the use of radioisotopes. The pH shifts can be overcome by manual or automatic pH control, though it is worth noting that most pH electrodes leak sufficient KCl to interfere with K^+ and Cl^- flux measurements. Furthermore, the experimenter must keep careful record of the ions and acidic or basic equivalents added with the acids and bases used to control pH, so as not to confuse their 'appearances' in the water with endogenous fluxes originating from the fish. Another aspect of this same problem is the mucus and fecal matter added to the water by the fish which can be extremely efficient at complexing many metals, as can the surfaces of the flux chamber itself (see below). Again, this a particular concern with Al which can only be overcome by working in continuously flowing systems (Booth et al., 1988; Wood et al., 1988b).

(ii) Adsorption and precipitation

The surfaces of many materials such as unsealed glass, plexiglass, certain plastics and polyethylenes have a high ion exchange and/or adsorption capacity for electrolytes as does fish mucus itself. Appearance or disappearance of electrolytes in the water during a flux period can originate from these surfaces, as well as from exchanges at the gills. In practice, this is rarely a problem when measuring J_{net} values for Na^+, Cl^-, and K^+, as long as unsealed glass is avoided. However when measuring unidirectional fluxes with radioisotopes, it is important to wait a few minutes for isotopic equilibrium prior to taking samples for the start of a flux measurement.

For many polyvalent cations (*e.g.* Ca^{2+} - Perry and Wood, 1985; Cu^{2+} - Laurén and McDonald, 1986; Zn^{2+} - Spry and Wood, 1989), the problem is much more serious when using radiotracers. Indeed the major portion of apparent 'influx' may be due to adsorption on the box walls, the fish's surface, or mucous precipitates, rather than to true entry into the fish. There is no simple way to overcome the problem; the only satisfactory solutions appear to be methods which measure radiotracer *appearance in the fish.* These may involve radio-counting the whole body after displacement of surface-bound radiotracer with a 'swamp' (Perry and Wood, 1985; Reid and Mc-

Donald, 1988) or else radio-counting of blood plasma samples. For the latter, it is first necessary to calibrate the relationship between J_{in} and blood radioactivity in some fashion; Perry and Flik (1988) and Spry and Wood (1989) provide details on two such methods.

There is no practical radiotracer for Al, but for this metal, measurements of even J_{net} are problematical. Based on total Al measurements in plasma and tissues (Booth et al., 1988), Al entry into fish is negligible during short-term exposures, despite considerable Al extraction from the water passing over the gills (Playle and Wood, 1989). This discrepancy is explained by the finding that only 10% of this extracted Al remains bound to the gills, apparently on the external surface; the other 90% is rapidly sloughed off as a precipitate bound to mucus (Playle and Wood, 1991). It remains unknown whether a similar phenomenon occurs for other metals with a high binding affinity for fish mucus.

(iii) Extra-branchial sites of flux

It is commonly assumed that flux measurements reflect exchanges at the gills; the assumption appears safe for J_{in}^{Na} and J_{in}^{Cl}, but not necessarily true for other substances. For example, up to 50% of J_{in}^{Ca} may occur through the skin in rainbow trout (Perry and Wood, 1985). O_2 uptake (and presumably CO_2 excretion) through the skin may account for 13% (rainbow trout) to 35% (eel, *Anguilla anguilla*) of the total (Kirsch and Nonnotte, 1977). While there are no data for other substances at present, the possibility of transcutaneous fluxes should be kept in mind.

A variety of electrolytes may appear in the water when fecal material is discharged, thereby contributing to J_{out} (C.M. Wood, unpublished data); this problem can be virtually eliminated by starving the fish for a few days prior to test. The kidney is a normal route of outflux for many substances. Measurements of renal J_{out} obtained by bladder catheterization suggest that for most electrolytes, the flux through this pathway is less than 20% of branchial J_{out} (McDonald and Wood, 1981; Spry and Wood, 1985; Wood, 1988). Ca^{2+} is an important exception; urinary and branchial J_{out}^{Ca} are of similar magnitude (Hobe et al., 1984a; Perry et al., 1988). Recent measurements using a new external catheterization technique which allows the bladder to function normally show that true urinary losses of Na^+ and Cl^- are only about half of those determined earlier by bladder catheterization (Curtis and Wood, 1991). Other substances do not appear to be reabsorbed or secreted by the bladder to any great extent. Under toxicant stress, renal outfluxes of all electrolytes tend to increase in concert with diuresis, but branchial outfluxes often increase at this time also, so the *relative contribution* of the kidney remains similar. In tests where there is a need to quantify or eliminate renal contributions to J_{out}, the most practical solution is bladder catheterization allowing external collection of the urine flow (e.g. Spry and Wood, 1985). Some disturbance to the fish is unavoidable, but in general the pro-

cedure seems to cause less perturbation of normal physiology than does arterial catheterization.

IV. O_2 AND CO_2 FLUXES AND TOXICANT ACTION

O_2 consumption ($\dot{M}_{O2}$) and CO_2 production ($\dot{M}_{CO2}$) can be measured simultaneously with other net fluxes as long as the flowthrough method is used, the system is sealed, and aeration is avoided. Branchial gas exchange occurs by simple transcellular diffusion of O_2 from water to red blood (RBC), and of CO_2 from RBC to water along partial pressure gradients (Fig. 5). The major route is probably through pavement (respiratory) cells which comprise up to 95% of the total branchial surface area. The most important factors affecting these fluxes at the level of the epithelium itself are the diffusion distance and the total surface area. During exercise, mean diffusion distance decreases, surface area increases, and the flows of blood and water past the epithelium are elevated, thereby improving the partial pressure gradients. All these factors help elevate O_2 and CO_2 fluxes (see Randall and Daxboeck, 1984; Wood and Perry, 1985; Perry and Wood, 1989 for reviews). Catecholamine mobilization plays a key coordinating role in these responses and additionally improves RBC O_2 carrying ability while contributing to plasma acidosis (cf. Nikinmaa, 1991, this symposium). Catecholamine mobilization is also a generalized response to stress (Mazeaud and Mazeaud, 1981).

At environmentally realistic levels, most metals and pH surges appear to stimulate, rather than inhibit respiratory gas exchange, due to stress-induced activity and/or mobilization of catecholamines. However there is little data on the latter point, and therefore a clear need for catecholamine measurements during *sublethal* exposures. The recent study of Witters et al. (1991) is a notable exception, showing substantial increases in plasma adrenaline and noradrenaline in fish exposed to sublethal low pH plus Al, but not sublethal low pH alone. At unnaturally high, *acutely toxic* concentrations, most metals and extreme pH's inhibit branchial O_2 and CO_2 fluxes by increasing the blood to water diffusion distance due to a stimulation of mucus secretion, oedematous swelling of the epithelium itself, and sometimes frank epithelial separation (e.g. Skidmore and Tovell, 1972; Daye and Garside, 1976; Lievestad, 1982; Mallat, 1985; Evans, 1987). These appear to be generalized inflammatory responses to any noxious agent. Only Al causes this effect at environmentally realistic levels (Wood et al., 1988a; Walker et al., 1988; Playle et al., 1989; Witters et al., 1991).

V. Na^+ AND Cl^- FLUXES AND TOXICANT ACTION

The uptake of Na^+ and Cl^- are traditionally viewed as active, independent, electroneutral exchange processes mediated by carrier molecules on the apical membranes of the transporting cells (Fig. 5; see McDonald et al., 1989b; Wood, 1991 for recent reviews). Na^+ is exchanged on a 1-for-1 basis for an acidic equivalent (H^+ or NH_4^+)

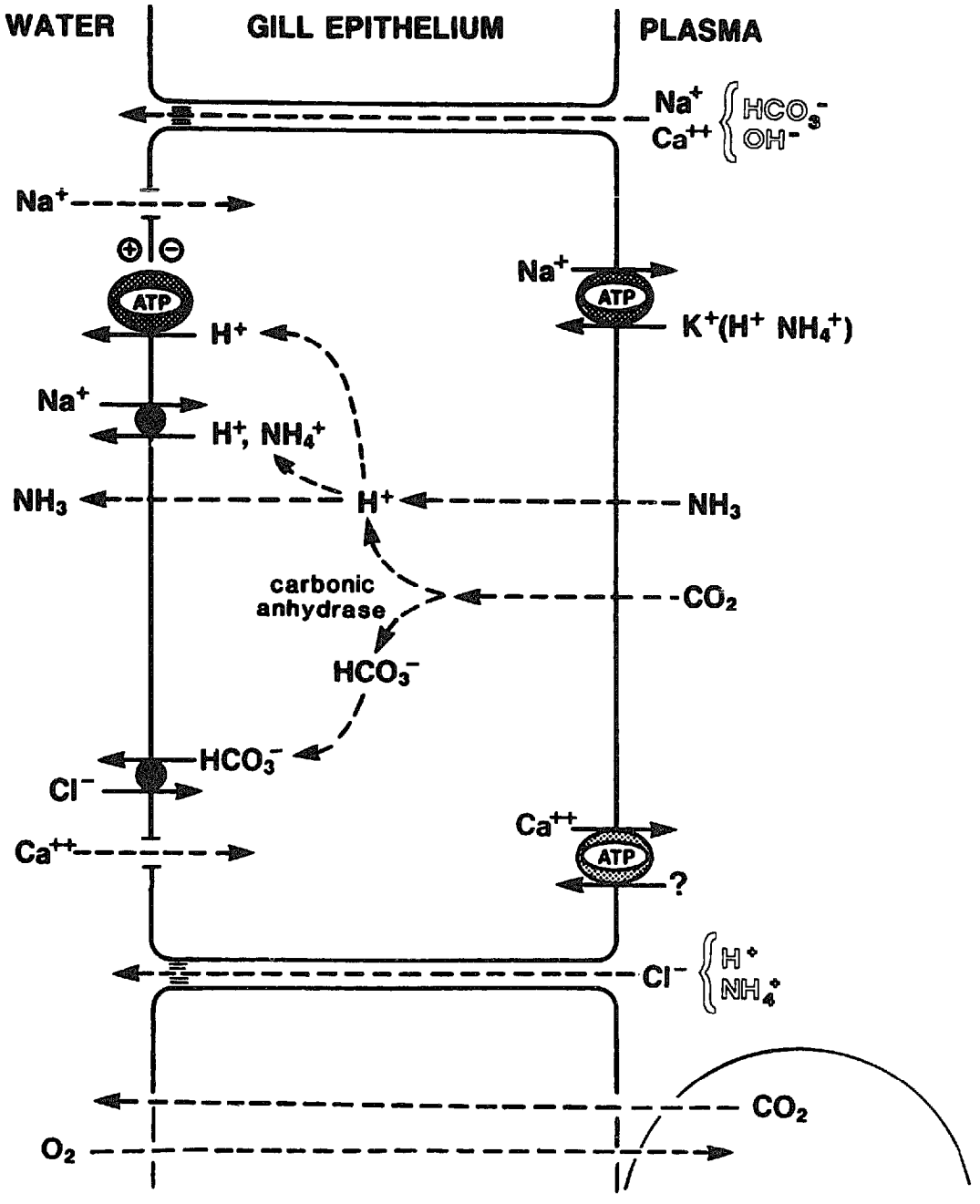

Fig. 5. A model incorporating current ideas about transport mechanisms between the external water and the internal blood plasma in the gills of freshwater fish. Carrier-mediated processes are indicated by solid arrows, diffusive processes by dashed lines. Paracellular movements of HCO_3^-, OH^-, H^+, NH_4^+ (acid-base equivalents) are shown in white to indicate that they may be dependent variables constrained by differential strong ion fluxes (Na^+, Cl^-, Ca^{2+} *etc.*). K^+ has been omitted as there is no information at present on its mechanism(s) of movement.

and Cl^- for a basic equivalent (HCO_3^-). The hydration of respiratory CO_2, catalyzed by intracellular carbonic anhydrase, is the immediate source of these acidic and basic equivalents. Clearly, any agent which differentially alters the rates of Na^+/'acid'

versus Cl^-/'base' exchange will change the net flux of acidic equivalents across the gills, and therefore the fish's acid-base status, unless balanced by differential Na^+ versus Cl^- outfluxes (Section VI).

Na^+/K^+ ATPase pumps on the basolateral membrane are the major source of energy input into the transport system, but these may not be the only source. Na^+/H^+, NH_4^+-dependent (Balm et al., 1988) and Cl^-/HCO_3^--dependent ATPases (Kerstetter et al., 1974; de Renzis and Bornancin, 1977; Battram, 1989) have also been detected in gills, though their precise localization, and linkage with the transport mechanism remains uncertain. Recently, a revision of the traditional model has been suggested by Avella and Bornancin (1989), based on analogy to the frog skin (Ehrenfeld et al., 1985). An active, electrogenic extrusion of H^+ ions across the apical membrane by a H^+ATPase would create an electrochemical gradient for the entry of Na^+ through a separate Na^+-selective channel. As yet there is no evidence to support this model in the fish gill. Whether this new idea, or the traditional Na^+/H^+, NH_4^+ exchanger eventually proves correct, both provide a 1:1 coupling of Na^+ uptake to acidic equivalent excretion.

By either model, the well-known immediate inhibitory effect of low environmental pH on J_{in}^{Na} (Figs. 1, 4, 6; see Wood and McDonald, 1982; McDonald, 1983a; Wood, 1989 for reviews) can be simply explained as a direct competitive action of external H^+ on the apical transporter. On the other hand, the inhibitory effect of Cu on J_{in}^{Na}, which is insensitive to external $[H^+]$ and develops progressively with time (Fig. 4; Laurén and McDonald, 1985, 1986; Reid and McDonald, 1988), is probably due to its demonstrated inhibition of Na^+/K^+ ATPase (Stagg and Shuttleworth, 1982; Laurén and McDonald, 1987b). Al has little immediate effect on J_{in}^{Na} (McDonald and Milligan, 1988) but inhibition increases with time (Fig. 2; Booth et al., 1988; McDonald et al., 1991b); as with Cu, its action may be largely by blockade of the transport enzymes (Staurnes et al., 1983). The same may be true with Hg which reduces Na^+ uptake in long term (Renfro et al., 1984) but not short term exposures (Stinson and Mallatt, 1989), and is known to inhibit Na^+/K^+ATPase in vivo (Lock et al., 1981) and in vitro (Miura and Imura, 1987). The mechanism by which high environmental pH quickly reduces J_{in}^{Na} remains unknown (Wright and Wood, 1985). The accompanying reduction in J_{in}^{Cl} caused by all these agents is well documented (Figs. 1, 6), but its mechanism(s) also remains obscure (McDonald et al., 1983; Laurén and McDonald, 1985; Audet et al., 1988; Stinson and Mallat, 1989; Battram, 1988, 1990; M. Wilkie and C.M. Wood, unpublished results). Certainly these observations suggest that J_{in}^{Na} and J_{in}^{Cl} may not be independent, in contrast to current belief (Fig. 5).

Metals for which the primary toxic action is related to an inhibition of Ca^{2+} transport (Section VII) generally do not inhibit Na^+ uptake. Indeed many of them actually stimulate J_{in}^{Na}. This includes La (Eddy and Bath, 1979), Zn (Fig. 3; Spry and Wood, 1985), Mn (Reader and Morris, 1988), and Cd (stimulation – Reader and Morris, 1988; no effect – Reid and McDonald, 1988; Verbost et al., 1989). Exposure to low

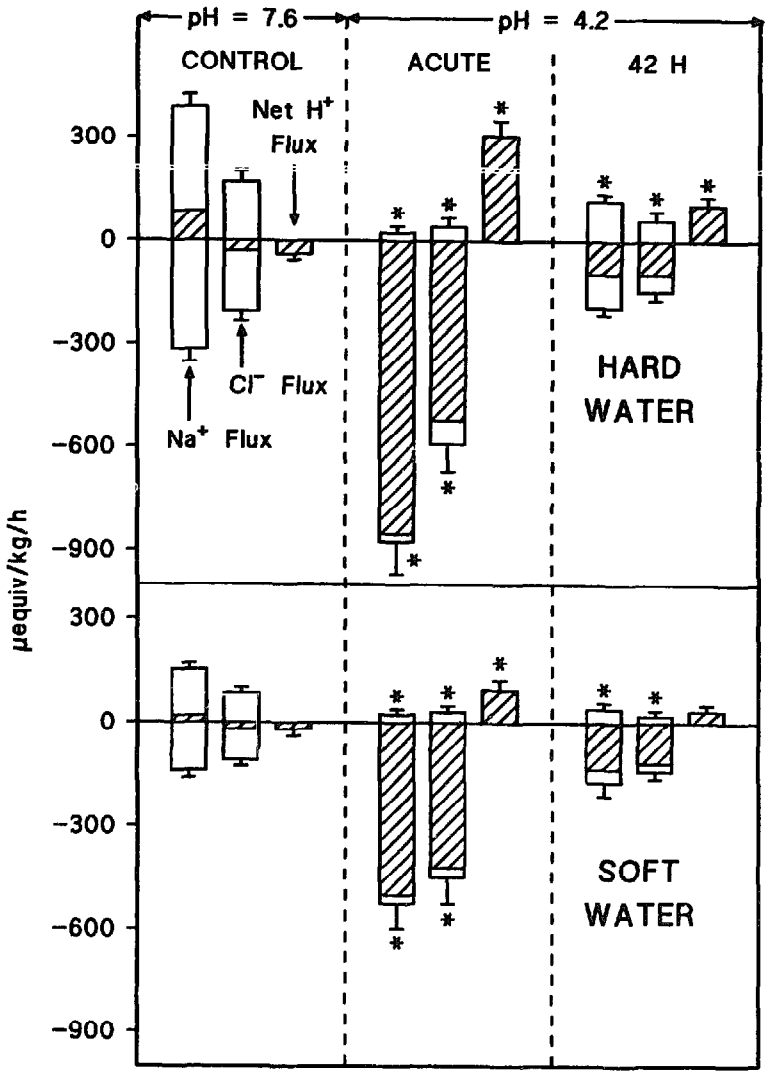

Fig. 6. Unidirectional (J_{in}, J_{out}; open bars) and net fluxes (J_{net}; stippled bars) of Na^+, Cl^-, and 'H^+' (acidic equivalents) across the gills of rainbow trout upon acute exposure to pH 4.2 (first 4 h) and after 42 h of continuous exposure in very hardwater (upper panel) and softwater (lower panel). Means ± 1 SEM ($N = 4$). Asterisks indicate means significantly different ($p<0.05$) from the pre-acid control value. Data of McDonald et al. (1983), redrawn from Wood (1989).

water [Ca^{2+}] and Ca^{2+} removal from the gill by chelating agents also tend to stimulate Na^+ uptake (Cuthbert and Maetz, 1972; McDonald and Rogano, 1986). This raises the interesting possibility that access to Na^+-binding sites on the apical transporter is shielded by bound Ca^{2+}.

Most studies suggest that the mitochondrial-rich chloride cells are the cell type responsible for both Na^+ and Cl^- uptakes, but the evidence is at best correlational (e.g. Avella et al., 1987; Perry and Laurent, 1989; Laurent and Perry, 1990). Two very recent studies (Goss et al., 1991a,b) provide further support for the chloride cell as the major site of Cl^-/base exchange, but suggest that the pavement cells are more im-

portant in Na^+/acid exchange. Again the evidence is based on correlations between J_{in}^{Na} and J_{in}^{Cl} and changes in cell numbers and morphology. Further work is required before any definitive conclusions can be drawn.

Proliferation and/or increased surface exposure of chloride cells is a well-documented response to chronic exposure to several agents known to inhibit branchial Na^+ and Cl^- uptake (low pH - Leino and McCormick, 1984; Leino et al., 1987; Wendelaar Bonga et al., 1990; Laurent and Perry, 1991; high pH - M. Wilkie and C.M. Wood, unpublished results; Al at low pH - Chevalier et al., 1985; Karlsson-Norggren et al., 1986a,b; Tietge et al., 1988; Mueller et al., 1991). Cortisol is a likely mediator of this response. The hormone is mobilized during exposures to low pH (Brown et al., 1984) and low pH plus Al (Goss and Wood, 1988; Brown et al., 1989) and is known to increase chloride cell fractional area on the gills (Perry and Wood, 1985; Perry and Laurent, 1989; Laurent and Perry, 1990). This effect may explain the partial recovery of J_{in}^{Na} and J_{in}^{Cl} seen during longer term exposures (Figs. 1, 6; McDonald et al., 1983, 1991b; Audet et al., 1988; McDonald and Milligan, 1988).

The major route of diffusive loss for Na^+ and Cl^- (and probably also Ca^{2+}) is thought to be through the paracellular channels and tight junctions (McDonald et al., 1991a; Fig. 5). Ca^{2+} plays a critical role in stabilizing these junctions and reducing permeability (Oschman, 1978; McWilliams, 1983; Freda and McDonald, 1988). Therefore water [Ca^{2+}] ('hardness') is particularly important in protecting against toxic agents which act to increase paracellular permeability (e.g. Fig. 2). These include elevated environmental [H^+] (McDonald et al., 1983; Wood, 1989), Al (Booth et al., 1988; Wood et al., 1988a,b; Playle et al., 1989), but not Cu (Fig. 4; Laurén and McDonald, 1985, 1986). The latter observation suggests that Cu disrupts tight junctions by a mechanism other than simple displacement of bound Ca^{2+}.

These are the same agents which inhibit Na^+ and Cl^- uptake, though higher threshold concentrations are needed to stimulate diffusive outflux (e.g. Fig. 4). The relative contribution of J_{in} inhibition and J_{out} stimulation to net losses will therefore depend on the concentration of toxicant. For example, compare the acute responses of rainbow trout to moderate acidity (pH 4.8, Fig. 1) and severe acidity (pH 4.2, Fig. 6). With time J_{out} values often return to control levels (or lower), while recovery of J_{in} is at best partial (Figs. 1, 6). This suggests that fish have an ability to reduce paracellular permeability, either hormonally (prolactin is a likely candidate - Wendelaar Bonga and Balm, 1989) or through mechanisms intrinsic to the gills (such as cell swelling -McDonald and Rogano, 1986).

VI. ACID-BASE AND AMMONIA FLUXES AND TOXICANT ACTION

Na^+ and Cl^- are the two strong ions present in greatest concentration in the blood plasma; they exhibit by far the highest flux rates across the gills. Any differential net flux of strong cations versus strong anions will have an acid-base effect. Strong Ion Difference Theory (Stewart, 1983), as well as the constraints of electroneutrality,

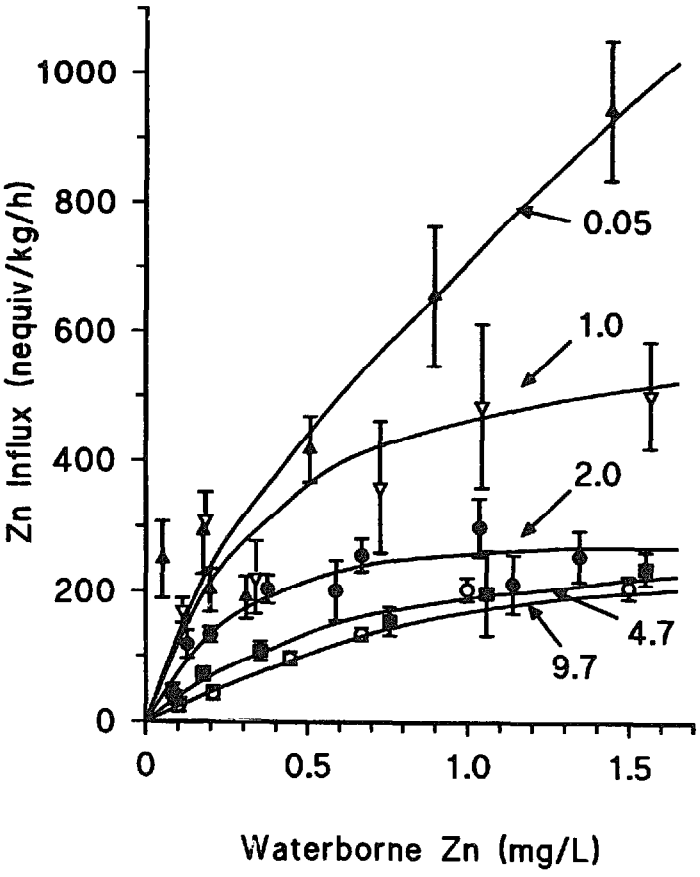

Fig. 7. Relationships between unidirectional Zn influx (J_{in}) and external Zn concentration in rainbow trout at different levels of water [Ca^{2+}]. Arrows indicate [Ca^{2+}] in mEq/l. Means ± 1 SEM (N = 3–12 at each point; different fish were used for each Zn/Ca^{2+} combination, N = 168 in total). The lines were fitted using a one-substrate Michaelis-Menten model, except at [Ca^{2+}] = 0.05 mEq/l, where the line was fitted by eye. Redrawn from Spry and Wood (1989).

dictates that net acidic equivalent flux (note: base = negative acid) must equal the difference between strong cation minus strong anion (SID) flux in the opposite direction. In particular, an excess of strong cation (Na^+) loss over strong anion (Cl^-) loss will constrain a net loss of basic equivalents, while the reverse will constrain a net loss of acidic equivalents. In terms of net acid-base balance, it is immaterial whether these equivalents (H^+, NH_4^+, HCO_3^-, OH^-) actually move through the paracellular channels accompanying differential Na^+ versus Cl^- outflux (Fig. 5), are transported by Na^+/H^+, NH_4^+ and Cl^-/HCO_3^- exchangers (Fig. 5), or are merely consumed/generated by the aqueous medium (an infinite source or sink of H^+ and OH^-) in response to a changing SID flux.

Several agents (Zn - Spry and Wood, 1985; high environmental pH - M. Wilkie and C.M. Wood, unpublished results) disturb acid-base balance by effects on branchial fluxes, but for only one, acute low environmental pH, has the mechanism been worked out (McDonald et al., 1980, 1983; McDonald, 1983; Wood, 1989). Here, acid

exposure in hard water (high $[Ca^{2+}]$) blocks J_{in}^{Na} and J_{in}^{Cl} equally, but promotes a greater stimulation of J_{out}^{Na} than J_{out}^{Cl}, resulting in net acidic equivalent uptake and internal acidosis (Fig. 6). The same exposure in soft water (low $[Ca^{2+}]$), causes almost equimolar increases in J_{out}^{Na} and J_{out}^{Cl} and negligible acid-base flux. Presumably, higher environmental $[Ca^{2+}]$ at low pH tends to make the paracellular channels more cation selective.

The mechanism(s) of ammonia excretion are still widely disputed in freshwater fish; the issue is beyond the scope of this review. Suffice it to say that there are proponents of passive transcellular NH_3 diffusion, passive paracellular NH_4^+ diffusion, apical Na^+/NH_4^+ exchange, and active transport by the K^+ site on basolateral Na^+/K^+ ATPase as the principal mechanisms in different species under different circumstances. All are represented in Fig. 5. Cameron and Heisler (1983), Wright and Wood (1985), Evans and Cameron (1986) and McDonald et al. (1989b) provide a representative selection of current views. NH_4^+ outflux represents acidic equivalent excretion, while NH_3 outflux does not contribute to acid-base balance. Present technology cannot distinguish between NH_4^+ outflux and NH_3 outflux *plus* H^+ outflux or HCO_3^- uptake, though fortunately they are the same in terms of *net* acid-base balance (McDonald and Wood, 1981).

Acute exposure to low pH (McDonald et al., 1983; Hobe et al., 1984b; Audet and Wood, 1988), high pH (Wright and Wood, 1985; Wilkie and Wood, 1991), and Cu (Laurén and McDonald, 1985) all impair J_{net}^{Amm}. Note that low pH and high pH both inhibit, even though the former would be expected to promote NH_3 diffusion, and the latter retard it via effects on the transbranchial P_{NH3} gradient. All three treatments simultaneously reduce J_{in}^{Na} (Section V). In total, these data suggest that a linkage of NH_4^+ outflux to Na^+ influx is blocked. During longer term exposures to low pH (McDonald and Wood, 1981; McDonald, 1983b; Audet et al., 1988) and Zn (Spry and Wood, 1985), J_{net}^{Amm} increases above control levels; this is probably attributable to increased rates of protein metabolism and ammonia production stimulated by cortisol mobilization in stressed fish.

VII. Ca^{2+} FLUXES AND TOXICANT ACTION

The present model of Ca^{2+} uptake (Flik et al., 1985; Perry and Flik, 1988) holds that Ca^{2+} enters the transport cells from the water by passive diffusion along the electrochemical gradient through voltage-independent Ca^{2+} selective channels (Fig. 5). Ca^{2+}-binding proteins within the cell keep intracellular activities extremely low. At the basolateral membrane, Ca^{2+} is actively transported to the blood by a high affinity Ca^{2+}ATPase which is calmodulin-dependent. It is unknown whether this ATPase co-or counter-transports other ions. The chloride cell appears to be the site of Ca^{2+} uptake and treatments such as cortisol injection which elevate chloride cell numbers and apical exposure have a marked stimulatory effect on J_{in}^{Ca} (Perry and Wood, 1985; Flik and Perry 1989). Though this evidence is again correlational, it is reinforced by

histochemical localization of Ca^{2+}-precipitates in chloride cells (Isihara and Mugiya, 1987) and of La, a specific Ca^{2+}-transport antagonist, on their apical surfaces (Perry and Flik, 1988).

J_{in}^{Ca} is specifically inhibited by Cd (Verbost et al., 1987, 1989; Reid and McDonald, 1988; Reader and Morris, 1988), La (Verbost et al., 1987, 1989; Perry and Flik, 1988), Zn (Fig. 3; Spry and Wood, 1985), and Mn (Reader and Morris, 1988). Two other polyvalent metals, Cu (Reid and McDonald, 1988) and Al (Reader and Morris, 1988; Booth et al., 1988) which are very effective in blocking Na^+ uptake (Section V) seem to have only minor effects on Ca^{2+} uptake. However low environmental pH does appear to inhibit J_{in}^{Ca}, though the effect may be only transient (Hobe et al., 1984a,b; Reader and Morris, 1988; Reid and McDonald, 1988). High pH has not been tested. Not surprisingly, elevations in water $[Ca^{2+}]$ generally protect against inhibition of J_{in}^{Ca} (Reid and McDonald, 1988) and reduce the entry of metals such as Cd and Zn into fish (e.g. Fig. 7; Spry and Wood, 1989; Wicklund, 1990). The transport of Zn (an essential micro-nutrient) and Ca^{2+} across the gills interacts in a simple competitive fashion (Section IX) and there is correlational evidence that the chloride cell is also the site of Zn uptake (Spry and Wood, 1988). It is unknown whether this competition occurs at the apical channels, the basolateral ATPase, or both. However Cd, which is not a micronutrient, appears to block J_{in}^{Ca} by inhibiting the basolateral high affinity Ca^{2+}ATPase rather than directly competing at the apical channel; the effect develops slowly (Verbost et al., 1987, 1988, 1989). On the other hand, La (another non-micro-nutrient), immediately lowers J_{in}^{Ca} by blocking the apical channels (Verbost et al., 1987, 1989; Perry and Flik, 1988).

Ca^{2+} outflux appears to occur passively through the paracellular channels (Fig. 5). Agents which increase the diffusive permeability of these channels to Na^+ and Cl^- (Section V) also stimulate J_{out}^{Ca}. These include low environmental pH (Hobe et al., 1984a; Reader and Morris, 1988; Reid and McDonald, 1988) and Cu (Reid and McDonald, 1988), whereas specific Ca^{2+} transport blockers such as Cd and Mn have no effect on J_{out}^{Ca} (Verbost et al., 1987; Reid and McDonald, 1988; Reader and Morris, 1988).

VIII. K^+ FLUXES AND TOXICANT ACTION

K^+ has been omitted from Fig. 5 because at present we know nothing about its mechanisms of movement through the gills, apart from the fact that K^+ can be taken up from freshwater by apparent active transport (Eddy, 1985). Negative K^+ balance at the gills is a common observation for fish under a variety of stresses (e.g. Fig. 3), but again the route(s) of loss is unknown. In contrast to Na^+, Cl^-, and Ca^{2+} for which the intracellular concentrations in branchial epithelial cells are likely quite low, intracellular $[K^+]$ is much higher than plasma levels (Wood and LeMoigne, 1991). Plasma $[K^+]$ on the other hand is quite low, comparable to plasma $[Ca^{2+}]$ and only about 3% of plasma $[Na^+]$ and $[Cl^-]$ (Table 1). A variety of toxicants including low pH

(McDonald and Wood, 1981; McDonald 1983b), Zn (Fig. 3; Spry and Wood, 1985), Cu (Laurén and McDonald, 1985), and Al (Booth et al., 1988) have been reported to induce net K^+ losses from the gills which are much higher than Ca^{2+} losses, and approach the same magnitude as Na^+ and Cl^- losses. This strongly suggests that at least a portion of J^K_{out} is transcellular due to leakage from the epithelial cells themselves. Laurén and McDonald (1985) have made the interesting suggestion that the ratio of K^+ to Na^+ losses could be used as an index of the relative permeabilities of transcellular versus paracellular pathways. The physiology of K^+ transport across the gills and the effects of toxicants thereon should be a rich area for future research.

IX. KINETIC ANALYSIS OF FLUXES

A method which has been employed with great success in the last few years is the kinetic analysis of fluxes (e.g. Laurén and McDonald, 1987a; Freda and McDonald, 1988; Spry and Wood, 1989; Gonzalez and Dunson, 1989), based on the same principles as used in enzymology. While the technique itself is certainly not new (Shaw, 1959) and is widely used in basic ionoregulatory studies (Evans, 1984; Wood, 1991), its application to toxicological work is a recent development. Experimentally, the external ion ('substrate') is set to a number of different concentrations for brief periods, and the unidirectional influx rate J_{in} ('velocity') measured with radiotracers at each concentration. This is done either by sequentially increasing the concentration of both 'hot' and 'cold' substrate in the external water of a single fish, or by using different fish for each concentration. The relationship between J^X_{in} and the external ion concentration $[X]_{ext}$ is usually hyperbolic (Fig. 7) and can be approximated by the Michaelis-Menten equation for one substrate reactions:

$$J^X_{in} = \frac{J^X_{max} \cdot [X]_{ext}}{K_m + [X]_{ext}} \tag{4}$$

where J^X_{max} is the maximum influx rate and K_m is the value of $[X]_{ext}$ at 1/2 J^X_{max}. The K_m is an index of the binding affinity of the operational site for transport (i.e. apical channel or exchanger; Fig. 5) while J^X_{max} is traditionally considered to be an index of the number of such sites available.

This approach can be used to evaluate a number of issues. For example, comparison of kinetic constants amongst different species can reveal why some are more sensitive to a particular toxicant than are others. Freda and McDonald (1988) found that the K_m for Na^+ transport was directly correlated and J_{max} inversely correlated with acid tolerance in three species, which supports the belief that external H^+ directly competes for the apical Na^+ transport mechanism (Section V). Determination of kinetic constants in the presence of the toxicant can directly test this sort of idea. Interference with affinity (i.e. altered K_m) without alteration of the number of sites (i.e. unchanged J^X_{max}) would indicate simple competitive inhibition, whereas alteration of J^X_{max} without effects on K_m would indicate non-competitive inhibition (e.g. destruc-

tion of transport sites). For example, in the rainbow trout, Spry and Wood (1989) demonstrated that water [Ca^{2+}] acts mainly as a competitive inhibitor of Zn transport (large increases in K_m), with only small effects on J^{Zn}_{max} (Fig. 7). The inhibitor constant for Ca^{2+} is similar to its K_m for transport (Perry and Wood, 1985), strongly supporting the idea that the Zn^{2+} and Ca^{2+} compete for a common uptake pathway (Section VII).

The kinetic approach can also be used to examine the time course and mechanism of recovery during chronic exposure to a toxicant. Laurén and McDonald (1987a) found an immediate increase in K_m and decrease in J_{max} for Na^+ uptake in trout exposed to sublethal Cu for 24 h (i.e. mixed competitive and non-competitive inhibition). However, during 28 days of chronic exposure, J^{Na}_{max} recovered almost completely, while K_m recovered only partially. The short term decrease and long term restoration of J^{Na}_{max} were paralleled by comparable changes in total Na^+/K^+ ATPase activity in the gill tissue (Laurén and McDonald, 1987b). These data reinforce the view that the principal action of Cu against J_{in} is an inhibition of the basolateral Na^+/K^+ ATPase (Section V).

Most recently, Goss and Wood (1991) have found that the rates of both Na^+ and Cl^- uptake in rainbow trout respond not only to the concentrations of external 'substrate', but also to the concentrations of the putative *internal* 'substrates' or counter-ions, H^+ and HCO_3^- respectively (Fig. 5). A modified Michaelis-Menten model for a two-substrate system adequately described the responses to experimental manipulation of internal acid-base status. These observations provide further support for the presence of Na^+/'acid' and Cl^-/'base' exchanges in the gills (Fig. 5). However, at the same time the two-substrate analysis carries the important implication that availability of internal counter-ions (H^+, HCO_3^- and perhaps NH_4^+) constitute a second factor governing apparent J^X_{max}, in addition to the actual number of transport sites. This is an important consideration for future flux studies at low and high pH. For example if HCO_3^- levels in gill transport cells are reduced by external acidity, it would account for the previously unexplained inhibition of J^{Cl}_{in} caused by exposure to low pH (Section V; Figs. 1, 6).

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